Monatshefte für Chemie 120, 1021 – 1028 (1989)

Monatshefte für Chemie
Chemical Monthly

On the Hypothetical Protein F154 of the TTV1 Virus/*Thermoproteus Tenax*. Part I: Synthesis of a Dodecapeptide Amide Related to the Protein Sequence 73–84***

Erich Wünsch* and **Daniel Krois****

Max-Planck-Institut für Biochemie, Abteilung für Peptidchemie, D-8033 Martinsried, Federal Republic of Germany

Summary. DNA sequence analysis of the virus TTV1 identified in archaebacterium *Thermoproteus tenax* revealed an open reading frame possibly encoding for a protein. To assess the expression of this hypothetical protein F154 via immunochemical methods, related synthetic fragments should allow for production of protein specific antisera. For this purpose the dodecapeptide amide H-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Ser-Pro-Thr-Pro-NH_2 related to a characteristic repeat in the protein primary structure was prepared by conventional methods of peptide synthesis.

Keywords. TTV1 virus; Peptide synthesis; Poly-(Thr-Pro)-peptides.

Zur Hypothese eines TTV1 Virus/*Thermoproteus tenax* F154 Proteins. Teil I: Synthese eines Dodekapeptidamids der Proteinsequenz 73–84

Zusammenfassung. Sequenzanalyse des Genoms des Virus TTV1, der im Archaebakterium *Thermoproteus tenax* aufgefunden wurde, läßt die Expression eines Proteins zu, dessen tatsächliche Existenz aber nicht gesichert ist. Antiseren gegen synthetische Fragmente dieses hypothetischen Proteins sollten dessen Identifizierung und somit indirekt den Nachweis der Expression im Bakterium ermöglichen. Zu diesem Zweck erfolgte die Synthese des Dodekapeptidamids H-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Ser-Pro-Thr-Pro-NH_2, eines charakteristischen Fragments dieses Proteins, mit Hilfe konventioneller Methoden.

Abbreviations

Standard abbreviations as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature are used for amino acids and related derivatives; HO*SU*, N-hydroxysuccinimide; HO*Bt*, 1-hydroxybenzotriazole; *DCC*, dicyclohexylcarbodiimide; *DCHA*, dicyclohexylamine; *DMF*, dimethylformamide; *Me*OH, methanol; *THF*, tetrahydrofuran; *TEA*, triethylamine; tlc, thin layer chromatography; hplc, high performance liquid chromatography.

** *Present address:* Institut für Organische Chemie, Universität Wien, A-1090 Wien, Austria

*** Dedicated to Prof. Dr. Karl Schlögl on occasion of his 65th birthday

Introduction

A family of four viruses, TTV1–TTV4, had been found to exist within populations of the extremely thermophilic sulfur reducing archaebacterium *Thermoproteus tenax* [1, 2]. Nucleotide sequence analysis of one of the DNA fragments obtained by restriction enzymes from the TTV1 genom revealed an open reading frame possibly encoding for a protein, the hypothetical protein F154 [1] (Scheme 1). It is not known whether this protein represents a regulatory element during virus assembly or a structure unit of the virus itself or whether it is expressed at all. The primary structure of this hypothetical protein F154 displays characteristic sequence repeats, i.e. poly-(Thr-Pro)-regions with a limited number of conservative point mutations (Ser for Thr), corresponding to the sequence portions 69–84, 96–115, and 127–148 and twice an undecapeptide sequence, i.e. Thr-Tyr-Asp-Ile-Thr-Tyr-Val-Val-Phe-Asp-Val, corresponding to positions 85–95 and 116–126, in the latter case with one conservative mutation (Ile for Val in position 123).

Scheme 1. Amino acid sequence of the hypothetical protein F-154 from TTV1 virus as predicted by nucleotide sequence analysis (cf. Ref. [1]). Poly(Thr-Pro)-repeats (full line) and the undecapeptide repeats (dotted line) are specially indicated

1	Met-Tyr-Leu-Ser-Ile-Asn-Gly-Ser-Thr-Ala-Asn-Val-Lys-Val-Tyr-Lys-Gln-Gly-Ser-Asn-	20
21	Ile-Gly-Thr-Val-Ser-Gly-Asn-Tyr-Ser-Thr-Thr-Pro-Tyr-Gly-Asn-Pro-Ser-Met-Ala-Gly-	40
41	Tyr-Gly-Thr-Val-Asp-Lys-His-Tyr-Ala-Asn-Phe-Ile-Val-Leu-Pro-Tyr-Glu-Pro-Asp-Pro-	60
61	Gln-Val-Thr-Val-Thr-Pro-Ile-Ser-Ser-Pro-Ser-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-	80
81	Thr-Pro-Thr-Pro-Thr-Tyr-Asp-Ile-Thr-Tyr-Val-Val-Phe-Asp-Val-Thr-Pro-Ser-Pro-Thr-	100
101	Pro-Thr-Pro-Thr-Leu-Thr-Ser-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Tyr-Asp-Ile-Thr-	120
121	Tyr-Val-Ile-Phe-Asp-Val-Thr-Pro-Ser-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-	140
141	Thr-Pro-Thr-Pro-Thr-Pro-Thr-Ser-Thr-Thr-Ser-Ser-Asn-Ile	

The aim of the present study is to prove the expression and possibly the function of the protein F154 by means of specific antiprotein antisera raised against conjugates of synthetic protein fragments [3]. In this context the two repeats mentioned above appear the most promising antigenic sites. The poly-(Thr-Pro)-stretches are likely to adopt rigid ordered structures in the folded protein, whereas the undecapeptide repeats as connecting sequences may well represent surface-exposed loops. Correspondingly, a synthetic peptide related to the undecapeptide repeat and having both at the N- and C-terminus a poly-(Thr-Pro)-extension, could possibly mimic in an efficient way characteristic immunepitops of the hypothetical protein. Following this working hypothesis, we decided to synthesize both possible antigenic sites, i.e. the poly-(Thr-Pro)-repeat as well as the undecapeptide extended in a fashion described above.

In the present communication the synthesis of the dodecapeptide amide H-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Ser-Pro-Thr-Pro-NH_2 related to the poly-(Thr-Pro)-regions is described, whereby in view of its selective conjugation to the carrier protein [4] at its N-terminus, the C-terminus was amidated. Additionally, end-group effects on the possible folding of the hapten molecule at the carrier surface

should be prevented. The synthesis of the second repeat will be described in the following communication [5].

Results and Discussion

The synthesis of the dodecapeptide amide, i.e. H-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Ser-Pro-Thr-Pro-NH_2, was accomplished by conventional methods following the strategy of maximum side chain protection and of fragment condensation, whereby a combination of the N^{α}-benzyloxycarbonyl group for the chain elongation steps with *tert*-butyl ethers for the hydroxylic functions of serine and threonine was used.

Following the synthetic route outlined in Scheme 2, the C-terminal dipeptide derivative ⟨11–12 a⟩ (for the synthetic work, the numbering is referred to the dodecapeptide and not to the protein sequence positions) was readily obtained by coupling of H-Pro-NH_2 with *Z*-Thr(*tBu*)-OH by the *DCC*/HO*Bt* procedure; its N^{α}-deprotection by catalytic hydrogenolysis under titrimetric addition of aqueous HCl yielded ⟨11–12 b⟩ hydrochloride as a homogeneous compound. To prevent the observed facile diketopiperazine formation, the free amine dipeptide derivative ⟨11–12 b⟩ was prepared in situ by neutralizing the hydrochloride with triethylamine at low temperature and then rapidly acylated with *Z*-Ser(*tBu*)-Pro-OH ⟨9–10⟩ via the mixed anhydride procedure. Subsequent N^{α}-deprotection of the resulting tetrapeptide amide derivative ⟨9–12 a⟩ followed by condensation of the resulting ⟨9–12 b⟩ with *Z*-Thr-(*tBu*)-Pro-OH ⟨7–8⟩ by the *DCC*/HO*Bt* method led to the hexapeptide amide derivative ⟨7–12 a⟩. Final N^{α}-debenzyloxycarbonylation produced the C-terminal fragment A, i.e. H-Thr(*tBu*)-Pro-Ser(*tBu*)-Pro-Thr(*tBu*)-Pro-$NH_2 \cdot$HCl, ⟨7–12 b · HCl⟩ in 33% overall yield (6 steps) as a crystalline homogeneous compound.

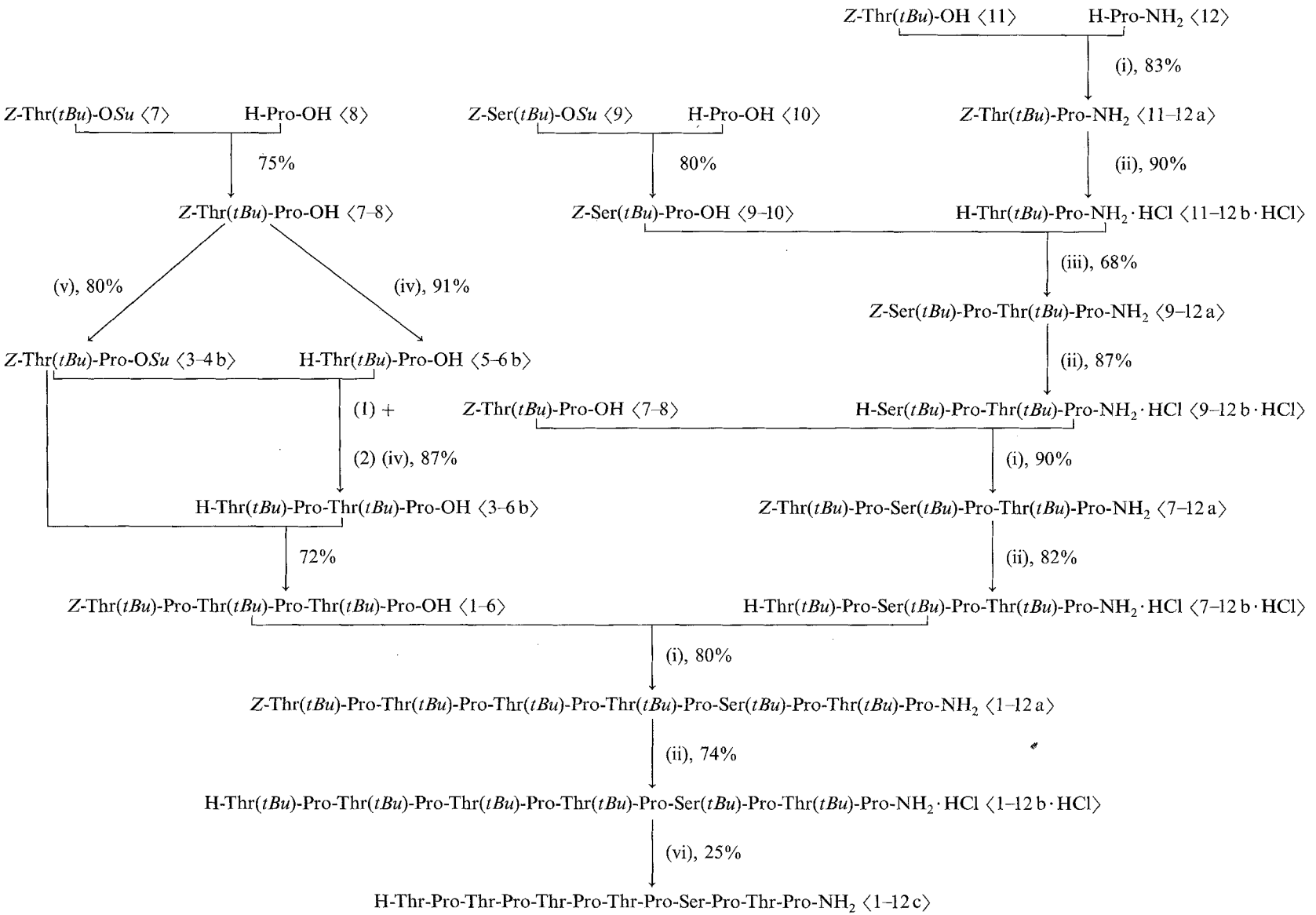
The N-terminal fragment B was synthesized in stepwise fragment condensations, starting from H-Thr(*tBu*)-Pro-OH ⟨5–6 b⟩ and using twice the dipeptide derivative *Z*-Thr(*tBu*)-Pro-O*Su* (positions 3–4 and 1–2, respectively) as acylating agent with intermediate N^{α}-deprotection of the tetrapeptide amide derivative ⟨3–6 a⟩ by catalytic hydrogenolysis. The hexapeptide derivative *Z*-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-OH ⟨1–6⟩ was obtained in 43% overall yield (5 steps), again as analytically well characterized compound.

Condensation of the fragments A, ⟨7–12 b⟩, and B, ⟨1–6⟩, by the *DCC*/HO*Bt* method produced the fully protected dodecapeptide amide ⟨1–12 a⟩ as homogeneous compound in good yields. Subsequent catalytic hydrogenolysis of the N^{α}-benzyloxycarbonyl group, followed by exposure of the resulting ⟨1–12 b⟩ to 95% aqueous trifluoroacetic acid and gel filtration of the crude deprotection product led to the desired dodecapeptide amide ⟨1–12 c⟩ in 25% yield as highly homogeneous material as determined by various indicative analytical assays. On the use of this peptide for the preparation of conjugates and related immunization experiments will be reported elsewhere [6].

Acknowledgements

The authors thank Mrs. A. Bernert, Mrs. R. Scharf, and Mrs. B. Guth for their skillful technical assistance. Elemental analyses were performed in the microanalytical laboratory (Dr. E. Jaeger).

Scheme 2. Synthesis of the dodecapeptide amide ⟨1–12 c⟩; reagents used: (i) *DCC*/1-hydroxybenzotriazole; (ii) H_2/Pd, titration with HCl; (iii) ethylchloroformate (M.A.); (iv) H_2/Pd; (v) N-hydroxysuccinimide + *DCC*; (vi) 95% trifluoroacetic acid followed by gel chromatography



Experimental

Materials and Methods

Melting points were determined on a Tottoli's capillary melting point apparatus and are uncorrected. Optical rotations were measured in a jacketed 1 dm cell on a Perkin Elmer polarimeter (model 241). Hydrolyses were conducted in 6 *M* HCl at 110°C in evacuated sealed ampoules or with aminopeptidase M (Boehringer) at 37°C. Amino acid analyses were obtained on a Biotronic analyzer (LC 6001). Gas chromatographic racemization tests were carried out on a Fractovap (Carlo Erba, 4160) with Chirasil-Val glass capillary columns according to Ref. [7]. For tlc silica gel 60 plates (Merck AG, Darmstadt) were used with various solvent systems, the following being the most indicative: (1) ethyl acetate/1-butanol/acetic acid/water, 5:3:1:1; (2) 1-butanol/acetic acid/water, 3:1:1; (3) ethyl acetate/1-butanol/water/pyridine/acetic acid, 50:27:11:9:3; (4) 1-butanol/water/pyridine/acetic acid, 55:22:18:5; (5) *n*-heptane/*tert*-butanol/acetic acid, 3:2:1; (6) *n*-heptane/*tert*-butanol/acetic acid, 5:1:1; (7) cyclohexane/chloroform/acetic acid, 45:45:10; (8) dichloromethane/ethyl acetate/*Me*OH, 3:2:1; (9) chloroform/trifluoroethanol/propionic acid/water, 46:22:22:10; (10) chloroform/trifluoroethanol/80% propionic acid, 70:15:15; (11) 1-butanol/water/pyridine/acetic acid, 50:22:18:10; (12) 1-pentanol/water/2-propanol/acetic acid/pyridine, 30:26:17:17:10. Peptidic compounds were visualized with chlorine/tolidine and ninhydrine reagents. Hplc was performed on Waters instruments.

The amino acid derivatives used in the syntheses were prepared according to standard procedures [8].

Synthesis of the Dodecapeptide Amide

Z-Thr(*tBu*)-Pro-NH_2 ⟨11–12 a⟩

A solution of H-Pro-NH_2 ⟨12⟩ (5.7 g, 50 mmol), *Z*-Thr(*tBu*)-OH ⟨11⟩ (15.47 g, 50 mmol) and HO*Bt* (6.76 g, 50 mmol) in *DMF* (200 ml) was combined at 0°C with DCC (10.3 g, 50 mmol) in *DMF* (80 ml) and stirred for 24 h at room temperature. Then the solvent was removed and the residue distributed between 0.1 *M* $KHSO_4$ and ethyl acetate. The organic layer was washed successively with water, 0.5 *M* Na_2CO_3, and water and dried over Na_2SO_4. After evaporation of the solvent the resulting residue was reprecipitated from ether with petroleum ether. Yield: 16.8 g (83%); m.p. 65°C; $[\alpha]^{20}_D = -12.77°$ and $[\alpha]^{20}_{546} = -15.44°$ (*c* 2, ethanol); tlc: 6, 8. Anal. calcd. for $C_{21}H_{31}N_3O_5$ (405.49): C 62.20, H 7.71, N 10.37; found: C 62.31, H 7.82, N 10.16.

H-Thr(*tBu*)-Pro-$NH_2 \cdot HCl$ ⟨11–12 b · HCl⟩

The dipeptide derivative ⟨11–12 a⟩ (16.5 g, 41 mmol) was hydrogenated over palladium/charcoal (Pd/C) in *Me*OH-water (3:1 *v/v*, 400 ml) under titrimetric addition of 1 *M* HCl (*pH* 4.0) at room temperature. The reaction mixture was worked up in usual manner (filtration, complete evaporation of the solvents under reduced pressure) and the solid residue was precipitated from *Me*OH (15 ml) with ether (100 ml). Yield: 11.62 g (90%); m.p. 175°C (dec.); $[\alpha]^{20}_D = -29.1°$ and $[\alpha]^{20}_{546} = -35.0°$ (*c* 1, ethanol); tlc: 1, 5; gas chromatographic racemization test: *D-allo*-Thr 0.7%; *D*-Pro 0.6%. Anal. calcd. for $C_{13}H_{25}N_3O_3 \cdot HCl \cdot 0.5\,H_2O$ (316.83): C 49.28, H 8.59, N 13.27, Cl 11.19; found: C 48.85, H 8.73, N 13.14, Cl 11.45.

Z-Ser(*tBu*)-Pro-OH ⟨9–10⟩

H-Pro-OH ⟨10⟩ (46.1 g; 0.4 mol) in 1 *M* NaOH (400 ml) and dioxane (300 ml) was reacted under cooling with an ice bath with *Z*-Ser(*tBu*)-O*Su* ⟨9⟩ (78.5 g, 0.2 mol) in dioxane (100 ml). After 24 h the bulk of dioxane was evaporated, ethyl acetate and 0.2 *M* $KHSO_4$ were added and the organic layer was washed with water and dried over Na_2SO_4. The solvent was removed under reduced pressure and the product was precipitated from ether/petroleum ether and recrystallized from ethyl acetate. Yield:

63.0 g (80%); m.p. 116–118°C; $[\alpha]^{20}_D = -48.48°$ and $[\alpha]^{20}_{546} = -57.58°$; tlc: 1, 6; gas chromatographic racemization test: *D*-Ser < 0.2%, *D*-Pro 1%. Anal. calcd. for $C_{20}H_{28}N_2O_6$ (392.46): C 61.21, H 7.19, N 7.14; found: C 61.12, H 7.29, N 7.10.

Z-Ser(*tBu*)-Pro-Thr(*tBu*)-Pro-NH_2 ⟨9–12 a⟩

To a chilled solution of ⟨9–10⟩ (11.8 g, 30 mmol) in *THF* (150 ml), *TEA* (4.3 ml, 30 mmol) and ethyl chloroformate (3.1 ml, 30 mmol) were added at −20°C. After 10 min the reaction mixture was combined with a suspension of ⟨11–12 b · HCl⟩ (11.0 g, 34.7 mmol) and *TEA* (5.0 ml, 34.7 mmol) in chloroform (50 ml) at −20°C. The temperature was allowed to reach 0°C in 2 h, then the solvents were removed under reduced pressure and the residue was worked up as described for ⟨11–12 a⟩. The product was obtained by precipitation from ether with petroleum ether. Yield: 13.2 g (68%); m.p. 95–98°C; $[\alpha]^{20}_D = -48.90°$ and $[\alpha]^{20}_{546} = -58.64°$ (*c* 1, ethanol); tlc: 1, 6; amino acid analysis (24 h, 6 *M* HCl): Thr 0.97 (1), Ser 1.04 (1), Pro 1.99 (2); gas chromatographic racemization test: *D-allo*-Thr 0.4%, *D*-Ser < 0.4%, *D*-Pro 0.8%. Anal. calcd. for $C_{33}H_{51}N_5O_8$ (645.79): C 61.37, H 7.96, N 10.85; found: C 60.47, H 7.92, N 10.27.

H-Ser(*tBu*)-Pro-Thr(*tBu*)-Pro-NH_2 · HCl ⟨9–12 b · HCl⟩

⟨9–12 a⟩ (9.2 g, 14.2 mmol) was hydrogenated over Pd/C in *Me*OH-water (3 : 1 *v/v*, 280 ml), worked up as described for ⟨11–12 b · HCl⟩ and recrystallized from methanol-ether. Yield: 7.0 g (87%); m.p. 167–169°C; $[\alpha]^{20}_D = -52.9°$ and $[\alpha]^{20}_{546} = -63.5°$ (*c* 2, ethanol); tlc: 1, 2, 4, 5; amino acid analysis (24 h, 6 *M* HCl): Thr 0.94 (1), Ser 0.99 (1), Pro 2.07 (2). Anal. calcd. for $C_{25}H_{45}N_5O_6 \cdot HCl \cdot H_2O$ (566.15): C 53.03, H 8.55, N 12.37, Cl 6.26; found: C 53.08, H 8.27, N 11.95, Cl 6.10.

Z-Thr(*tBu*)-Pro-OH ⟨7–8⟩

H-Pro-OH ⟨8⟩ (13.8 g, 0.12 mol) in 1 *M* NaOH (120 ml) and dioxane (80 ml) was reacted with *Z*-Thr(*tBu*)-O*Su* ⟨7⟩ (40.6 g, 0.1 mol) in dioxane (80 ml) under addition of $NaHCO_3$ (8.4 g, 0.1 mol) and worked up after 24 h as described for ⟨9–10⟩. Homogeneous product was obtained upon recrystallization from ether-petroleum ether. Yield: 30.6 g (75%); m.p. 104–106°C; $[\alpha]^{20}_D = -44.89°$ and $[\alpha]^{20}_{546} = -54.33°$ (*c* 2, ethanol); tlc: 6, 7, 8; gas chromatographic racemization test: *D-allo*-Thr 0.4%, *D*-Pro 1.2%. Anal. calcd. for $C_{21}H_{30}N_2O_6$ (406.48): C 62.05, H 7.44, N 6.89; found: C 61.74, H 7.43, N 6.73.

Z-Thr(*tBu*)-Pro-Ser(*tBu*)-Pro-Thr(*tBu*)-Pro-NH_2 ⟨7–12 a⟩

To a solution of ⟨7–8⟩ (4.7 g, 11.5 mmol), ⟨9–12 b · HCl⟩ (6.5 g; 11.5 mmol) and HO*Bt* (1.55 g, 11.5 mmol) in *DMF* (200 ml), *TEA* (1.6 ml, 11.5 mmol) was added followed by *DCC* (2.5 g, 12 mmol) in *DMF* (50 ml) at 0°C. After 60 h at room temperature *DMF* was evaporated under reduced pressure, the residue was worked up as described for ⟨11–12 a⟩ and the product was reprecipitated from ether/petroleum ether. Yield: 9.3 g (90%); m.p. 107–113°C; $[\alpha]^{20}_D = -72.46°$ and $[\alpha]^{20}_{546} = -87.13°$ (*c* 1, ethanol); tlc: 1, 3, 5; amino acid analysis (24 h, 6 *M* HCl): Thr 2.06 (2), Ser 1.03 (1), Pro 2.91 (3); gas chromatographic racemization test: *D-allo*-Thr < 0.6%, *D*-Ser < 0.2%, *D*-Pro 0.7%. Anal. calcd. for $C_{46}H_{73}N_7O_{11}$ (900.11): C 61.38, H 8.17, N 10.90; found: C 60.15, H 7.99, N 10.55.

H-Thr(*tBu*)-Pro-Ser(*tBu*)-Pro-Thr(*tBu*)-Pro-NH_2 · HCl ⟨7–12 b · HCl⟩

⟨7–12 a⟩ (9.3 g, 10.3 mmol) was hydrogenated over Pd/C in *Me*OH-water (3 : 1, *v/v*, 280 ml) and worked up as described for ⟨11–12 b · HCl⟩. The homogeneous product was isolated by recrystallization from ethyl acetate (containing traces of *Me*OH)-ether. Yield: 6.8 g (82%); m.p. 165–170°C (dec.); $[\alpha]^{20}_D = -72.95°$ and $[\alpha]^{20}_{546} = -87.71°$ (*c* 2, ethanol); tlc: 3, 4. Anal. calcd. for $C_{38}H_{67}N_7O_9 \cdot HCl \cdot 1.5\,H_2O$ (829.47): C 55.02, H 8.63, N 11.82, Cl 4.27; found: C 54.97, H 8.69, N 11.41, Cl 4.07.

H-Thr(*tBu*)-Pro-OH ⟨5–6 b⟩

The dipeptide derivative *Z*-Thr(*tBu*)-Pro-OH ⟨5–6 a⟩ (= ⟨7–8⟩) (20 g, 50 mmol) was hydrogenated over Pd/C in *Me*OH-water (4 : 1 *v/v*, 650 ml). The reaction mixture was worked up as usual and the residue was washed with ethyl acetate. Yield: 12.4 g (91%); m.p. 147–149°C; $[\alpha]^{20}_{D} = -59.54°$ and $[\alpha]^{20}_{546} = -71.68°$ (*c* 1, MeOH); tlc: 1. Anal. calcd. for $C_{13}H_{24}N_2O_4$ (272.34): C 57.33, H 8.88, N 10.29; found: C 57.26, H 8.75, N 10.14.

Z-Thr(*tBu*)-Pro-O*Su* ⟨3–4 b⟩

To a solution of *Z*-Thr(*tBu*)-Pro-OH ⟨3–4 a⟩ (= ⟨7–8⟩) (40.6 g, 0.1 mol) and HO*SU* (11.5 g, 0.1 mol) in dioxane (300 ml) *DCC* (20.6 g, 0.1 mol) in dioxane (100 ml) was added at 0°C. After 24 h the dicyclohexylurea was filtered off and the dioxane was evaporated. The residue was distributed between ethyl acetate and 0.5 *M* $NaHCO_3$. After washing with water the organic layer was dried over Na_2SO_4 and evaporated to dryness. The product was recrystallized from 2-propanol. Yield: 40.3 g (80%); m.p. 115–117°C; $[\alpha]^{20}_{D} = -60.74°$ and $[\alpha]^{20}_{546} = -72.36°$ (*c* 1, dioxane); tlc: 5. Anal. calcd. for $C_{25}H_{33}N_3O_8$ (503.55): C 59.62, H 6.61, N 8.35; found: C 59.65, H 6.57, N 8.24.

H-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-OH ⟨3–6 b⟩

⟨5–6 b⟩ (13.6 g, 50 mmol) in 1 *M* NaOH (50 ml) and dioxane (20 ml) was reacted with *Z*-Thr(*tBu*)-Pro-O*Su* ⟨3–4 b⟩ (26.2 g, 52 mmol) in dioxane (200 ml) and $NaHCO_3$ (4.4 g, 52 mmol) in water (70 ml). After 24 h at room temperature the bulk of dioxane was evaporated, the residue was worked up as described for ⟨9–10⟩ and the resulting solid was washed with petroleum ether to yield crude *Z*-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-OH ⟨3–6 a⟩ (homogeneous according to tlc: 1, 5).

This product was hydrogenated over Pd/C in *Me*OH-water (4 : 1 *v/v*, 700 ml) and worked up in usual manner. The residue was treated with ether and filtered off. Yield: 23.6 g (87%); m.p. 159–161°C (dec.); $[\alpha]^{20}_{D} = -59.40°$ and $[\alpha]^{20}_{546} = -71.66°$ (*c* 0.6, *Me*OH); tlc: 2, 4; gas chromatographic racemization test: *D-allo*-Thr 0.4%, *D*-Pro 0.9%. Anal. calcd. for $C_{26}H_{46}N_4O_7 \cdot H_2O$ (544.68): C 57.33, H 8.88, N 10.29; found: C 57.70, H 8.90, N 10.28.

Z-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-OH ⟨1–6⟩

⟨3–6 b⟩ (16.3 g, 30 mmol) in 1 *M* NaOH (30 ml) and dioxane (50 ml) was reacted with *Z*-Thr(t*Bu*)-Pro-O*Su* ⟨1–2 b⟩ (= ⟨3–4 b⟩) (15.0 g, 30 mmol) in dioxane (100 ml) and $NaHCO_3$ (2.52 g, 30 mmol) and worked up as described for ⟨3–6 a⟩. The resulting residue was reprecipitated from ether with petroleum ether and washed with boiling hexane-cyclohexane. Yield: 19.4 g (72%); m.p. 120–122°C; $[\alpha]^{20}_{D} = -87.02°$ and $[\alpha]^{20}_{546} = -104.22°$ (*c* 1, *Me*OH); tlc: 5, 6; gas chromatographic racemization test: *D-allo*-Thr 0.2%; *D*-Pro 1.2%. Anal. calcd. for $C_{47}H_{74}N_6O_{12}$ (915.12): C 61.68, H 8.15, N 9.19; found: C 61.38, H 7.97, N 9.03.

Z-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Ser(*tBu*)-Pro-Thr(*tBu*)-Pro-NH_2 ⟨1–12 a⟩

To a chilled solution of ⟨7–12 b · HCl⟩ (1.25 g, 1.5 mmol), ⟨1–6⟩ (1.37 g, 1.5 mmol) and HO*Bt* (204 mg, 1.5 mmol) in *DMF* (13 ml), *TEA* (0.205 ml, 1.5 mmol) and *DCC* (350 mg, 1.5 mmol) in *DMF* (2 ml) were added. After 3 days at room temperature the dicyclohexylurea was filtered off and the filtrate was diluted with ethyl acetate and aqueous $KHSO_4$-solution (0.1 *M*). The organic layer was washed with water and evaporated. The residue was reprecipitated from ethyl acetate/ether/petroleum ether. Yield: 2.0 g (80%); m.p. 140–145°C; $[\alpha]^{20}_{D} = -81.37°$ and $[\alpha]^{20}_{546} = -93.63°$ (*c* 1, ethanol); tlc: 1, 3, 8. Amino acid analysis (24 h, 6 *M* HCl): Thr 5.01 (5), Ser 1.06 (1), Pro 5.94 (6); gas chromatographic racemization test: *D-allo*-Thr < 0.5%, *D*-Ser < 0.5%, *D*-Pro 0.9%. Anal. calcd. for $C_{85}H_{139}N_{13}O_{20}$ (1 663.10): C 61.38, H 8.42, N 10.95; found: C 59.86, H 8.32, N 10.38.

H-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Pro-Ser-Pro-Thr-Pro-NH_2 ⟨1–12 c⟩

(a) ⟨1–12 a⟩ (1.65 g, 0.99 mmol) was hydrogenated over Pd/C in *Me*OH-water (4 : 1 *v/v*, 250 ml) by titrimetric addition of 1 *M* HCl (*pH* 4.5). The reaction mixture was worked up in usual manner and the residue was reprecipitated from *Me*OH with ether. Yield of H-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Thr(*tBu*)-Pro-Ser(*tBu*)-Pro-Thr(*tBu*)-Pro-NH_2 · HCl, ⟨1–12 b · HCl⟩: 1.14 g (74%), m.p. 175°C (dec.); tlc: 2, 3, 10.

(b) ⟨1–12 b · HCl⟩ (615 mg, 0.39 mmol) was treated with trifluoroacetic acid (4.5 ml) containing 5% water for 2.5 h at room temperature. On addition of ether-petroleum ether (10 : 1, 90 ml) the precipitate was filtered off and reprecipitated from 2-propanol-ether and dried over KOH pellets. The crude product was chromatographed on Fractogel HW-40 S (Merck) (column 130 × 4 cm, flow rate: 50 ml/h) with 0.05 *M* ammonium acetate buffer (*pH* 4.8) as eluent. Yield: 190 mg (25% calcd. for a peptide content of 85%). The product behaved homogeneously on tlc [solvent systems: 9, 11, 12] and hplc [μ-bondapak C18 (30 × 0.4 cm) eluent: acetonitrile/0.01 *M* sodium phosphate buffer, *pH* 5.4 (14 : 86 *v/v*), flow rate 1.7 ml/min, uv-detection: 210 nm]; amino acid analysis of the acid hydrolysate (24 h, 6 *M* HCl): Thr 4.94 (5), Ser 1.05 (1), Pro 6.01 (6) (peptide content: 85% calcd. for M_r = 1 192.3); amino acid analysis of the enzymatic digest (aminopeptidase M): Thr 4.99 (5), Ser 1.01 (1), Pro 5.99 (6) (recovery: 84%; calcd. for M_r = 1 192.3).

References

[1] Zillig W., Gropp F., Henschen A., Neumann H., Palm P., Reiter W. D., Rettenberger M., Schnabel H., Yeats S. (1986) System. Appl. Microbiol. **7**: 58

[2] Janekovic D., Wunderl S., Holz I., Zillig W., Gierl A., Neumann H. (1983) Mol. Gen. Genet. **192**: 39

[3] For reviews concerning the use of antipeptide antisera see: Lerner R. A. (1982) Nature **299**: 592; Sutcliff J. G., Shinnick T. M., Green N., Lerner R. A. (1983) Science **219**: 660; Walter G. (1986) J. Immunol. Meth. **88**: 149

[4] Wünsch E., Moroder L., Nyfeler R., Kalbacher H., Gemeiner M. (1985) Biol. Chem. Hoppe-Seyler **366**: 53; Moroder L., Mourier G., Dufresne M., Bovermann G., Göhring W., Gemeiner M., Wünsch E. (1987) Biol. Chem. Hoppe-Seyler **368**: 839

[5] Krois D., Simonetti M., Wünsch E. (1989) Monatsh. Chem. **120**: 1029

[6] Krois D., Dufresne M., Neumann H., Moroder L., Wünsch E. (in preparation)

[7] Frank H., Nicholson G. J., Bayer E. (1977) J. Chromatogr. Sci. **15**: 17

[8] Wünsch E. (ed.) In: Houben-Weyl (1974) Methoden der Organischen Chemie: Synthese von Peptiden, Vol. 15. Thieme, Stuttgart

Received April 21, 1989. Accepted May 17, 1989