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ORIGINAL ARTICLE

Comparative syntheses of peptides and peptide thioesters derived from mouse and human prion proteins

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Abstract Prions are suspected as causative agents of several neuropathogenic diseases, even though the mode of their action is still not clear. A combination of chemical and recombinant syntheses can provide suitable probes for explanation of prions role in pathogenesis of neurodegenerative diseases. However, the prions contain several difficult sequences for synthesis by Fmoc/tBu approach. For that reason, the peptide thioesters as the key building blocks for chemical syntheses of proteins by native chemical ligation were employed. A scan of the mouse prion domain 93–231 was carried out in order to discover availability of derived thioesters as the suitable building blocks for a total chemical synthesis of the prion protein based probes. The synthesis on 2-chlorotritylchloride resin was utilized and after a deprotection of the samples for analysis, the peptide segments were purified and characterized. If the problems were detected during the synthesis, the segment was re-synthesized either using the special pseudoproline dipeptides or by splitting its molecule to two or three smaller segments, which were prepared easier. The protected segments, prepared correctly without any deletion and in sufficient amounts, were coupled either with EtSH after DIC/DMAP activation or with p-Ac-NH-Ph-SH using PyBOP activation to yield corresponding thioesters. In some special cases, the other techniques of thioester formation, like sulfonamide-safety catch and/or trimethylaluminium approach were utilized.

Keywords Prion protein segments · Classical synthesis · Chemical ligation synthesis · Peptide thioesters

Abbreviations

AA	Amino acid analysis
ACN	Acetonitrile
Asi	Aspartic acid imide
Boc	*tert*-Butoxycarbonyl
ClTrt	2-Chlorotrityl
DIC	*N,N′*-Diisopropylcarbodiimide
DCM	Dichloromethane
DIEA	*N,N*-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
Dmb	Dimethylbenzyl
DMF	*N,N*-Dimethylformamide
EDT	1,2-Ethanedithiol
ESI MS	Electro-spray ionization mass spectrometry
Et	Ethyl
Fmoc	[(Fluoren-1-yl-methoxy]carbonyl
HBTU	O-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HFIP	Hexafluoroisopropanol
HOBt	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
MALDI TOF	Matrix-assisted laser desorption ionization time of flight
Mpa	Mercaptopropionic acid
NMP	N-Methylpyrrolidone

The nomenclature and symbols of the amino acids follow the Recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature. Eur J Biochem (1984) 138:9–37.

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PyBOP	(Benzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate
Sec	Selenocysteine
SPPS	Solid-phase peptide synthesis
tBu	*tert*-Butyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thz	Thiazolidine-4-carboxylic acid
TIS	Tri-isopropylsilane
TMS	Trimethylsilyl

Introduction

Transmissible spongiform encephalopathies are fatal neurodegenerative diseases of the central nervous system characterized by loss of motor control, dementia, and paralysis (Cohen and Prusiner 1998; Weissman 1999; Caughey 2000). They include scrapie of sheep and goats, bovine spongiform encephalopathy of cattle, and several human diseases, such as Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker syndrome, and fatal familial insomnia (Will et al. 1996; Prusiner 1998; Bons et al. 1999). The "protein-only" hypothesis formulated by Prusiner and co-workers (Cohen and Prusiner 1998; Prusiner 1998) considers that the infectious form of the prion (PrP^{Sc} from scrapie) has an amino acid sequence identical to a normal host protein (PrP^{C}) and infection of organism with PrP^{Sc} would result in the conversion of PrP^{C} into a conformational isomer of itself resulting in PrP^{Sc} aggregation in the brain. It has been proposed that a predominantly alpha-helical structure in the PrP^{C} converts into beta-sheet rich PrP^{Sc}, especially in the region 90–145 (Cohen and Prusiner 1998; Pan et al. 1993; Huang et al. 1996; Jackson et al. 1999; Supattapone et al. 1999; Kaneko et al. 2000; Forloni et al. 1993). Recently, Legname et al. (2004) showed that a recombinant mouse PrP(89–230) with the P101L mutation folded in amyloid fibrils "in vitro" induced prion-like disease in transgenic mouse over expressing identical PrP. In addition, methods of "protein misfolding cyclic amplification" succeed in generation of infectious PrPSc "in vitro" (Castilla et al. 2005). The protein-only theory gained great support with the finding that PrP knockout mice do not propagate prion and are completely resistant to prion infection (Bueler et al. 1992). Even the mechanism of prion conversion remains unknown, most models predict an autocatalytic process (Cohen and Prusiner 1998; Weissman 1999; Prusiner 1998; Jarrett and Lansbury 1993; Come et al. 1993). However, to gain more knowledge about the structure of prions and the mechanism of their transmission, additional studies and new tools are necessary to unravel remaining uncertainties. One such a tool could be synthetic prion protein with precisely positioned fluorescent labels allowing testing of PrP^{Sc} structure by fluorescent method. However, prions posses more than 200 amino acid residues, which strongly disfavor straightforward peptide synthesis. It requires syntheses of shorter fragments, which are finally linked together. This "small building blocks approach" would also simplify the synthesis of prion protein with any modification, including fluorescent amino acid labels. The Boc/Bzl strategy previously provided prion polypeptides of up to 112 amino acids with some point mutations (Ball et al. 2001), i.e. the biggest prion chemically synthesized was the bovine PrP(90–200) with one more Lys residue.

In this work, our interest was focused on syntheses of building blocks of the C-domain of mouse prion protein MoPrP(93–231) by Fmoc/tBu strategy. Thioesters of corresponding peptide segments could play a role of the building blocks for native chemical ligation method for the total chemical synthesis of proteins (Dawson and Kent 2000; Milton et al. 1992). We intended to check this approach firstly in the synthesis of the building blocks from the human prion-derived peptide HuPrP106–126 (Fig. 1) as a model pattern, because its sequence was found to be a difficult one when using the classical Fmoc synthetic strategy (Jobling et al. 1999), which provided 9% yield only (Šebestik et al. 2006; Table 2, method A). We optimized the syntheses of various sequences with aim to find the best synthetic route for corresponding thioesters suitable for a final ligation steps. Finally, we applied these results in the syntheses of peptide thioesters from the sequence of the mouse prion domain 93–231 (MoPrP93–231) (Fig. 2).

Materials and methods

General methodologies

The solvents DCM, DMF, cleaving reagent TFA and other chemicals were purchased from Sigma-Aldrich–Fluka corporations (Prague, Czech Republic), the HPLC solvent ACN was from Scharlau, Germany, the 2-chlorotrityl chloride resin (substitution 1.2 mmol/g), Wang polystyrene resin (substitution 0.8 mmol/g), 4-sulfamylbutyryl AM resin (0.69 mmol/g), DIC, HOBt and other reagents for amino acid couplings were purchased from Merck-Novabiochem, Czech Republic and protected amino acids from Iris Biotech GMBH, Germany. The indole-3-carboxaldehyde-polystyrene resin (1.03 mmol/g; Šebestik et al. 2004) was prepared in our laboratory. Peptides were synthesized by Fmoc/tBu method (Fields and Noble 1990) either manually in plastic syringes (5–10 ml) with Teflon

(Cys) (Sec)
H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-**Ala-Ala-Ala-Ala-Gly**-**Ala-Val-Val-Gly-Gly-Leu-Gly**-OH
106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126

Fig. 1 Sequence of human prion derived peptide H-HuPrP(106–126)-OH

Fig. 2 Sequence of mouse prion domain MoPrP(93–231)

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 93    100         110         120         130         140         150
  GTHNQWNK PSKPKTNLKH VAGAAAAGAV VGGLGGYMLG SAMSRPMIHF GNDWEDRYYR

       160         170         180         190         200         210
ENMYRYPNQV YYRPVDQYSN QNNFVHDCVN ITIKQHTVTT TTKGENFTET DVKMMERVVE

       220          231
QMCVTQYQKE SQAYYDGRRS S
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filter, or by automatic solid-phase synthesizer ABI 433A (Applied Biosystems, Foster City, CA, USA). During manual synthesis, progress of coupling in peptide synthesis was monitored by Kaiser (Kaiser et al. 1970; Sarin et al. 1981), chloranil (Vojkovský 1995) or bromophenol blue tests (Krchňák et al. 1988a, b). Fmoc deprotection was monitored by precipitation of dibenzofulvene in water. An automated synthesizer used the FastMoc 0.1 mmol program (SynthAssist^{TM} version 3.1) with a double coupling: 10 eq excess of protected amino acids and HBTU coupling reagent and 20 eq excess of DIEA were used. Molecular weights of the compounds prepared were determined by mass spectrometry using either an ESI technique (Agilent 5975B MSD) from Agilent Technologies, USA, or MALDI-TOF spectra, recorded on mass spectrometer Lasermat (Finnigan, USA) with Lasermat 2000 program. For semipreparative HPLC, a Spectra Physics SP8800 pump with a TSP Chrom Jet SP4290 integrator and a Spectra 100 UV detector were used with a Vydac RP-18, 25 × 1 cm, 10 µm column (Separations Group, Hesperia, CA, USA) of flow rate 3 mL/min and detection at 220 nm using a 0–100% gradient of ACN in 0.05% aqueous TFA over 120 min unless stated otherwise. For analytical HPLC, Agilent 1200 instrument with quaternary pump, thermostat, diode array detector and reverse phase C_{18} columns were used with a flow rate of 1 mL/min, detection at 220 nm. HPLC columns and gradients are enlisted in Table 1. When necessary, the NMR spectra were recorded on a Bruker Avance I^{TM} 400 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Products were dried in a vacuum drying box (Salvis AG, Emmenbrűcke, Lucern, Switzerland) at room temperature for 16 h.

Glycine trithioorthoester hydrochloride **1**

The compound **1** was prepared according to Brask et al. (2003). Briefly, to a mixture of dry DCM (80 mL) and $AlMe_3$ (2 M heptane solution, 52 mL), EtSH (23.5 mL) was added drop wise under argon at 0°C. To this mixture, the solution of H-Gly-OtBu acetate (2.08 g; 11 mmol) in DCM (15 mL) was slowly added. The reaction mixture was left stirred over night and then poured to ice (300 mL). The reaction flask was washed with additional DCM (120 mL) and the combined organic phases were separated and washed with 1% HCl (4 × 200 mL). The aqueous solution was alkalized with solid NaOH (10.5 g) to pH 11 and then washed with DCM (3 × 200 mL), as well. The combined DCM solutions were finally dried with Na_2SO_4, separated from drying agent and evaporated to dryness. The oily residue was dissolved in 1% HCl (30 mL), the excess of HCl removed in vacuum at 0°C and the solution was lyophilized with a yield 0.8 g (27%) of the white hygroscopic solid **1**. ^{1}H-NMR (400 MHz, dmso-d6): δ 8.35 (bs, 3H, NH_3^+), δ 3.22 (s, 2H, α-CH_2), δ 2.71 (q, 6H, ^3J = 7.5 Hz, CH_2), δ 1.20 (t, 9H, ^3J = 7.5 Hz, CH_3).

The syntheses of peptides 2–5 and peptide thiosters 6–15 of HuPrP(106–126), (Fig. 1) using methods A–E (Tables 1, 2; Fig. 3)

Method A

Synthesis on Wang polystyrene resin (200 mg; substitution of the Fmoc-Gly was 0.26 mmol/g of the resin 0.052 mmol) was performed by the Fmoc/Boc strategy, using the 4 eq. excess of corresponding Fmoc-amino acids (0.21 mmol) and coupling reagents DIC (33 µL; 0.21 mmol) and HOBt (28 mg; 0.21 mmol) in DMF (2 mL), followed by deprotection with our standard mixture **M:** TFA (90%)–TIS (2.5%)–H_2O (2.5%)–EDT (5%), 4 mL.

Obtained peptides

H-[Sec^{120}]HuPrP(120–126)-OH—H-Sec-Val-Val-Gly-Gly-Leu-Gly-OH (**2**)—Yield 26 mg (65%). HPLC retention time (RT; Ecli25) was 8.0 min (95%). For $C_{25}H_{45}N_7O_8Se$ (651.25) found ESI-MS, m/z: 673.2 (M + Na^+).

Table 1 Parameters used in HPLC analysis of peptides and peptide thioesters 2–65

Code	ACN concentration (%)	Time (min)	Temperature (°C)
Disc25[a]	5–50–100	0–20–25	40
Ecli25[b]	5–50–100	0–20–25	60
Poro15a[c]	8–15–100	0–10–15	40
Poro15b[c]	5–25–100	0–10–15	40
Poro15 c[c]	5–50–100	0–10–15	40
Poro15d[c]	12–25–100	0–10–15	40
Poro18[c]	5–20–100	0–10–18	40
Poro25a[c]	20–57–100	0–20–25	40
Poro25b[c]	5–50–100	0–20–25	40
Poro40[c]	5–50–100	0–30–40	40
PoroIso[c]	5–5–100	0–10–15	40

Flow 1 mL/min of ACN in 0.05% aqueous TFA

[a] Discovery C_{18} 5 µm column, 4.6 x 150 mm (Supelco, Bellefonte, PA, USA)

[b] Eclipse XDB-C_{18} 5 µm column, 4.6 x 150 mm (Agilent Technologies, Santa Clara, CA, USA)

[c] PoroShell 120 SB-C_{18} 2.7 µm column, 4.6 x 150 mm (Agilent Technologies, Santa Clara, CA, USA)

H-[Sec^{115}]HuPrP(115–126)-OH—H-Sec-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly-OH (**3**)—Yield 5.8 mg (10%). HPLC RT (Ecli25) was 11.4 min (96%). For $C_{39}H_{68}N_{12}O_{13}Se$ (992.42) found MALDI-MS, m/z: 1,015.2 ($M + Na^+$), 1,031.2 ($M + K^+$).

H-[Cys^{120}]HuPrP(120–126)-OH—H-Cys-Val-Val-Gly-Gly-Leu-Gly-OH (**4**)—Yield 6.0 mg (16%). HPLC RT (Ecli25) was 8.5 min (94%). For $C_{25}H_{45}N_7O_8S$ (603.31) found ESI-MS, m/z: 604.2 ($M + H^+$), 626.3 ($M + Na^+$).

H-[Cys^{115}]HuPrP(115–126)-OH—H-Cys-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly-OH (**5**)—Yield 7.2 mg (13%). HPLC RT (Ecli25) was 13.8 min (97%). For $C_{39}H_{68}N_{12}O_{13}S$ (944.47) found ESI-MS, m/z: 945.3 ($M + H^+$), 967.5 ($M + Na^+$).

Method B

Synthesis on Wang polystyrene resin was carried out by method **A**. However, the Boc-Lys(Boc)-OH was coupled as the last amino acid. The cleavage of the peptides from the resin and their simultaneous thiolation were carried out by Hilvert's method (Sewing and Hilvert 2001) with a mixture of $AlMe_3$ (1 mmol; 500 µL 2 M $AlMe_3$ in heptane), EtSH (3 mmol; 222 µL) in dry DCM (5 mL) under inert conditions of argon, followed by final deprotection with our standard mixture **M**.

Obtained peptides

H-[Asi^{108}]HuPrP(106–114)-SEt—H-Lys-Thr-Asi-Met-Lys-His-Met-Ala-Gly-SEt (**6**)—Yield 7.0 mg (9%). HPLC RT (Ecli25) was 8.0 min (95%). For $C_{43}H_{73}N_{13}O_{11}S_3$ (1,043.47) found ESI-MS, m/z: 1,044.1 ($M + H^+$).

H-[ΔThr^{107}, Asi^{108}]HuPrP(106–119)-SEt—H-Lys-Asi-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-SEt (**7**)—Yield 10.0 mg (11%). HPLC RT (Ecli25) was 7.9 min (98%). For $C_{53}H_{89}N_{17}O_{14}S_3$ (1,283.59) found MALDI-

Table 2 Syntheses of the difficult sequence H-HuPrP(106–126)-OH and related subsequences

Item	Peptide	Yield[a] (%)	Method
1	H-HuPrP(106–126)-OH	<0.1 (Jobling et al. 1999)	Jobling et al. (1999)
2	H-HuPrP(106–126)-OH	9 (Šebestik et al. 2006)	Šebestik et al. (2006)
C-terminal part			
3	H-[Sec^{120}]HuPrP(120–126)-OH (**2**)	65	A
4	H-[Sec^{115}]HuPrP(115–126)-OH (**3**)	10	A
5	H-[Cys^{120}]HuPrP(120–126)-OH (**4**)	16	A
6	H-[Cys^{115}]HuPrP(115–126)-OH (**5**)	13	A
N-terminal part			
7	H-[Asi^{108}]HuPrP(106–114)-SEt (**6**)	9	B
8	H-[ΔThr^{107},Asi^{108}]HuPrP(106–119)-SEt (**7**)	11	B
9	H-HuPrP(106–114)-SEt (**8**)	12	C
10	H-HuPrP(106–119)-SEt (**9**)	10	C
11	H-HuPrP(106–114)-Mpa-OnBu (**10**)	6	D
12	H-HuPrP(106–119)-Mpa-OnBu (**11**)	2	D
13	H-HuPrP(106–114)-SEt (**12**)	30	E
14	H-HuPrP(106–119)-SEt (**13**)	6	E
Middle part			
15	H-[Cys^{115}]HuPrP(115–119)-SEt (**14**)	54	E
16	H-[Thz^{115}]HuPrP(115–119)-SEt (**15**)	52	E

Chemical reactions B–E are depicted in Fig. 2

[a] Yield after HPLC purification

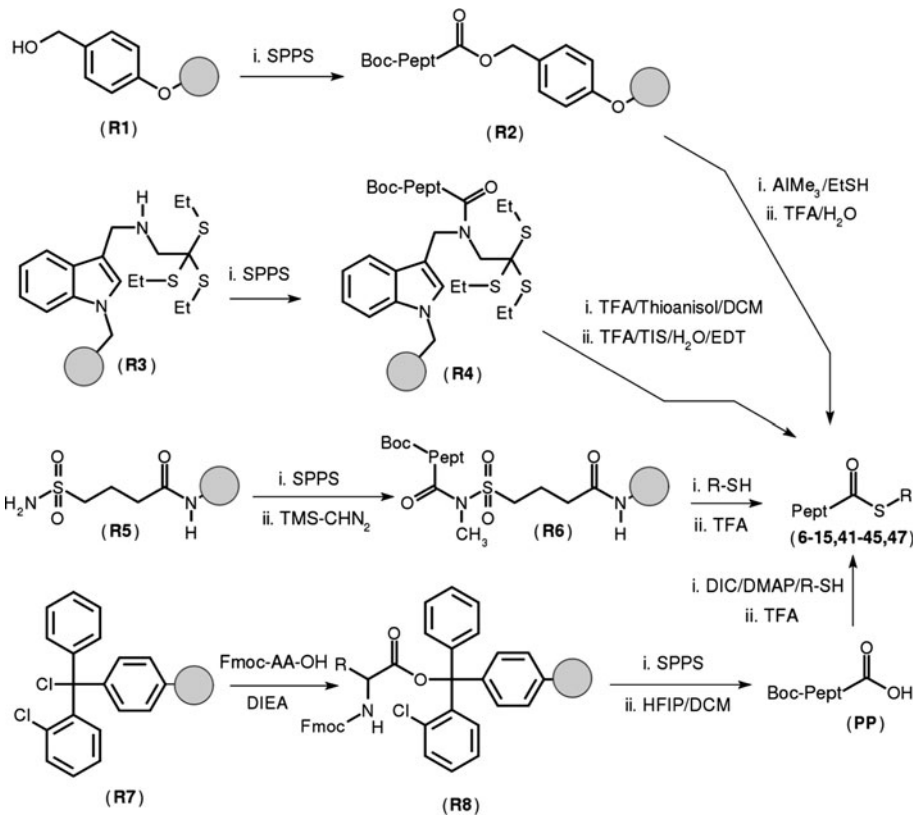

Fig. 3 The scheme of investigated syntheses of the peptide thioesters. Method **B** starts from resin (**R1**). Method **C** is using the resin (**R3**). In the method **D**, the resin (**R5**) serves as a starting compound. The most effective method **E** for short peptides begins with the 2-chlorotrityl chloride resin (**R7**)

MS, m/z: 1,283.4 (M^+), 1,305.4 ($M + Na^+$), 1,321.5 ($M + K^+$).

Method C

The trithioorthoester masking of thioester function on the C-terminal Gly residue was applied (Brask et al. 2003). An indole-3-carboxaldehyde-polystyrene resin (100 mg; substitution 1.06 mmol/g of the resin) with backbone acid labile linker was loaded with compound **1** (0.42 mmol; 111 mg) by the procedure described by Šebestik et al. (2004) with a yield 112 mg (the substitution 0.45 mmol/g was determined by elemental analysis as a content of sulfur). The peptide chain was elongated using coupling procedure described in Method **A,** with the Boc-Lys(Boc)-OH coupled as the last amino acid. The cleavage of peptide thioesters from the resin was performed by a TFA-thioanisole-DCM (2:1:20, 3 mL) mixture and the final deprotection with the standard mixture **M** (3 mL).

Obtained peptides

H-HuPrP(106–114)-SEt—H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-SEt (**8**)—Yield 9.2 mg (12%). HPLC RT (Ecli25) was 9.6 min (98%). For $C_{43}H_{76}N_{14}O_{11}S_3$ (1,060.50) found MALDI-MS, m/z: 1,060.5 (M^+).

H-HuPrP(106–119)-SEt—H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-SEt (**9**)—Yield 9.4 mg (10%). HPLC RT (Ecli25) was 11.4 min (95%). For $C_{57}H_{99}N_{19}O_{16}S_3$ (1,401.67) found MALDI-MS, m/z: 1,402.7 ($M + H^+$), 1,424.8 ($M + Na^+$).

Method D

Synthesis on 4-sulfamylbutyryl AM resin (150 mg) loaded with Fmoc-Gly-OH (0.18 mmol/g) (Backes and Ellman 1999) used the coupling procedure described in Method **A** with the Boc-Lys(Boc)-OH coupled as the last amino acid. After extensive washing with freshly dried THF, a solution of $TMS\text{-}CHN_2$ (15 mmol; 7.5 mL of 2 M solution in hexane) in dry THF (8 mL) was added to a swollen resin. After gentle stirring for 2 h, the resin was washed with dry THF (3 × 10 mL) and DCM (4 × 10 mL) and was dried under vacuum for 6 h. The peptide segments were cleaved from the resin and thiolated with Mpa-OnBu (18 μL; 0.11 mmol) in DMF (2 mL) at 55°C for 3–7 days. The protecting groups were finally removed by the standard mixture **M** (3 mL).

Obtained peptides

H-HuPrP(106–114)-Mpa-OnBu—H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Mpa-OnBu (**10**)—Yield 2.6 mg (6%). HPLC RT (Ecli25) was 10.0 min (95%). For $C_{48}H_{84}N_{14}O_{13}S_3$ (1,160.55) found MALDI-MS, m/z: 1,183.6 ($M + Na^+$).

H-HuPrP(106–119)-Mpa-OnBu—H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Mpa-OnBu (**11**)—Yield 1.1 mg (2%). HPLC RT (Ecli25) was 10.4 min (97%). For $C_{62}H_{107}N_{19}O_{18}S_3$ (1,501.72) found MALDI-MS, m/z: 1,524.8 ($M + Na^+$).

Method E

Synthesis on 2-chlorotrityl chloride resin (250 mg, the substitution 0.27 mmol/g of the resin) used the coupling procedure described in the method **A,** with the Boc-Lys(Boc)-OH coupled as the last amino acid. The protected peptides were cleaved from the resin by HFIP (1 mL) in DCM (3 mL), 5 min, and then treated with an excess of EtSH (97 µL; 1.35 mmol) in the presence of the DIC (12 µL; 75 µmol) and DMAP (0.82 mg; 6.75 µmol) mixture in DCM (3 mL). The resulting thioesters were finally deprotected with the standard reagent **M** (4 mL).

Obtained peptides

H-HuPrP(106–114)-SEt—H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-SEt (**12**)—Yield 30.7 mg (30%). HPLC RT (Ecli25) was 9.6 min (98%). For $C_{43}H_{76}N_{14}O_{11}S_3$ (1,060.50) found MALDI-MS, m/z: 1,060.5 (M^+).

H-HuPrP(106–119)-SEt—H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-SEt (**13**)—Yield 7.5 mg (6%). HPLC RT (Ecli25) was 11.5 min (96%). For $C_{57}H_{99}N_{19}O_{16}S_3$ (1,401.67) found MALDI-MS, m/z: 1,402.7 ($M + H^+$), 1,424.8 ($M + Na^+$).

H-[Cys^{115}]HuPrP(115–119)-SEt—H-Cys-Ala-Ala-Ala-Gly-SEt (**14**)—Yield 20 mg (54%). HPLC RT (Ecli25) was 6.2 min (95%). For $C_{16}H_{29}N_5O_5S_2$ (435.16) found ESI-MS, m/z: 458.1 ($M + Na^+$), 436.2 ($M + H^+$).

H-[Thz^{115}]HuPrP(115–119)-SEt—H-Thz-Ala-Ala-Ala-Gly-SEt (**15**)—Yield 19.7 mg (52%). HPLC RT (Ecli25) was 6.7 min (95%). For $C_{17}H_{29}N_5O_5S_2$ (447.16) found ESI-MS, m/z: 470.2 ($M + Na^+$).

Syntheses of peptide segments 16–39 of the mouse prion domain MoPrP (93–231, Table 3)

The peptide segments **16–39** from the mouse prion domain 93–231 (Table 3) were assembled on automated solid phase synthesizer as specified in the General methodologies (in some cases a combination with manually driven approach was chosen) using 2-chlorotritylchloride resin (200–400 mesh, 0.65 mmol/g). Fully protected peptides were cleaved from the 2-chlorotrityl resin by HFIP (1 mL)

Table 3 Availability of sequences from Mouse Prion Protein MoPrP(93–231)

Item	Sequence	Yield[a] in %	Yield[a,b] in %	Yield[a,c] in %
1	H-MoPrP(93–104)-OH (**16**)	77	–	–
2	H-[Thz^{105}]MoPrP(105–113)-OH (**17**)	62	–	–
3	H-[Thz^{114}]MoPrP(114–118)-OH (**18**)	>99	–	–
4	H-[Thz^{119}]MoPrP(119–131)-OH (**19**)	82	–	–
5	H-[Thz^{132}]MoPrP(132–141)-OH (**20**)	69	–	–
6	H-[Thz^{142}]MoPrP(142–152)-OH (**21**)	61	–	–
7	H-[Thz^{153}]MoPrP(153–164)-OH (**22**)	67	–	–
8	H-[Thz^{165}]MoPrP(165–177)-OH (**23**)	81	–	–
9	H-[Thz^{178}]MoPrP(178–194)-OH **24** (**25**) **26**	12 (32)[d,e]	58	–
10	H-[Thz^{195}]MoPrP(195–212)-OH (**28**) (**29**)	(31)[d,f] (27)[d,c]	–	–
	(**31**) (**32**) (**33**) **34**	–	(7)[d,g] (8)[d,h] (43)[d,i]	41
11	H-MoPrP(213–231)-OH **37** (**38**) **39**	10 (63)[d,j]	68	–

[a] HPLC yield of desired peptide

[b] The synthesis utilized pseudo-proline and/or DMB protection

[c] Manual synthesis from 12th residue

[d] HPLC yield of significant by-product

[e] Deletion peptide without Asn

[f] Deletion of Thr and Val

[g] Deletion of Glu-Thr

[h] Deletion of Thr-Glu-Thr

[i] MS Δm/z ca 1,259 Da is missing

[j] Formation of aspartimide

in DCM (3 mL) to obtain protected substrates for peptide thioesters preparation. The small portions of these protected peptides (mostly 50 mg) were fully deprotected by the standard mixture **M:** (TFA (90%)–TIS (2.5%)–H_2O (2.5%)–EDT (5%), and characterized with HPLC/MS as described in General methodologies.

H-MoPrP(93–104)-OH—H-Gly-Thr-His-Asn-Gln-Trp-Asn-Lys-Pro-Ser-Lys-Pro-OH (**16**)—150 mg Fmoc-Pro-O-ClTrt-resin (0.37 mmol g^{-1}). HFIP treatment yielded 292 mg of the protected peptide. By deprotection of the sample with **M** and HPLC purification, the 7.5 mg of **16** was prepared. HPLC retention time (RT) (Poro15a) was 3.69 min (crude 77%; after HPLC purification 98%). For $C_{61}H_{92}N_{20}O_{18}$ (1,392.69) found MALDI-MS, m/z: 1,393.6 ($M + H^+$), 1,415.7 ($M + Na^+$).

H-[Thz^{105}]MoPrP(105–113)-OH—H-Thz-Thr-Asn-Leu-Lys-His-Val-Ala-Gly-OH (**17**)—150 mg Fmoc-Gly-O-ClTrt-resin (0.59 mmol g^{-1}). HFIP treatment yielded 420 mg of the protected peptide that after deprotection of the sample with **M** and purification afforded peptide **17** (5.5 mg). HPLC RT (Poro15b) was 3.92 min (crude 62%; after HPLC purification 98%). For $C_{40}H_{67}N_{13}O_{12}S$ (953.48) found ESI-MS, m/z: 954.4 ($M + H^+$), 477.7 ($M + 2\ H^+/2$).

H-[Thz^{114}]MoPrP(114–118)-OH—H-Thz-Ala-Ala-Ala-Gly-OH (**18**)—150 mg Fmoc-Gly-O-ClTrt-resin (0.59 mmol g^{-1}). After treatment with HFIP, the protected peptide (300 mg) was obtained. Deprotection of the sample with **M** and purification yielded peptide **18** (4.5 mg) with HPLC RT (PoroIso) 0.5 min (crude 84%; after HPLC purification >99%). For $C_{15}H_{25}N_5O_6S$ (403.15) found ESI-MS, m/z: 404.1 ($M + H^+$), 426.1 ($M + Na^+$).

H-[Thz^{119}]MoPrP(119–131)-OH—H-Thz-Val-Val-Gly-Gly-Leu-Gly-Gly-Tyr-Met-Leu-Gly-Ser-OH (**19**) –120 mg Fmoc-Ser(OtBu)-O-ClTrt-resin (0.40 mmol g^{-1}). HFIP treatment yielded the protected peptide (262 mg). After the deprotection of the sample with **M** and purification the peptide **19** (12.5 mg) was obtained. HPLC RT (Poro15c) was 5.58 min (crude 82%; after HPLC purification 99%). For $C_{53}H_{85}N_{13}O_{16}S_2$ (1,223.57) found MALDI-MS, m/z: 1,246.4 ($M + Na^+$), 1,262.4 ($M + K^+$).

H-[Thz^{132}]MoPrP(132–141)-OH—H-Thz-Met-Ser-Arg-Pro-Met-Ile-His-Phe-Gly-OH (**20**)—150 mg Fmoc-Gly-O-ClTrt-resin (0.59 mmol g^{-1}). After the peptide-resin treatment with HFIP, the protected peptide (312 mg) was obtained. The sample was deprotected by **M** and purified with a yield of peptide **20** (6.7 mg). HPLC RT (Poro15c) was 4.63 min (crude 69%; after HPLC purification 97%). For $C_{51}H_{79}N_{15}O_{12}S_3$ (1,189.52) found MALDI-MS, m/z: 1,190.3 ($M + H^+$).

H-[Thz^{142}]MoPrP(142–152)-OH—H-Thz-Asp-Trp-Glu-Asp-Arg-Tyr-Tyr-Arg-Glu-Asn-OH (**21**)—150 mg Fmoc-Asn(Trt)-O-ClTrt-resin (0.52 mmol g^{-1}). HFIP treatment afforded 297 mg of protected peptide. After the sample deprotection by **M** and purification the 12.4 mg of peptide **21** was obtained. HPLC RT (Poro15c) was 3.66 min (crude 61%; after HPLC purification 98%). For $C_{67}H_{89}N_{19}O_{23}S$ (1,559.61) found MALDI-MS, m/z: 1,560.6 ($M + H^+$).

H-[Thz^{153}]MoPrP(153–164)-OH—H-Thz-Tyr-Arg-Tyr-Pro-Asn-Gln-Val-Tyr-Tyr-Arg-Pro-OH (**22**)—150 mg Fmoc-Pro-O-ClTrt-resin (0.37 mmol g^{-1}). After treatment of the peptide-resin with HFIP, the protected peptide (241 mg) was obtained. Deprotection by **M** and purification of its sample afforded 17.5 mg of **22**. HPLC RT (Poro15d) was 4.11 min (crude 67%; after HPLC purification 95%). For $C_{76}H_{104}N_{20}O_{19}S$ (1,632.75) found MALDI-MS, m/z: 1,633.7 ($M + H^+$).

H-[Thz^{165}]MoPrP(165–177)-OH—H-Thz-Asp-Gln-Tyr-Ser-Asn-Gln-Asn-Asn-Phe-Val-His-Asp-OH (**23**)—150 mg Fmoc-Asp(OtBu)-O-ClTrt-resin (0.32 mmol g^{-1}). HFIP treatment yielded 114 mg of the protected peptide. After deprotection of the sample by **M** and purification the 7.8 mg of **23** was obtained. HPLC RT (Poro15c) was 3.14 min (crude 81%; after HPLC purification 99%). For $C_{66}H_{90}N_{20}O_{25}S$ (1,594.61) found MALDI-MS, m/z: 1,595.6 ($M + H^+$), 1,617.6 ($M + Na^+$), 1,633.7 ($M + K^+$).

H-[Thz^{178}]MoPrP(178–194)-OH—H-Thz-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-OH (**24**)—First, the Fmoc-MoPrP180–194-O-ClTrt-Resin was assembled by automated peptide synthesis on 150 mg Fmoc-Gly-O-ClTrt-resin (0.60 mmol g^{-1}). Then, remaining amino acids were coupled by manual Fmoc/tBu synthesis with monitoring of coupling and Fmoc removal in each step. After HFIP treatment, the sample of the protected peptide was deprotected by **M** to yield peptide **24** HPLC RT (Poro15c) was 4.46 min (crude 12%). For $C_{79}H_{137}N_{23}O_{26}S$ (1,855.98) found MALDI-MS, m/z: 1,857.1 ($M + H^+$), 1,879.1 ($M + Na^+$), 1,895.1 ($M + K^+$).

H-[Thz^{178},ΔAsn^{180}]MoPrP(178–194)-OH—H-Thz-Val-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-OH (**25**)—By-product of synthesis of the peptide **24** was isolated and characterized with Asn^{180} residue missing. HPLC RT (Poro15c) was 4.20 min (crude 32%). For $C_{75}H_{131}N_{21}O_{24}S$ (1,741.94) found MALDI-MS, m/z: 1,743.0 ($M + H^+$), 1,765.0 ($M + Na^+$), 1,781.0 ($M + K^+$).

H-[Thz^{178}]MoPrP(178–194)-OH—H-Thz-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-OH (**26**)—To increase the yield of protected peptide corresponding to sequence **24,** the synthesis was carried out with pseudoproline dipeptides: Fmoc-Ile-Thr($\psi^{Me,Me}$pro)-OH and Fmoc-Val-Thr($\psi^{Me,Me}$pro)-OH. The peptide Fmoc-[Thr($\psi^{Me,Me}$pro)^{182,189}]MoPrP180–194-OClTrt-resin was assembled by automated peptide synthesis on 150 mg Fmoc-Gly-O-ClTrt-resin (0.60 mmol g^{-1}). The remaining four amino acids were coupled manually with a monitoring of the coupling and N^{α}-deprotection after each step. HFIP treatment yielded 622 mg of protected peptide. The sample

was deprotected by **M** and free peptide **26** purified. HPLC RT (Poro15c) was 4.45 min (crude 58%). For $C_{79}H_{137}N_{23}O_{26}S$ (1,855.98) found MALDI-MS, m/z: 1,857.2 ($M + H^+$), 1,879.2 ($M + Na^+$), 1,895.2 ($M + K^+$).

H-[Thz^{195}]MoPrP(195–212)-OH—H-Thz-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**27**)—Synthesis was carried analogously to **24**, Fmoc-MoPrP198–212-OClTrt-resin was built automatically and the remaining part by manual synthesis. However, desired product was not found in HPLC of crude mixture.

H-[Thz^{195},ΔThr^{198}, ΔVal^{202}]MoPrP(195–212)-OH—H-Thz-Asn-Phe-Glu-Thr-Asp-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**28**)—By-product of **27**. HPLC RT (Poro15c) was 6.81 min (crude 31%). For $C_{82}H_{132}N_{22}O_{28}S_4$ (2,000.85) found MALDI-MS, m/z: 2,002.1 ($M + H^+$).

H-[Thz^{195},ΔAsn^{196}]MoPrP(195–212)-OH—H-Thz-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**29**)—By-product of **27** HPLC RT (Poro15c) was 7.24 min (crude 27%). For $C_{87}H_{142}N_{22}O_{29}S_4$ (2,086.92) found MALDI-MS, m/z: 2,087.7 ($M + H^+$).

H-[Thz^{195}]MoPrP(195–212)-OH—H-Thz-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**30**)—Automatically prepared Fmoc-[Thr($\psi^{Me,Me}$pro)^{200}]MoPrP198–212-OClTrt-Resin was extended by manual synthesis. However, the synthesis did not provide desired peptide according to HPLC (Poro 25a).

H-[Thz^{195}, ΔGlu-Thr$^{198-199}$]MoPrP(195–212)-OH—H-Thz-Asn-Phe-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**31**)—By-product of **30**. HPLC RT (Poro25a) was 8.47 min (crude 7.1%). For $C_{82}H_{134}N_{22}O_{26}S_4$ (1,970.87) found MALDI-MS, m/z: 1,971.6 ($M + H^+$). Sequence was determined by Edman degradation.

H-[Thz^{195},ΔThr-Glu-Thr$^{198-200}$]MoPrP(195–212)-OH—H-Thz-Asn-Phe-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**32**)—By-product of **30**. HPLC RT (Poro25a) was 8.63 min (crude 8.4%). For $C_{78}H_{127}N_{21}O_{24}S_4$ (1,869.82), found MALDI-MS, m/z: 1,870.5 ($M + H^+$). Sequence was determined by Edman degradation.

Compound 33. By-product of **30**. HPLC RT (Poro25a) was 8.87 min (Crude 43%). Found MALDI-MS, m/z: 1,009.3.

H-[Thz^{195}]MoPrP(195–212)-OH—H-Thz-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**34**)—120 mg Fmoc-Met-O-ClTrt-resin (0.42 mmol g^{-1}). Automatically built Fmoc-MoPrP201–212-OClTrt-resin was extended by manual synthesis, which used Fmoc-Glu(OtBu)-Thr($\psi^{Me,Me}$pro)-OH in the first coupling of this extension to the peptide sequence desired. HFIP treatment resulted in protected peptide (189 mg). Its sample was deprotected by **M** to yield peptide **34**. HPLC RT (Poro25b) was 15.7 min (crude 41%). For $C_{91}H_{148}N_{24}O_{31}S_4$ (2,200.96) found MALDI-MS, m/z: 2,204.2 ($M + 3H^+$).

Intermediates

H-MoPrP(201–212)-OH—H-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**35**)—HPLC RT (Poro25b) was 12.1 min (59%). For $C_{61}H_{107}N_{17}O_{20}S_3$ (1,493.70) found MALDI-MS, m/z: 1,494.70 ($M + H^+$), 1,516.7 ($M + Na^+$).

H-MoPrP(198–212)-OH—H-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-OH (**36**)—HPLC RT (Poro25b) was 13.1 min (44%). For $C_{74}H_{128}N_{20}O_{27}S_3$ (1,824.84) found MALDI-MS, m/z: 1,825.8 ($M + H^+$), 1,847.8 ($M + Na^+$).

H-MoPrP(213–231)-OH—H-Cys-Val-Thr-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-Ala-Tyr-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-OH (**37**)—150 mg Fmoc-Ser(tBu)-O-ClTrt-resin (0.32 mmol g^{-1}). After direct treatment of the assembled protected peptide on the resin with **M** and HPLC purification the peptide **37** was obtained (25 mg). HPLC RT (Poro18) was 9.15 min (crude 10%; after HPLC purification 98%). For $C_{95}H_{145}N_{29}O_{34}S$ (2,268.02) found MALDI-MS, m/z: 2,269.0 ($M + H^+$), 2,291.0 ($M + Na^+$), 2,307.0 ($M + K^+$).

H-[Asi^{226}]MoPrP(213–231)-OH—H-Cys-Val-Thr-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-Ala-Tyr-Tyr-Asi-Gly-Arg-Arg-Ser-Ser-OH (**38**)—By-product of **37**. HPLC RT (Poro18) was 9.0 min (crude 63%; after HPLC purification 98%). For $C_{95}H_{143}N_{29}O_{33}S$ (2,251.40) found MALDI-MS, m/z: 2,251.1 (M^+).

H-MoPrP(213–231)-OH—H-Cys-Val-Thr-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-Ala-Tyr-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-OH (**39**)—With aim to increase the yield of the peptide **37**, the Fmoc-Asp(OtBu)-(Dmb)Gly-OH was used in automated peptide synthesis. HPLC RT (Poro18) was 9.15 min (crude 68%; after HPLC purification 98%). For $C_{95}H_{145}N_{29}O_{34}S$ (2,268.02) found MALDI-MS, m/z: 2,269.0 ($M + H^+$), 2,291.0 ($M + Na^+$), 2,307.0 ($M + K^+$).

Synthesis of thioesters 40–65 from MoPrP(93–231) sequence

Method Ea

Protected peptide (2 mg) was added to EtSH (20 eq), DIC (1.1 eq), DMAP (0.1 eq) in DCM 50 µl. After 2 h, reaction mixture was evaporated to dryness and analyzed by HPLC with subsequent MS analyzes. If necessary, the reaction time was extended up to 24 h. Deprotection of resulting thioesters was carried out with the reagent **M**.

Obtained thioesters

H-[Asp(S-Et)^{96,99}]MoPrP(93–104)-S-Et—H-Gly-Thr-His-Asp(S-Et)-Gln-Trp-Asp(S-Et)-Lys-Pro-Ser-Lys-Pro-S-Et (**40**)—HPLC RT (Poro25b) was 11.1 min (60%). For $C_{67}H_{102}N_{18}O_{17}S_3$ (1,526.68) found MALDI-MS, m/z: 1,527.6 ($M + H^+$), 1,549.7 ($M + Na^+$), 1,565.8 ($M + K^+$).

H-[Thz^{105}]MoPrP(105–113)-S-Et—H-Thz-Thr-Asn-Leu-Lys-His-Val-Ala-Gly-S-Et (**41**)—HPLC RT (Disc25) was 9.6 min (22%). For $C_{42}H_{71}N_{13}O_{11}S_2$ (997.48) found ESI-MS, m/z: 998.5 ($M + H^+$), 1,020.6 ($M + Na^+$).

H-[Thz^{114}]MoPrP(114–118)-S-Et—H-Thz-Ala-Ala-Ala-Gly-S-Et (**42**)—HPLC RT (Poro25b) was 7.04 min (89%). For $C_{17}H_{29}N_5O_5S_2$ (447.16) found ESI-MS, m/z: 470.2 ($M + Na^+$).

H-[Thz^{119}]MoPrP(119–131)-S-Et—H-Thz-Val-Val-Gly-Gly-Leu-Gly-Gly-Tyr-Met-Leu-Gly-Ser-S-Et (**43**)—HPLC RT (Poro40) was 8.4 min (20%). For $C_{55}H_{89}N_{13}O_{15}S_3$ (1,267.58) found ESI-MS, m/z: 1,268.6 ($M + H^+$), 1,290.6 ($M + Na^+$).

H-[Thz^{132}]MoPrP(132–141)-S-Et—H-Thz-Met-Ser-Arg-Pro-Met-Ile-His-Phe-Gly-S-Et (**44**)—HPLC RT (Poro25b) was 16.6 min (47%). For $C_{53}H_{83}N_{15}O_{11}S_4$ (1,233.53) found MALDI-MS, m/z: 1,235.3 ($M + 2H^+$).

H-[Thz^{142}]MoPrP(142–152)-S-Et—H-Thz-Asp-Trp-Glu-Asp-Arg-Tyr-Tyr-Arg-Glu-Asn-S-Et (**45**)—HPLC RT (Poro25b) was 12.0 min (43%). For $C_{69}H_{93}N_{19}O_{22}S_2$ (1,603.62) found MALDI-MS, m/z: 1,604.5 ($M + H^+$).

H-[Thz^{153},Asp(S-Et)^{158}]MoPrP(153–164)-S-Et—H-Thz-Tyr-Arg-Tyr-Pro-Asp(S-Et)-Gln-Val-Tyr-Tyr-Arg-Pro-S-Et (**46**)—HPLC RT (Poro25b) was 14.0 min (66%). For $C_{80}H_{111}N_{19}O_{18}S_3$ (1,721.75) found ESI-MS, m/z: 1,783.6 ($M + Na^+ K^+$).

H-[Thz^{165}]MoPrP(165–177)-S-Et—H-Thz-Asp-Gln-Tyr-Ser-Asn-Gln-Asn-Asn-Phe-Val-His-Asp-S-Et (**47**)—HPLC RT (Disc25) was 7.6 min (5%). For $C_{68}H_{94}N_{20}O_{24}S_2$ (1,638.62) found MALDI-MS, m/z: 1,639.4 ($M + H^+$).

Method Eb

Protected peptide (2 mg) was added to PyBOP (3 eq) and DIEA (4 eq) in 50 uL of DCM. After 2 min stirring, EtSH (3 eq) was added. Work-up was according to the method **Ea**.

Obtained peptides

H-[Thz^{178}]MoPrP(178–194)-S-Et—H-Thz-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-S-Et (**48**)—Peptide thioester desired was not identified by HPLC analysis (Poro 25b).

H-[Thz^{178}]MoPrP(178–194)-pyrrolidine—H-Thz-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-pyrrolidine (**49**)—By product of **48**. HPLC RT (Poro25b) was 9.1 min (51%). For $C_{83}H_{144}N_{24}O_{25}S$ (1,909.05) found MALDI-MS, m/z: 1,909.8 (M+), 1,932.0 ($M + Na^+$).

H-[Thz^{195}]MoPrP(195–212)-S-Et (50)—H-Thz-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-S-Et—Peptide thioester was not identified by HPLC analysis (Poro 25b).

H-[Thz^{195}]MoPrP(195–212)-pyrrolidine—H-Thz-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-pyrrolidine (**51**)—By product of **50**. HPLC RT (Poro25b) was 16.0 min (60%). For $C_{95}H_{155}N_{25}O_{30}S_4$ (2,254.03) found MALDI-MS, m/z: 2,252.9 ($M - H^+$).

Method Ec

This method was modified according to von Eggelkraut-Gottanka et al. (2003) method. Corresponding peptide (2 mg) was added to PyBOP (3 eq), DIEA (5 eq) in 50 µl DCM. After 2 min stirring, *p*-acetamidothiophenol (3 eq) was added. After 2 h, reaction mixture was processed in accordance with the procedure **Ea**.

Obtained peptides

H-MoPrP(93–104)-S-Ph-NHCOCH$_3$—H-Gly-Thr-His-Asn-Gln-Trp-Asn-Lys-Pro-Ser-Lys-Pro-S-Ph-NHCOCH$_3$ (**52**)—HPLC RT (Poro25b) was 9.32 min (68%). For $C_{69}H_{99}N_{21}O_{18}S$ (1,541.72) found MALDI-MS, m/z: 1,542.6 ($M + H^+$), 1,564.7 ($M + Na^+$), 1,580.7 ($M + K^+$).

H-[Thz^{114}]MoPrP(114–118)-S-Ph-NHCOCH$_3$—H-Thz-Ala-Ala-Ala-Gly-S-Ph-NHCOCH$_3$ (**53**)—HPLC RT (Poro25b) was 8.68 min (47%). For $C_{23}H_{32}N_6O_6S_2$ (552.18) found ESI-MS, m/z: 553.2 ($M + H^+$).

H-[Thz^{114}]MoPrP(114–118)-pyrrolidine—H-Thz-Ala-Ala-Ala-Gly-pyrrolidine (**54**)—By-product of **53** synthesis. HPLC RT (Poro25b) was 5.40 min (31%). For $C_{19}H_{32}N_6O_5S$ (456.22) found ESI-MS, m/z: 457.2 ($M + H^+$), 479.2 ($M + Na^+$).

H-[Thz^{119}]MoPrP(119–131)-S-Ph-NHCOCH$_3$—H-Thz-Val-Val-Gly-Gly-Leu-Gly-Gly-Tyr-Met-Leu-Gly-Ser-S-Ph-NHCOCH$_3$ (**55**)—HPLC RT (Poro25b) was 15.9 min (34%). For $C_{61}H_{92}N_{14}O_{16}S_3$ (1,372.60) found MALDI-MS, m/z: 1,395.7 ($M + Na^+$), 1,262.4 ($M + K^+$).

H-[Thz^{119}]MoPrP(119–131)-pyrrolidine—H-Thz-Val-Val-Gly-Gly-Leu-Gly-Gly-Tyr-Met-Leu-Gly-Ser-pyrrolidine (**56**)—By-product of **55**. HPLC RT (Poro25b) was 14.6 min (25%). For $C_{57}H_{92}N_{14}O_{15}S_2$ (1,276.63) found MALDI-ESI-MS, m/z: 1,299.7 ($M + Na^+$), 1,315.7 ($M + K^+$).

H-[Thz^{142}]MoPrP(142–152)-S-Ph-NHCOCH$_3$—H-Thz-Asp-Trp-Glu-Asp-Arg-Tyr-Tyr-Arg-Glu-Asn-S-Ph-NHCOCH$_3$ (**57**)—HPLC RT (Poro25b) was 9.4 min (65%). For

$C_{75}H_{96}N_{20}O_{23}S_2$ (1,708.64) found MALDI-MS, m/z: 1,709.7 ($M + H^+$).

H-[Thz^{142}]MoPrP(142–152)-pyrrolidine—H-Thz-Asp-Trp-Glu-Asp-Arg-Tyr-Tyr-Arg-Glu-Asn-pyrrolidine (**58**)—By-product of **57**. HPLC RT (Poro25b) was 12.1 min (10%). For $C_{71}H_{96}N_{20}O_{22}S$ (1,612.67) found MALDI-MS, m/z: 1,613.6 ($M + H^+$).

H - [T h z ^{153}]MoPrP(153–164)-S-Ph-NHCOCH$_3$—H-Thz-Tyr-Arg-Tyr-Pro-Asn-Gln-Val-Tyr-Tyr-Arg-Pro-S-Ph-$NHCOCH_3$ (**59**)—HPLC RT (Poro25b) was 12.5 min (56%). For $C_{84}H_{111}N_{21}O_{19}S_2$ (1,781.78) found MALDI-MS, m/z: 1,783.1 ($M + H^+$).

H-[Thz^{165}]MoPrP(165–177)-S-Ph-NHCOCH$_3$—H-Thz-Asp-Gln-Tyr-Ser-Asn-Gln-Asn-Asn-Phe-Val-His-Asp-S-Ph-$NHCOCH_3$ (**60**)—Peptide was not identified using HPLC analysis (Poro25b).

H-[Thz^{165}]MoPrP(165–177)-pyrrolidine—H-Thz-Asp-Gln-Tyr-Ser-Asn-Gln-Asn-Asn-Phe-Val-His-Asp-pyrrolidine (**61**)—By product of **60** synthesis. HPLC RT (Poro25b) was 9.0 min (33%). For $C_{70}H_{97}N_{21}O_{24}S$ (1,647.67) found MALDI-MS, m/z: 1,648.5 ($M + H^+$), 1,671.0 ($M + Na^+$), 1,686.5 ($M + K^+$).

H-[Thz^{178}]MoPrP(178–194)-S-Ph-NHCOCH$_3$—H-Thz-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-S-Ph-$NHCOCH_3$ (**62**)—HPLC RT (Poro25b) was 10.6 min (26%). For $C_{87}H_{144}N_{24}O_{26}S_2$ (2,005.01) found MALDI-MS, m/z: 2,006.0 ($M + H^+$).

H-[Thz^{178}]MoPrP(178–194)-pyrrolidine—H-Thz-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-pyrrolidine (**63**)—By product of **62** synthesis. HPLC RT (Poro25b) was 9.1 min (19%). For $C_{83}H_{144}N_{24}O_{25}S$ (1,909.05) found MALDI-MS, m/z: 1,909.9 ($M + H^+$).

H-[Thz^{195}]MoPrP(195–212)-S-Ph-NHCOCH$_3$—H-Thz-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-S-Ph-$NHCOCH_3$ (**64**)—HPLC RT (Poro25b) was 15.1 min (23%). For $C_{99}H_{155}N_{25}O_{31}S_5$ (2,349.99) found MALDI-MS, m/z: 2,352.0 ($M + 2+$), 2,372.9 ($M + Na^+$).

H-[Thz^{195}]MoPrP(195–212)-pyrrolidine—H-Thz-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-pyrrolidine (**65**)—By product of **64** synthesis. HPLC RT (Poro25b) was 12.9 min (18%). For $C_{95}H_{155}N_{25}O_{30}S_4$ (2,254.03) found MALDI-MS, m/z: 2,255.0 ($M + H^+$), 2,276.9 ($M + Na^+$).

Results and discussion

To increase the overall yield of the HuPrP106–126 (Fig. 1), we decided to check the chemical ligation strategy for its synthesis. Therefore, this sequence was split to smaller fragments (Table 2), which were subjected to thioesters formation for their subsequent ligation (Dawson et al. 1994) and desulfurization (Wan and Danishefsky 2007) procedure. Two splitting sites in the molecule, between Gly^{114}-Ala^{115} and Gly^{119}-Ala^{120} residues, were designed (chosen) to avoid racemization in corresponding peptide thioesters with C-terminal Gly residue. An introduction of the retro synthetically functional group was applied on the N-terminal Ala^{120} residue, so that the Ala to Cys, resp. to Sec transformation was carried out (Wan and Danishefsky 2007). However, the syntheses of the segments with 12 (the items **4** and **5**) and 14 (the items **8**, **10**, **12**, **14**) residues, due to low yields, were non-competitive to the divergent Fmoc approach described (Šebestik et al. 2006; Table 2, method A). Only the syntheses of selenocysteine derivative in the C-terminus (120–126, item **3**), and N-terminal peptide thioester (106–114, item **13**) on 2-chlorotrityl chloride resin (von Eggelkraut-Gottanka et al. 2003) were found to be competitive. The limited availability of the difficult peptide segments due to their low yields led us to design the shorter ones of the central part (115–119) using the Kent's modular approach (Bang and Kent 2004), in which the both splitting sites were used in one synthetic scheme. Therefore, the middle part of the peptide was prepared according to adapted version of von Eggelkraut-Gottanka et al. (2003) with a protection of the N-terminal Cys as thiazolidine-4-carboxylic acid. This method provided two peptides (items **15** and **16**) in very competitive yields to divergent peptide synthesis (item **2**). From the yields of the items **3**, **11**, and **14**, the synthesis of N-terminal peptide thioester (item **3**) could be viewed as the yield-limiting step for the synthesis of the above peptide.

In general, our study on preparation of the peptide thioester segments for the HuPrP106–126 synthesis by chemical ligation have found the adopted version of von Eggelkraut-Gottanka et al. (2003) strategy as the method of choice because of the presence of the C-terminal Gly residue in HuPrP106–126.

Consequently, above method was employed for a scan of the mouse prion domain 93–231 (Fig. 2). The first step included the sequences assembling from the corresponding Fmoc amino acids on the 2-chlorotrityl resin as described in the method E with the exception of attachment of the last amino acid in the given sequence with Boc protection. These sequences were selected as follows: H-MoPrP(93–104)-OH, H-[Thz^{105}]MoPrP(105–113)-OH, H-[Thz^{114}]MoPrP(114–118)-OH, H-[Thz^{119}]MoPrP(119–131)-OH, H-[Thz^{132}]MoPrP(132–141)-OH, H-[Thz^{142}]MoPrP(142–152)-OH, H-[Thz^{153}]MoPrP(153–164)-OH, H-[Thz^{165}]MoPrP(165–177)-OH, H-[Thz^{178}]MoPrP(178–194)-OH, H-[Thz^{195}]MoPrP(195–212)-OH and H-MoPrP(213–231)-OH. A usage of Thz residues introduced several mutations into prion protein: Lys^{105}Thz, Ala^{114}Thz, Ala^{119}Thz, Ala^{132}Thz, Asn^{142}Thz, Met^{153}Thz, Val^{165}Thz, and Glu^{195}Thz. Danishefsky's reduction (Wan and Danishefsky 2007) can remove

the Ala mutations (Ala^{114}Thz, Ala^{119}Thz, and Ala^{132}Thz). Thus only following mutations will remain: Lys^{105}Ala, Asn^{142}Ala, Met^{153}Ala, Val^{165}Ala, and Glu^{195}Ala. From synthetic point of view, these mutations will facilitate the protein synthesis. Since Glu^{195}Ala is very close to Asn^{196}, which glycosylation has no effect on biological properties of prion protein (Wollscheid et al. 2009), it can be speculated that from structural point of view the mutation cannot influence the conformation of prion protein, too. This can be confirmed by database search (GPCRDB: Swiss-Prot entry 2011) where the position 195 can contain both acidic Glu residue and basic Lys residue. Site 104 is involved in biologically active mutation: Pro^{104}Leu where the secondary structure breaking Pro is replaced with Leu (Spielhaupter and Schaetzl 2001). Thus, we can also suggest that Lys^{105}Ala mutation is not sensitive for overall structure changes. For sites 142, 153, and 165, there is no evidence for influence of these mutations on prion structure or function.

In the second step, the small portions of the protected peptide-resins were treated with a mixture **M** and cleaved peptides analyzed by HPLC/MS. HPLC yields at 220 nm were used as a criterion of the sequence availability (Table 3). The sequences shorter than 13 amino acids were available in a moderate (items **1**, **2**, **5**–**7**) to excellent (items **3**, **4**, **8**) purity when an automated peptide synthesis was employed. The moderate yields were probably caused by inappropriate sequence length: 9–11 amino acid residues (Hagenmaier 1970). The occurrence of deletion peptides in the sequences longer than 15 residues (items **9**–**11**) demanded employment of the special dipeptides, which prevent an undesirable hydrogen bond formation during the synthesis: either an amide bond protection with Dmb group (Zahariev et al. 2005) in the H-MoPrP(213–231)-OH preparation (item **11**) or pseudo-proline masking of Ser and Thr residues (White et al. 2004). The synthesis of the latter peptide without a usage of Fmoc-Asp-(Dmb)Gly-OH (Cardona et al. 2008) mostly provided corresponding aspartimide.

In the case of H-[Thz^{178}]MoPrP(178–194)-OH, an introduction of pseudo-proline Fmoc-Ile-Thr(ψMe,Mepro)-OH at positions 181–182 and 188–189, respectively, improved the HPLC yield nearly 5 times (item **9**). Since the automated synthesis of the sequence H-[Thz^{195}]MoPrP(195–212)-OH (item **10**) has failed, this segment was finally prepared manually, by continuation from its sequence 201–212, prepared on the synthesizer.

Sufficient availability of corresponding peptide segments opened a venue for syntheses of their thioesters (Table 4) with the exception of the last sequence H-MoPrP(213–231)-OH that was prepared as a free acid, because subsequent ligation was not necessary. The fully protected peptide segments were converted to thioesters either by an adopted version of von Eggelkraut-Gottanka et al. (2003) using DIC as a coupling agent (Table 4, column DIC/EtSH, Ea) or by the genuine version using PyBOP as a coupling agent and p-acetamidothiophenol as a thiolic source (Table 4, the last column, Ec).

The DIC coupling with poor nucleophilic EtSH require prolongated time for conversion of corresponding peptide to the peptide thioesters. During this extensive thiolic treatment a vulnerable group such as Asn and Asp(OtBu)

Table 4 Conversion of protected peptides to corresponding unprotected thioesters

Item	Sequence	Yield[a] (%)		
		DIC EtSH *Ea*	PyBOP EtSH *Eb*	PyBOP HS-Ph-NHCOCH$_3$ *Ec*
1	H-MoPrP(93–104)-R (**40**), **52**	(60)[b,c]	–	68
2	H-[Thz^{105}]MoPrP(105–113)-R **41**	22	–	–
3	H-[Thz^{114}]MoPrP(114–118)-R **42**, **53** (**54**)	89	–	47 (31)[b,d]
4	H-[Thz^{119}]MoPrP(119–131)-R **43**, **55** (**56**)	20	–	34 (25)[b,d]
5	H-[Thz^{132}]MoPrP(132–141)-R **44**	47	–	–
6	H-[Thz^{142}]MoPrP(142–152)-R **45**, **57** (**58**)	43	–	65 (10)[b,d]
7	H-[Thz^{153}]MoPrP(153–164)-R (**46**) **59**	(66)[b,c]	–	56
8	H-[Thz^{165}]MoPrP(165–177)-R **47** (**61**)	5	–	(33)[b,d]
9	H-[Thz^{178}]MoPrP(178–194)-R (**49**), **62** (**63**)	–	(51)[b,d]	26 (19)[b,d]
10	H-[Thz^{195}]MoPrP(195–212)-R (**51**), **64** (**65**)	–	(60)[b,d]	23 (18)[b,d]

Protected peptide was after thiolation treated with a mixture **M**: (TFA (90%)–TIS (2.5%)–H_2O (2.5%)–EDT (5%)

[a] HPLC yield of desired peptide

[b] HPLC yield of significant by-product

[c] Desired thioester with Asn to Asp(SEt) conversion

[d] Pyrrolidine by-product

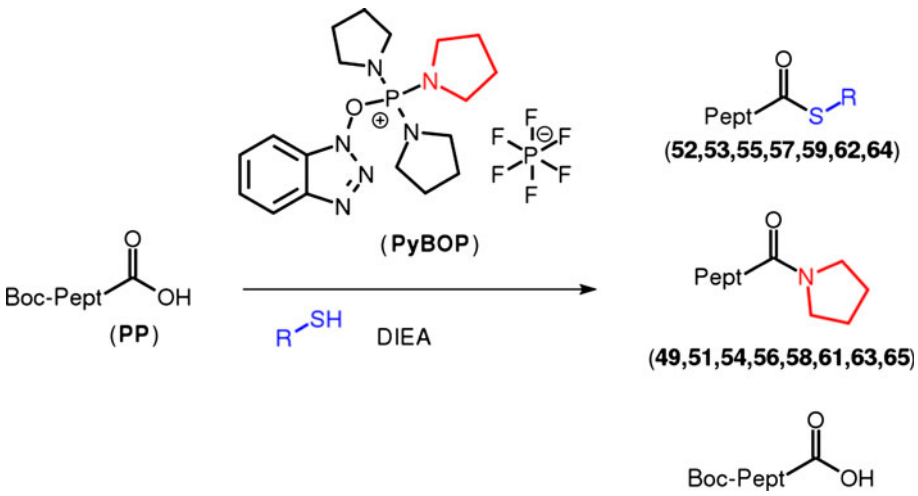

Fig. 4 Formation of by-products during PyBOP activation. Successfully prepared peptide thioesters (**52**, **53**, **55**, **57**, **59**, **62**, **64**) and obtained by-products (**49**, **51**, **54**, **56**, **58**, **61**, **63**, **65**)

can be converted to corresponding thioesters Asp(SEt) (Table 4, items **1** and **7**; compounds **40** and **46**). The longer peptides with poorer solubility did not provide corresponding thioesters at all (items **9** and **10**).

On the other hand, the genuine version of von Eggelkraut-Gottanka et al. (2003) method led to desired conversion of free acid to peptide thioesters almost in every evaluated case. The exception was synthesis of H-[Thz^{165}]MoPrP(165–177)-S-Ph(NH-Ac) (**60**) where only the corresponding H-[Thz^{165}]MoPrP(165–177)-pyrrolidone (**61**) was obtained accompanied with an unconverted free acid. A usage of PyBOP with a poorer nucleophile—EtSH—led to pyrrolidone by-products without formation of desired peptide thioester (Table 4, items **9** and **10**). In fact, the pyrrolidone derivative on C-terminus was obtained in almost all cases with PyBOP activation as either main product or by-product (Fig. 4).

The formation of H-[Thz^{165}]MoPrP(165–177)-SEt (**47**) was achieved with the adopted version of von Eggelkraut-Gottanka et al. (2003) method with very poor HPLC yield (ca 5%). It is a bottleneck of the conversion of assembled peptides to thioesters. For future, we suggest the problematic peptide should be prepared on resin directly without following conversion to thioester either by Boc/Bzl approach or another method of Fmoc/tBu one. The limitation of proposed strategy is C-terminal Asp residue vulnerable to several side reactions.

Conclusions

The comparative study carried on HuPrP106–126—the difficult sequence—revealed that an assembly of fully protected peptide with subsequent conversion to thioester is the favorable way for short thioester syntheses. This strategy was evaluated on peptide thioesters derived from C-domain of mouse PrP(93–231). With the exception of H-[Thz^{165}]MoPrP(165–177)-SEt (**47**), the peptide thioesters are easily available. The last mentioned sequence is accessible only in limited yield and poses a difficulty for future protein assembly by native chemical ligations. However, it may be synthesized by Boc/Bzl approach with decreased possibility for future modification of this site.

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