

Synthesis and Structural Characterization of Bioactive Peptide Conjugates using Thioether Linkage Approaches

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Abstract: Applications of cysteine-insertion and thioether linkage approaches to the preparation of a number of bioactive peptide conjugates are reported. Peptides containing epitopes from (i) herpes simplex virus type 1 glycoprotein D, (ii) a specific *N*-terminal β -amyloid epitope recognized by therapeutically active antibodies, and (iii) a GnRH-III peptide from sea lamprey with antitumour activity, were elongated with Cys residues and attached to a chloroacetylated tetratuftsin derivative carrier via a thioether linkage either directly, or by insertion of a spacer. The structures and molecular homogeneity of all the peptide conjugates were ascertained by HPLC, MALDI and electrospray mass spectrometry. The use of a spacer such as an oligoglycine or GFLG-tetrapeptide gave an increased yield in the conjugation reaction and enhanced reaction rates. In the formation of cysteinyl-thioether linkages, it was found that the position of flanking Cys residues markedly influenced the conjugation reaction and the formation of intermolecular epitope disulfide-dimers. *C*-terminal Cys residues gave thioether conjugates with significantly diminished epitope-dimerization, while Cys at the *N*-terminal caused rapid disulfide-dimerization, thereby preventing efficient conjugation. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Carrier molecules are frequently applied for the preparation of synthetic peptide-based antigens for vaccine and/or diagnostics development, and also for drug targeting to be used in cancer therapy [1].

Synthetic peptide-based vaccines have advantages, compared with traditional approaches, of being selective, chemically defined and safe [2,3].

In addition, large quantities of chemically purified peptide vaccines can be prepared. Short stretches of amino acid sequences are derived from the protein, containing epitopes able to elicit a protective immune response. This allows for the elimination of other epitopes, which may be responsible for inducing non-specific or undesirable stimulation of the immune system [4]. Peptide epitopes may be conjugated to an appropriate carrier molecule to increase immunogenicity. Carriers are usually chosen on the basis of size, number of reactive groups, solubility and their potential to conceal or even eliminate the anti-peptide response. The link between the peptide and carrier is chosen in such a way that the

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structure of the peptide is not perturbed or shielded by the carrier [5]. In general, peptides incorporating T_H- and B cell epitopes in multiple copies in branched architecture (topology) are better immunogens than linear oligomers [6].

In cancer therapy the attachment of an antitumour drug to a targeting molecule is a widely used combination [1]. The attachment of drug molecules to a carrier with or without a recognition moiety could lead to an increased solubility, better controlled drug-release, decreased toxicity and a prolonged therapeutic effect. Targeting molecules with high molecular mass, even without a recognition unit, are taken up by endocytosis of cancer cells [7]. Targeting molecules with a recognition unit (e.g. hormones) can bind specifically to receptors on the surface of tumour cells [8]. A wide variety of proteins are used as carriers. However, the usual carriers such as keyhole limpet hemocyanine (KLH), bovine serum albumin (BSA), tetanus toxoid (TT) and purified protein derivatives (PPD) are not, or are no longer, applicable in human therapy. This is due to immunological properties such as immunogenicity, cross-reactivity and induced tolerance [9–11]. To overcome these drawbacks, synthetic polymer and sequential polypeptide carriers have been developed and applied for the preparation of immunogens. Difficulties in the characterization (molecular mass, average degree of polymerization) of polymers and their production on an appropriate scale limit their use in human medicine. During the past decade, carriers with discrete molecular masses and defined sequences have been introduced and successful applications have been reported: Tam *et al.* have described the conjugates of a multiple antigenic peptide (MAP) containing a branching lysine core [2,12], and Tsikaris *et al.* have reported a sequential oligopeptide carrier (SOC) with the sequence Ac-(Lys-Aib-Gly)_n-OH (*n* = 2–7) which has regular secondary structure (3₁₀-helix) [13].

A new group of sequential oligopeptides has been developed in our laboratory recently [14]: oligotuftsins consisting of tandem pentapeptide repeat unit [TKPKG]_n (*n* = 2,4,6,8) based on the canine tuftsins sequence TKPK. These compounds are non-toxic, non-immunogenic and exhibit tuftsins-like biological properties, e.g. immunostimulatory activity and chemotactic activity on monocytes [15,16]. For the present study the tetratuftsins derivative was used. This carrier has eight Lys residues with free ε-NH₂ side-chain functional groups as conjugation sites.

There are several methods for coupling peptide to carrier. Amide bond, disulfide bridge and thioether bond formation are the most widely used. An amide bond between the peptide and carrier can be created by stepwise total synthesis of the conjugate (usually leading to a very poor yield of the synthesis), or by the attachment of protected or semiprotected peptide fragments. For the preparation of protected peptides, special resins and/or protecting schemes are needed; consequently the cost is higher.

Protected peptides can also be insoluble resulting in purification difficulties. Unprotected peptide derivatives can be used in the formation of disulfide or thioether bonds. The chemical and biological stability of the disulfide linkage are rather limited, especially in the case of conjugates containing asymmetrical S-S bonds [17]. In contrast, the thioether bond is a chemically and biologically stable linkage, which can be formed easily between the thiol group of a Cys residue and an haloacetylated amino group [18]. A further advantage of such a direct linkage is the non-antigenic character of the linkage compared with some of those introduced with heterobifunctional coupling agents [19].

This paper describes the preparation of several peptide conjugates containing thioether bonds. Unprotected peptides containing epitopes from glycoprotein D of herpes simplex virus type 1 (⁹LKNleADPNRFRGKDL²², ²⁷⁶SALLEDPVG²⁸⁴ [20,21]) and GnRH-III from sea lamprey (GlpHWSHDWKPG-NH₂) [22] with antitumour activity [23], were attached to the tetratuftsins derivative carrier directly or by the insertion of spacer between the peptide and the carrier. The same approach was also used for conjugation of a specific β-amyloid peptide epitope (⁴FRHDSGY¹⁰), which is recognized in a mouse model by therapeutically active antibodies against Alzheimer's disease, to modify the immunological response of a specific B-cell epitope [24,25] by the tetratuftsins carrier. The influence of the spacer on the efficacy and kinetics of the conjugation reactions was also studied, with special emphasis on disulfide formation, a side-reaction leading to peptide dimers. It was found that the positioning of either N- or C-terminal Cys residues markedly influenced the selectivity of conjugate formation.

MATERIALS AND METHODS

All amino acid derivatives were purchased from Reanal (Budapest, Hungary) or Novabiochem

(Läufelfingen, Switzerland). MBHA and NovaSyn TGR resins were obtained from Novabiochem (Läufelfingen, Switzerland). Scavengers, coupling agents and cleavage reagents were from Fluka (Buchs, Switzerland) except for PyBOP, which was obtained from Novabiochem (Läufelfingen, Switzerland). Solvents were obtained from Reanal (Budapest, Hungary).

Synthesis of Chloroacetylated Tetratuftsins Derivatives: Ac-(TKPK(CIAC)G)₄-NH₂, Ac-(TKPK(CIAC-GFLG)G)₄-NH₂

Chloroacetylated (CIAC-) tetratuftsins derivatives were synthesized manually, by SPPS, according to the Boc/Bzl strategy using MBHA resin (1.04 mmol/g capacity) as a support. The following Boc-protected amino acid derivatives were used: Boc-Gly-OH, Boc-Lys[Z(2Cl)]-OH, Boc-Lys(Fmoc)-OH, Boc-Pro-OH, Boc-Thr(Bzl)-OH, Boc-Leu-OH·H₂O and Boc-Phe-OH. The protocol was as follows: (i) deprotection with 33% TFA/DCM (2 + 20 min); (ii) DCM washing (5 × 0.5 min); (iii) neutralization with 10% DIEA/DCM (4 × 1 min); (iv) DCM washing (4 × 0.5 min); (v) coupling of 3 equiv of Boc-amino acid derivative — DCC — HOBt in DCM-DMF 4 : 1 or 1 : 4 (v/v) mixture, depending on the solubility of Boc-amino acid derivatives (60 min); (vi) DMF washing (2 × 0.5 min); (vii) DCM washing (2 × 0.5 min); (viii) ninhydrin or isatin assay. The acetylation of the *N*-terminal was carried out with acetic anhydride (1 ml) and DIEA (1 ml) in DMF. The *N*^ε-amino group of Lys residues was chloroacetylated using 5 equiv/Lys of chloroacetic acid pentachlorophenyl ester in DMF-DCM (1 : 1, v/v) after removal of the *N*^ε-Fmoc groups with a 2% DBU and 2% piperidine mixture in DMF (2 + 2 + 5 + 10 min). The coupling reaction was carried out for 2–3 h. In case of the tetratuftsins derivative with a GFLG spacer the tetrapeptides were built up on Lys side-chains first by using 12 equiv of Boc-amino acid derivative for the four Lys residues, and then the peptide was chloroacetylated. In both cases the chloroacetylated peptides were cleaved from the resin by liquid HF in the presence of *m*-cresol and *p*-thiocresol (HF-*m*-cresol-*p*-thiocresol = 10 ml: 0.5 ml: 0.5 g) for 1.5 h at 0 °C. The crude products were purified by HPLC. The purified peptides were analysed by RP-HPLC, amino acid analysis and ESI-MS (Table 1).

Synthesis of Cys-peptides Derived From Herpes Simplex Virus (HSV) gD-1: H-CLKNleADPNRFRG-KDL-NH₂ (Cys-(Nle¹¹)-(9-22)) and H-LKNleADPNRFRGKDL-NH₂ ((Nle¹¹)-(9-22)-Cys), and H-CSALLEDPVG-NH₂ (Cys-276-284)

The HSV gD-1 epitope peptides were synthesized manually by SPPS (Boc/Bzl strategy) on MBHA resin (1.1 mmol/g capacity) using the following side-chain protected amino acids Asp(OcHx), Arg(Tos), Cys[Bzl(4Me)], Glu(OcHx), Lys[Z(2Cl)] and Ser(Bzl). The syntheses were performed according to the protocol described above. The peptides were cleaved from the resin by liquid HF in the presence of *p*-cresol and 1,4-dithiothreitol (HF-*p*-cresol-DTT = 10 ml:1 g:0.1 g) for 1.5 h at 0 °C. The crude products were purified by RP-HPLC. The purified peptides were analysed by HPLC, amino acid analysis and ESI-MS (Table 1).

Synthesis of Cys-peptides of the β-amyloid-derived Epitope ⁴FRHDSGY¹⁰: H-CFRHDSGY-NH₂ (CAβ4-10), H-CGGGGFRHDSGY-NH₂ (CG₅Aβ4-10), H-FRHDSGYGGGGC-NH₂ (Aβ4-10G₅C), H-FRHDSGYC-NH₂ (Aβ4-10C),

Peptides H-CFRHDSGY-NH₂ (CAβ4-10), H-CGGGGFRHDSGY-NH₂ (CG₅Aβ4-10), H-FRHDSGYGGGGC-NH₂ (Aβ4-10G₅C) and H-FRHDSGYC-NH₂ (Aβ4-10C) were synthesized by SPPS on a NovaSyn TGR resin (0.18 mmol/g) using Fmoc/^tBu chemistry by a semi-automated Economy Peptide Synthesizer EPS-221 (ABIMED, Germany). The following side-chain protected amino acids were used: Tyr(^tBu), Ser(^tBu), Asp(O^tBu), His(Trt), Arg(Pbf) and Cys(Trt). The protocol was as follows: (i) DMF washing (3 × 1 min), (ii) deprotection with 2% DBU, 2% piperidine in DMF (15 min), (iii) DMF washing (6 × 1 min), (iv) coupling of 5 equiv of Fmoc amino acid : PyBOP : NMM in DMF (50 min), (v) DMF washing (3 × 1 min). After completion of the syntheses, the peptides were cleaved from the resin using TFA, triethylsilane and deionized water (95 : 2.5 : 2.5, v/v/v) for 2.5 h at room temperature. After cleavage, the crude product was precipitated with cold *tert*-butyl-methyl-ether, washed three times with diethyl ether and dissolved in 5% acetic acid (aqueous solution) prior to freeze-drying. The crude products were purified by RP-HPLC. Retention times, amino acid composition and MALDI-MS data are shown in Table 1.

Table 1 Characteristics of Tetratuftsins Derivatives and Cys-Peptides Prepared for Conjugation

Peptide (Code)	Yield (%)	MALDI-MS/ESI-MS ^a		Amino acid analysis ^b	HPLC ^c R _t (min)
		Calculated [M]	Found [M]		
Ac-[TKPK(ClAc)G] ₄ -NH ₂ (Ac-T20(ClAc))	68	2410.2	2410.5	Thr 3.9 (4); Gly 4.4 (4); Pro 3.7 (4); Lys 8.0 (8)	20.4
Ac-[TKPK(ClAc-GFLG)G] ₄ -NH ₂ (Ac-T20(ClAc-GFLG))	62	3908.4	3908.6	Thr 3.9 (4); Gly 12.8 (12); Pro 3.7 (4); Leu 4.2 (4); Phe 3.5 (4); Lys 7.9 (8)	27.8
H-CSALLEDPVG-NH ₂ (C276-284)	72	914.4	914.7	Asp 1.0 (1); Ser 0.9 (1); Glu 1.1 (1); Pro 0.9 (1); Gly 1.1 (1); Ala 1.0 (1); Val 0.9 (1); Leu 2.0 (2);	25.9
H-CLKNleADPNRFRGKDL-NH ₂ (C-[Nle ¹¹]-9-22)	55	1744.9	1745.2	Asp 2.9 (3); Pro 0.9 (1); Gly 1.1 (1); Ala 1.0 (1); Nle 1.0 (1); Leu 2.0 (2); Phe 0.9 (1); Lys 2.2 (2); Arg 2.0 (2)	23.5
H-LKNleADPNRFRGKDL-NH ₂ ([Nle ¹¹]-9-22-C)	61	1744.9	1745.4	Asp 3.0 (3); Pro 0.9 (1); Gly 1.1 (1); Ala 1.0 (1); Nle 1.0 (1); Leu 2.1 (2); Phe 0.9 (1); Lys 2.1 (2); Arg 1.9 (2)	23.6
H-CFRHDSGY-NH ₂ (CAβ4-10)	83	982.4	982.8 ^a	Asp 1.0 (1); Ser 1.1 (1); Gly 1.1 (1); Tyr 0.9 (1); Phe 0.9 (1); His 1.1 (1); Arg 1.0 (1)	17.1
H-FRHDSGYC-NH ₂ (Aβ4-10C)	84	982.4	983.1 ^a	Asp 1.1 (1); Ser 1.0 (1); Gly 1.1 (1); Tyr 0.9 (1); Phe 0.9 (1); His 1.0 (1); Arg 1.0 (1)	17.5
H-CGGGGFRHDSGY-NH ₂ (CG ₅ Aβ4-10)	79	1267.5	1267.5 ^a	Asp 1.0 (1); Ser 1.0 (1); Gly 6.2 (6); Tyr 0.9 (1); Phe 0.9 (1); His 1.1 (1); Arg 0.9 (1)	17.7
H-FRHDSGYGGGGC-NH ₂ (Aβ4-10G ₅ C)	81	1267.5	1267.8 ^a	Asp 1.0 (1); Ser 0.9 (1); Gly 6.3 (6); Tyr 0.9 (1); Phe 0.9 (1); His 1.0 (1); Arg 0.9 (1)	17.1
GlpHWSHDWK(H-C)PG-NH ₂ (GnRH-III(C))	60	1361.6	1362.0	Ser 0.9 (1); Glu 1.1 (1); Pro 1.0 (1); Gly 1.1 (1); His 1.9 (2) Lys 1.0 (1)	21.2
GlpHWSHDWK(Ac-C)PG-NH ₂ (GnRH-III(Ac-C))	65	1403.6	1404.2	Ser 0.9 (1); Glu 1.1 (1); Pro 0.9 (1); Gly 1.1 (1); His 2.0 (2) Lys 1.0 (1)	22.9

^a ESI-MS were performed on PE API 2000 triple quadrupole mass spectrometer, except Aβ related peptides (a) that were measured with MALDI-MS on a Bruker BiflexTM linear TOF mass spectrometer.

^b Amino acid analyses were made by using Beckman Model 6300 analyser after hydrolysis in 6 M HCL at 110 °C for 24 h.

^c RP-HPLC column: Phenomenex Synergy C₁₂ (4 μm, 80 Å, 250 × 4.6 mm); eluents: 0.1% TFA/water (A), 0.1% TFA/AcN-water 80 : 20, v/v (B); flow rate: 1 ml/min; gradient: 0 min 0% B, 5 min 0% B, 50 min 90% B.

Synthesis of Cys-GnRH-III Derivatives: GlpHWSHDWK(H-C)PG-NH₂ and GlpHWSHDWK(Ac-C)PG-NH₂

GnRH-III derivatives were prepared on an MBHA resin (1.04 mmol/g capacity) using mixed Boc and Fmoc chemistry. The first two amino acids were attached to the resin as Boc-protected derivatives. Then Fmoc-Lys(Boc)-OH was coupled to the

peptidyl-resin. After removal of the Boc protecting group from the εNH₂ of lysine residue, Boc-Cys[Bzl(4Me)]-OH was coupled to the side-chain of Lys. In the case of the N^α-Ac-Cys-peptide derivative, the Boc group was cleaved from the α-amino group of Cys[Bzl(4Me)] first and then the free amino group was acetylated with acetic anhydride and DIEA in DMF (1 : 1 : 3, v/v/v). The remaining part of the sequence was built up by the Fmoc strategy using

Fmoc-Trp-OH, Fmoc-Asp(O^tBu)-OH, Fmoc-His(Trt)-OH, and Fmoc-Ser(^tBu)-OH amino acid derivatives. Finally the pyroglutamic acid was attached to the *N*-terminus of the peptide without any protection. The Fmoc-protocol was as follows: (i) DMF washing (3 × 1 min); (ii) deprotection with 2% DBU, 2% piperidine in DMF (2 + 2 + 5 + 10 min); (iii) DMF washing (8 × 1 min); (iv) coupling with 3 equiv of Fmoc protected amino acid derivative: DIC:HOBt in DMF (60 min); (v) DMF washing (3 × 1 min); (vi) DCM washing (2 × 1 min); (vii) monitoring by ninhydrin assay. The removal of the side-chain protecting groups was carried out in two steps. First, Trt and ^tBu groups were cleaved with TFA-water-EDT (95:2.5:2.5, v/v/v) mixture (1 h at room temperature). The resin was washed with DCM (5 × 0.5 min), then it was neutralized with 10% DIEA/DCM (3 × 0.5 min), washed with DCM (4 × 0.5 min), then with ethanol (2 × 0.5 min) and dried in a desiccator overnight. The methylbenzyl (Bzl(4Me)) protecting group was cleaved simultaneously with the removal of the peptide from the resin using HF in the presence of *p*-cresol and DTT (HF-*p*-cresol-DTT = 10 ml: 1 g: 0.1 g). The crude products were purified by HPLC. The purified peptides were characterized by HPLC, amino acid analysis and ESI-MS (Table 1).

Oxidation Study of Cys-peptides

In order to study the oxidation reactions of peptides containing one cysteine residue with a free SH, the peptides were dissolved in 0.1 M Tris buffer, pH = 8.2 (at a concentration of 0.5 µg/µl). The reaction vessel was tightly closed and the mixture was stirred at 25 °C for 24 h. After each 60 min (for 8 h, then after 24 h) a 50 µl aliquot of sample was analysed

by analytical RP-HPLC, and the relative amount of reduced and oxidized Cys-peptide was calculated from the peak area (Table 2).

Conjugation of Cys-peptides to Chloroacetylated Tetrafluorinated Derivatives

Ac-[TKPK(ClAc)G]₄-NH₂ or Ac-[TKPK(ClAc-GFLG)-G]₄-NH₂ oligopeptide carriers were dissolved in 20 ml of Tris buffer (0.1 M, pH = 8.2) and the Cys-peptide in solid form was added to the solution in small portions (2–3 mg) every 10–15 min. The reaction mixture was stirred at room temperature in a tightly closed tube (20 ml volume) and the conjugation was followed by analytical RP-HPLC for 4–48 h (depending on the components). In some cases a small amount of the reaction mixture was analysed by HPLC, the peaks were separated and the mass values of the related peaks were measured by mass spectrometry. After completion of the reaction, the solution was acidified with TFA to pH 3. The conjugate containing four copies of the attached peptide (4-copy conjugate) was separated by semi-preparative or preparative RP-HPLC as described below. This procedure was used for the synthesis of all conjugates prepared.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Analytical RP-HPLC was performed on a Knauer (H. Knauer, Bad Homburg, Germany) system using a Phenomenex Synergy C₁₂ column (250 × 4.6 mm I.D.) with 4 µm silica (80 Å pore size) (Torrance, CA) or Vydac C₄ column (250 × 4.6 mm I.D.) with 5 µm silica (300 Å pore size) (Hesperia, CA) as a

Table 2 Oxidation of Cys-peptides

Time	Peptide (dimer)							
	C-[Ni ¹¹]- (9-22)	[Ni ¹¹]- (9-22)-C	CAβ4-10	CG ₅ Aβ4-10	Aβ4-10C	Aβ4-10G ₅ C	GnRH-III(C)	GnRH-III(Ac-C)
5 min	nd	nd	36% ^a	27%	2%	2%	31%	0%
1 h	22%	5%	86%	76%	3%	3%	90%	15%
2 h	43%	10%	93%	88%	4%	4%	98%	27%
4 h	68%	16%	100%	100%	nd	nd	100%	41%
6 h	90%	23%	nd	nd	10%	10%	nd	nd
8 h	100%	30%	nd	nd	nd	nd	nd	nd
24 h	nd	62%	nd	nd	15%	13%	nd	82%

nd, no data.

^a Percentage of dimer present in the reaction mixture according to area under the peak in HPLC chromatogram.

stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20 v/v)) was used at a flow rate of 1 ml/min at ambient temperature. Peaks were detected at $\lambda = 214$ nm. The samples were dissolved in eluent A. The crude products were purified on a semi preparative Phenomenex Jupiter C₁₈ column (250 × 10 mm I.D.) with 10 μm silica (300 Å pore size) (Torrance, CA) or on a Vydac C₄ column (250 × 10 mm I.D.) with 10 μm silica (300 Å pore size) (Hesperia, CA). The flow rate was 4 ml/min. The same eluents with an appropriate linear gradient were applied. The β-amyloid related peptides were purified on a preparative C₁₈ column (GROM-SIL 120 ODS-4 HE, 10 μm, 250 × 20 mm; Herrenberg-Kayh, Germany) using as mobile phases 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile–water (80:20, v/v) (eluent B) with a 10 ml/min flow rate.

Amino Acid Analysis

The amino acid composition of the peptides and conjugates was determined by amino acid analysis using a Beckman Model 6300 analyser (Fullerton, CA, USA) or OPA based amino acid analysis with precolumn derivation as described previously [26]. Prior to analysis samples were hydrolysed in 6 M HCl in an N₂ atmosphere at 110 °C for 20–24 h.

Mass Spectrometry

Positive ion electrospray ionization mass spectrometric (ESI-MS) analyses were performed on a PE API 2000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). The spray voltage was set to 4.8 kV and a 30 V orifice voltage was applied. Samples were dissolved in a methanol–water (1:1 v/v) mixture containing 0.1% acetic acid, and a 5 μl of sample was injected with a flow rate of 100 μl/min. The instrument was used in Q₁ scan mode in the range *m/z* 400–1700, with a step size of 0.3 amu and a dwell time of 0.5 ms.

MALDI-MS was carried out with a Bruker Biflex™ linear TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a 26-sample SCOUT source and video system, a nitrogen UV laser ($\lambda_{\text{max}} = 337$ nm) and a dual channel plate detector. 1 μl of the sample solution was applied to the target, and 1 μl of a freshly prepared saturated solution of 4-hydroxy- α -cyanocinnamic acid (HCCA) in acetonitrile–0.1% TFA in water (2:1 v/v) was

added. The acquisition of spectra was performed at an acceleration voltage of 20 kV, and a detector voltage of 1.9 kV.

ESI-FT-ICR MS was performed with a Bruker Daltonik Apex II FT-ICR spectrometer (Bremen, Germany) equipped with a 7.0 Tesla actively shielded superconducting magnet (Magnex, Oxford, UK), an APOLLO (Bruker Daltonik) electrospray ionization source, an API1600 ESI control unit and a UNIX based Silicon Graphics O2 workstation [27]. Acquisition of spectra was performed with the Bruker Daltonik software XMASS and corresponding programs for mass calculation, data calibration and processing. For sample preparation, methanol:acetic acid:water (50:2:48 v/v/v) was used.

RESULTS AND DISCUSSION

Synthesis of Tetratuftsins Derivatives

This work reports the synthesis of peptide conjugates, in which epitope peptides and GnRH-III were attached to the tetratuftsins derivatives through a thioether bond. Tetratuftsins derivatives considered as carriers in functional studies, consisting of four repeated units of TKPKG pentapeptide were used. This sequential oligopeptide was built up by solid phase methodology using Boc-chemistry and orthogonal protecting groups (for lysine side-chains: Z(2Cl) in position 2 and Fmoc in position 4 of the pentapeptide unit). The chloroacetyl group was attached either directly to the side-chain of Lys residues or to the N-terminal of GFLG enzyme labile spacer [28] built up on the N^ε-amino group of Lys residues at 'position 4'. The chloroacetylation using chloroacetic acid pentachlorophenyl ester was slower than that with chloroacetic anhydride, but led to a higher yield of the reaction and to cleaner crude product (data not shown). The chloroacetylated tetratuftsins derivatives were cleaved from the resin by the aid of HF in the presence of *m*-cresol and *p*-thiocresol [29]. The yields of the products Ac-[Thr-Lys-Pro-Lys(ClAc)-Gly]₄-NH₂ and Ac-[Thr-Lys-Pro-Lys(ClAc)-Gly-Phe-Leu-Gly]-Gly]₄-NH₂ were 68% and 62%, respectively (Table 1).

Synthesis of Cys-oligopeptides for Conjugation

Epitope peptides from glycoprotein D of herpes simplex virus, elongated with Cys at the

N-terminus were prepared by solid-phase peptide synthesis according to Boc-chemistry. Both H-CLKNleADPNRFRGKDL-NH₂ (Cys-[Nle¹¹]-9-22) and H-CSALLEDPVG-NH₂ (Cys-276-284) were produced with good yield. However, in the case of Cys-[Nle¹¹]-9-22, an additional peak was detected by HPLC, which was identified as a dimer. After 4 h the ratio of monomer to dimer was 70:30 in 0.1% TFA solution. This observation indicated that the peptide formed dimer at a much higher rate than conjugate. Therefore a new derivative was synthesized in which the cysteine was attached to the *C*-terminus of the epitope instead of the *N*-terminus. During the preparation and purification of this peptide (H-LKNleADPNRFRGKDL-NH₂), no oxidation product was detected.

In the same manner, the β -amyloid specific epitope peptides with or without a pentaglycine spacer (H-CFRHDSGY-NH₂, H-FRHDSGYC-NH₂, H-CGGGGGFRHDSGY-NH₂ and H-FRHDSGYGGGGGC-NH₂) were synthesized on NovaSyn TGR resin by Fmoc-chemistry, and cysteine residue as conjugation site was attached either to the *N*- or to the *C*-terminus. The orientation of the epitope in a conjugate can dramatically influence antibody recognition [30], particularly for the present A β -epitope, which is *N*-terminal to the transmembrane part of A β -peptide. With this consideration, several conjugation reactions were performed to prepare conjugates with either *N*- or *C*-terminal Cys residues. However, of these only

conjugates with *C*-terminal Cys could be prepared effectively with oligotuftsins derivatives, because of rapid dimerization of the *N*-terminal Cys peptides (Table 2).

For the synthesis of GnRH-III analogues, mixed Boc- and Fmoc-strategies were applied. The peptide was built up on an MBHA resin, and the first two amino acids were attached as Boc-protected derivatives. Fmoc-Lys(Boc)-OH was then coupled to the peptidyl-resin. Before continuing the synthesis of the GnRH-III sequence, Boc-Cys[Bzl(4Me)]-OH was attached to the side-chain of Lys after removal of its side-chain protecting group. The remaining part of the peptide was built up by Fmoc strategy. The final cleavage was carried out in two steps. Acid-labile protecting groups (Boc, ^tBu, Trt) were removed with TFA solution first, and then the peptide was cleaved from the resin with simultaneous removal of the Bzl(4Me) protecting group with liquid HF. Using this approach, peptide GlpHWSHDWK(H-C)PG-NH₂ was synthesized in fairly good yield. This GnRH-III derivative exhibited very fast dimerization and very low conjugation under the alkaline conditions employed. This might be explained by intermolecular ionic interaction between two peptides involving the β -carboxyl group of Asp and the α -amino group of Cys attached to the ϵ -amino group of Lys. To prevent this interaction, the α -amino group of Cys was blocked by acetylation. The acetylation was carried out after removal of Boc from the Boc-Cys[Bzl(4Me)] containing peptide. The acetyl

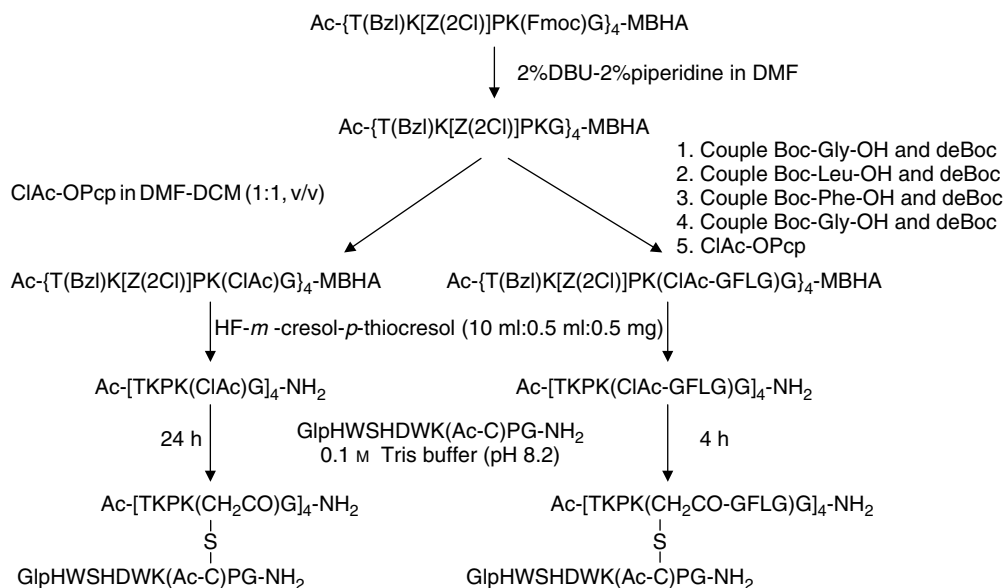


Figure 1 Conjugation of GnRH-III(Ac-C) to chloroacetylated tetra tuftsins derivatives.

derivative was used successfully in the conjugation with tetrafluoroborate derivatives (Figure 1).

Oxidation Study of Cys-peptides

Because the dimerization of Cys-peptides is a side reaction, competing with attachment to carrier, the oxidation reactions of the peptides were analysed. In all cases, the peptides were dissolved in 0.1 M Tris buffer (pH = 8.2) at $c = 0.5$ mg/ml in closed tubes and the oxidation reactions were followed by HPLC.

The Cys-[Nle¹¹]- (9-22) epitope peptide of herpes simplex virus type 1 with *N*-terminal Cys showed fast dimerization. After an 8 h reaction time, no monomer could be detected in the solution (Table 2). However, the same peptide except for the cysteine at the *C*-terminal ([Nle¹¹]- (9-22)-Cys) showed substantially less oxidation. After 2 h only 10% dimer was detected and even after 8 h less than

one-third of the peptide formed dimer by oxidation (Table 2).

The differences in the oxidation rate of the peptides containing cysteine either at the *N*- or *C*-terminus were found particularly pronounced in the case of the A β -related epitope peptides. Peptides with cysteine at the *N*-terminus with or without a spacer group (H-CFRHDSGY-NH₂ and H-CGGGGGFRHDSGY-NH₂) generally showed rapid dimerization. It was observed that even after dissolving the peptides in Tris-buffer a very high amount of dimer was detected. HPLC analysis after 5 min provided chromatograms with two peaks corresponding to the monomer and dimer (36% in the case of CA β 4-10 and 27% in the case of CG₅A β 4-10 respectively). After 2 h, only 7% of H-CFRHDSGY-NH₂ and 12% of H-CGGGGGFRHDSGY-NH₂ remained in the solution (Table 2 and Figure 2). By contrast, in the case of the

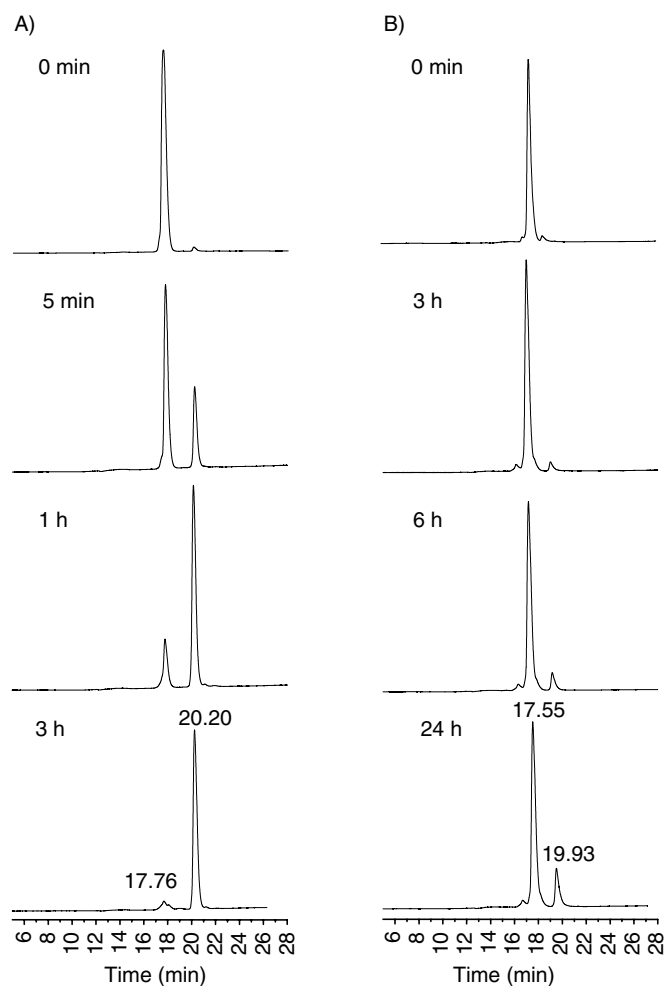


Figure 2 Oxidation of CG₅A β 4-10 (A) and A β 4-10G₅C (B) peptides followed by HPLC.

A β epitope peptides with cysteine at the C-terminal, no significant oxidation was observed. After 3 h only 4% of dimer was formed with both H-FRHDSGYC-NH₂ and H-FRHDSGYGGGGC-NH₂, and even after 24 h 15% and 13% dimer were found, respectively (Table 2 and Figure 2).

The GnRH-III peptide modified by Cys on the side-chain of the lysine residue (GlpHWSHDWK(H-C)PG-NH₂) was designed for conjugation with tetratuftsins derivatives. (It should be noted that in an earlier study the GnRH-III was attached to another carrier molecule through the ϵ -amino group of lysine [31]). However, no coupling of the peptide to the carrier could be detected due to rapid oxidation. HPLC analysis after dissolving the peptide in Tris buffer showed the presence of 31% dimer and complete dimerization after 2 h (Table 2). Interestingly, when the α -amino group of Cys was acetylated only a moderate level of dimer formation was observed, and this compound proved to be suitable for conjugation. The acetylated version of GnRH-III derivative gave 15% dimer after 1 h and 27% in 2 h in the oxidation study. Even after one day, some reduced peptide could be detected in the solution (Table 2).

Preparation of Conjugates with Tetratuftsins Derivative

Peptides containing single cysteine residues in their sequences were conjugated with chloroacetylated tetratuftsins derivatives by thioether bond formation. This study was initiated after experience in preparation of cyclopeptides and peptide conjugates with thioether bonds. In previous studies Cys-peptides were used at low concentration to prevent, or at least to decrease, disulfide bond formation between the two peptide chains. For this purpose, the chloroacetylated tetratuftsins derivative was dissolved in 1 M Tris buffer (pH 8.0–8.2) and the peptides, in solid form, were added to the carrier solutions in small portions at intervals [26, 32]. In the present study oxidation analysis of Cys-peptides was performed before conjugation to get a better insight about the role of molar excess. The attachment of the HSV epitope peptide Cys-276-284 (H-CSALLEDPVG-NH₂) to the tetratuftsins carrier (Ac-[Thr-Lys-Pro-Lys(ClAc)-Gly]₄-NH₂) was completed in a day. Two equivalents of epitope peptide for each chloroacetyl group of the carrier were needed to obtain conjugate containing four copies of the HSV peptide. However, the peptide C-[Nle¹¹](9-22) (H-CLKNleADPNRFRGKDL-NH₂), with different length and amino acid composition, derived

from another part of the same protein, did not react efficiently with the carrier. By using even 4 equivalents of the epitope peptide, conjugates containing one or two epitopes were detected as the main products after 48 h. A significant amount of peptide dimer was also present. The same peptide with the Cys at the C-terminal [Nle¹¹](9-22)-C (H-LKNleADPNRFRGKDL-NH₂) gave better results. The conjugation was relatively slow, but after 48 h it was almost complete and resulted in four epitope peptides on the carrier.

The same result was obtained with the A β related epitope peptides possessing N-terminal Cys. Both peptides (CA β 4–10 and CG₅A β 4–10) were found to oxidize very rapidly. The use of the C-terminal cysteine residue yielded successful conjugation with only slow dimerization. The conjugation was monitored by RP-HPLC on a C₄ column and MALDI-TOF (Figures 3 and 4). The mass spectra established the expected structure of the conjugates present in the mixture at different time points. The attachment

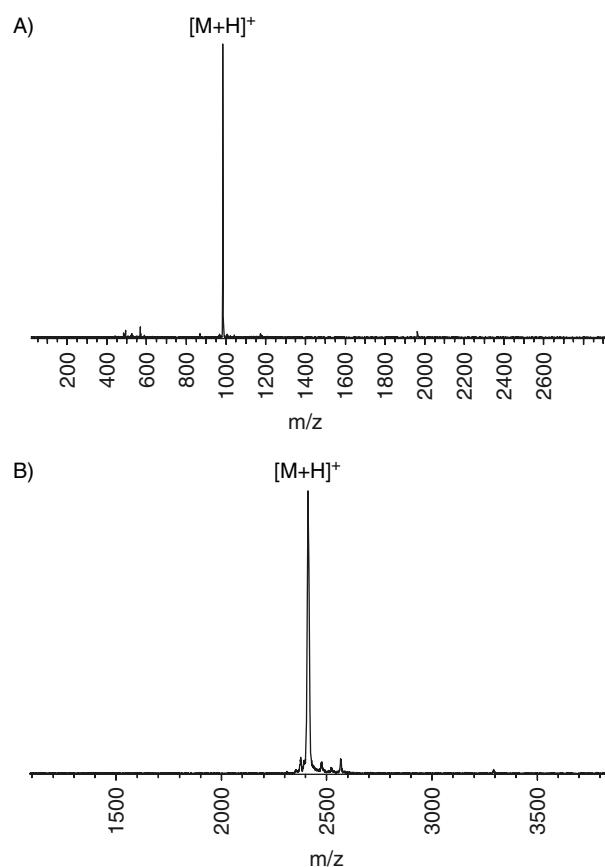


Figure 3 MALDI-TOF mass spectra of A β 4-10C epitope peptide (A) and ClAc-T20 carrier (B).

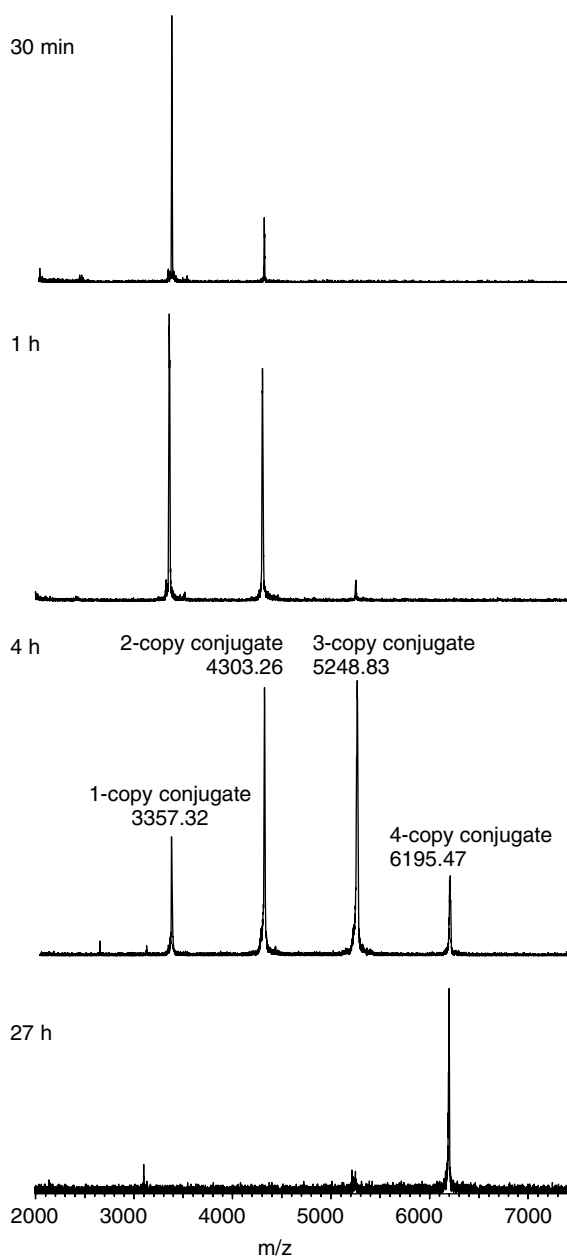


Figure 4 Conjugation of A β 4-10C epitope peptide to ClAc-T20 followed by MALDI-TOF MS.

of these peptides to Ac-[TKPK(ClAc-GFLG)G]-NH₂ carrier was fast, and the yield was high. After 3 h no peak corresponding to the unreacted carrier was present in the chromatograms. The peak corresponding to the 1-copy conjugate almost disappeared, and the intensity of the peak corresponding to the 4-copy conjugate increased significantly. After 4 h the reaction was complete. Because of the rapid conjugation and low dimerization of the peptides,

only a 0.5 equivalent excess of epitope peptide was needed.

As described above, the conjugation between Ac-[Thr-Lys-Pro-Lys(ClAc)-Gly]₄-NH₂ and the GnRH-III analogue GlpHWSHDWK(H₂N-C)PG-CNH₂ was unsuccessful. No change in the amount of chloroacetylated tetratuftsin derivative was observed, but the dimer of GnRH peptide was formed very rapidly. In contrast, the application of GnRH-III derivative with Ac-Cys at the Lys residue resulted in the 4-copy conjugate after 24 h. The conjugate was separated from the reduced and oxidized form of GnRH-III derivative only on C₄ column. As with the A β related peptides, the application of the carrier with GFLG side-chains resulted in very fast conjugation. The reaction was complete in 4 h and only a limited amount of dimer was formed (Figure 5). Only a low excess of GnRH-III derivative was therefore needed for preparation of the 4-copy conjugate.

The conjugates were characterized by RP-HPLC, amino acid analysis (data not shown) and mass spectrometry (Table 3). Mass spectra confirmed the calculated molecular mass of the conjugates and the presence of S-carboxymethyl-cysteine produced by the hydrolysis during amino acid analysis [26] from the linkage part were also detected.

CONCLUSIONS

This study reports the preparation of bioconjugates with well-defined structures that can be used as synthetic antigens or in tumour therapy. A 20-mer oligotuftsin derivative with eight Lys residues seems to be a good candidate as a carrier molecule. Using orthogonal protecting groups for lysine side-chains, the number and the position of the attached compounds can also be selectively manipulated. The conjugation *via* a thioether bond between the chloroacetylated form of oligotuftsin derivative and peptides elongated by Cys at the N- or C-terminal resulted in well-defined conjugates. Significant differences were found in the oxidation of Cys-containing peptides and also in the conjugation reaction depending on the position of Cys. The reaction of peptides with C-terminal Cys residues with tetratuftsin derivatives resulted in conjugates with good yields, while the presence of Cys at the N-terminal of the peptide leads predominantly to the formation of peptide dimers. The dimerization occurring during the conjugation procedure might be due to the structural differences and ionic interactions of the epitope peptide chains. Modification of the carrier (e.g. the presence of GFLG spacer)

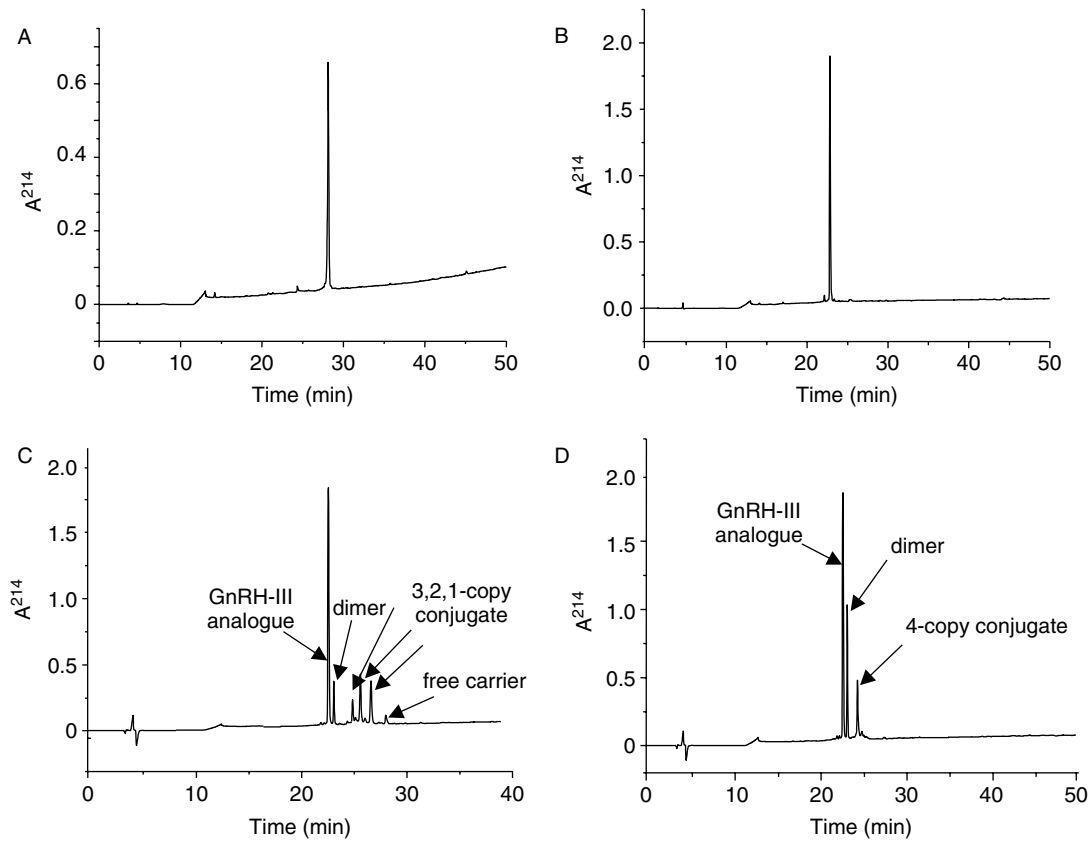


Figure 5 HPLC chromatograms of purified Ac-[TKPK(CIAc-GFLG)G]₄-NH₂ (A) and purified GlpHWSHDWK(Ac-C)PG-NH₂ (B); HPLC chromatograms of their conjugation mixture after 1 h (C) and 4 h (D).

Table 3 Characteristics of Conjugates with Oligotuftsin Carrier Molecules

Conjugate	Yield (%) ^a	MALDI-MS/ESI-MS ^b		HPLC ^c R _t (min)
		Calculated [M]	Found [M]	
Ac-T20(C276-284) ₄	81	5882.1	5882.4	27.6
Ac-T20(9-22C) ₄	78	9243.4	9243.9	24.0
Ac-T20(Aβ 4-10C) ₄	83	6194.5	6195.1	16.5
Ac-20(Aβ4-10G ₅ C) ₄	85	7334.9	7334.2	15.3
Ac-T20(Aβ4-10C-GFLG) ₄	90	7691.7	7691.8	21.1
Ac-T20(Aβ4-10G ₅ C-GFLG) ₄	91	8832.4	8832.3	20.4
Ac-T20(GnRH-III(Ac-C)) ₄	79	7883.6	7883.6	23.4
Ac-T20(GnRH-III(Ac-C)-GFLG) ₄	86	9381.4	9381.0	24.5

^a Calculated to the tetrauftsin derivatives.

^b β-amyloid related peptide-conjugates were measured by MALDI-TOF, while other conjugates were measured by ESI-MS. In these cases the deconvoluted molar mass data were calculated from the multicharged peaks.

^c β-amyloid related peptide-conjugates were measured on Vydac C₄ column (250 × 4.6 mm, 5 μm, 300 Å), while other conjugates were measured on a Phenomenex Synergy C₁₂ column (250 × 4.6 mm, 4 μm, 80 Å); Eluents: 0.1% TFA/water (A), 0.1% TFA/acetonitrile–water (80 : 20, v/v) (B); Gradient: 0 min 0%B, 5 min 0% B, 50 min 90% B; Flow rate: 1 ml/min.

or elongation of β -amyloid epitope peptide by pen-taglycine increased the speed of conjugation and consequently reduced the dimer formation.

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