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Synthesis and Antibody Recognition of Mucin 1 (MUC1)-α-Conotoxin Chimera

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Abstract: We synthesized and characterized new chimera peptides by inserting an epitope of the mucin 1 glycoprotein (MUC1) as a 'guest' sequence in the 'host' structure of α-conotoxin GI, a 13-residue peptide (ECCNPACGRHYSC) isolated from the venom of *Conus geographus*. The Pro-Asp-Thr-Arg (PDTR) sequence of MUC1 selected for these studies is highly hydrophilic and adopts a β-turn conformation. The α-conotoxin GI also contains a β-turn in the 8–12 region, which is stabilized by two disulphide bridges in positions 2–7 and 3–13. Thus, the tetramer sequence of α-conotoxin, Arg^{9}-His-Tyr-Ser^{12}, has been replaced by PDTR, comprising the minimal epitope for MUC1 specific monoclonal antibodies (MAbs) HMFG1 (PDTR) and HMFG2 (DTR). Synthesis of the chimera peptide was carried out by Fmoc strategy on (4-(2′,4′-dimethoxyphenyl-aminomethyl)phenoxy) (Rink) resin and either 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) or air oxidation was applied for the formation of the first Cys^{3}–Cys^{13} or Cys^{2}–Cys^{7} disulphide bridge, respectively. For the second disulphide bridge, three different oxidation procedures (iodine in acetic acid, 10% DMSO/1 M HCl or tallium trifluoroacetate ($Tl(tfa)_3$) in TFA) were utilized. The HPLC purified peptides were characterized by electrospray mass spectrometry (ES-MS) and amino acid analysis. The CD spectra of the bicyclic MUC1-α-[Tyr^{1}]-conotoxin chimera peptide showed partially ordered conformation with turn character. In antibody binding studies, the RIA data showed that both the linear and the bicyclic forms of MUC1-α-[Tyr^{1}]-conotoxin chimera were recognized by MAb HMFG1 specific for PDTR sequence, while no binding was observed between MAb HMFG2 and various forms of the chimera. MAb HMFG1, using synthetic epitope conjugates or native MUC1 as target antigens, recognizes the PDTR motif more efficiently in the linear than in the bicyclic compound, but no reactivity was found with the monocyclic forms of MUC1-α-[Tyr^{1}]-conotoxin chimera, underlining the importance of certain conformers stabilized by double cyclization. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: mucin 1 epitope; α-conotoxin chimera; synthesis of cyclic peptides; solution conformation; antibody binding; synthetic antigen

Abbreviations: Abbreviations of amino acids follow the revised recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, entitled 'Nomenclature and Symbolism for Amino Acids and Peptides'. *Eur. J. Biochem.* 1984; **138**: 9–37. All amino acids except glycine are of the L-configuration; AcOH, acetic acid; AgOTf, silver trifluoromethane sulphonate; AK, poly[Lys(DL-$Ala_{3.0}$)]; Bu^{t}, *tert*-butyl; DTNB, 5,5′-dithio-*bis*-(2-nitrobenzoic acid), Ellman's reagent; DTT, dithiotreitol; EDT, 1,2-ethanedithiol; EDTA, ethylenediaminetetraacetic acid; ES-MS, electrospray mass spectrometry; HBTU, *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexa-fluoro-phosphate; MeCN, acetonitrile; MUC1, mucin 1 glycoprotein; PBS, phosphate buffered saline; RT, room temperature; Tl(tfa)3, tallium trifluoracetate; Tx, toxin.

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INTRODUCTION

Recent immunological studies on various antigenic determinants indicate that modification and/or a change in the three-dimensional (3D) presentation of the epitope core peptide can significantly influence its immune recognition. For example, the introduction of conformational constrains (e.g. cyclization, attachment to macromolecules or anchoring to a Lys branch) in peptide antigens has very often resulted in increased binding to antibodies raised against the native protein or improved epitope-specific immunogenicity [1,2]. A new strategy for manipulation of short linear peptide epitope sequences is their insertion into a well-defined conformational part of a natural protein scaffold [3–5]. In these newly designed constructs, the replacement of surface residues in a β-turn or in a loop region of a 'host' structure by a 'guest' epitope sequence could result in a chimera with interesting immune recognition properties under *in vitro* and/or *in vivo* conditions.

MUC1 encoded by the MUC-1 gene on chromosome band 1q21 [6] is a member of the mucin family of large, highly glycosylated proteins. MUC1 is expressed and often secreted by epithelial cells and contains a polypeptide core consisting of variable number (30–100) repeats of the 20-amino acid sequence APDTRPAPGSTAPPAHGVTS [7–9]. Carcinoma cells produce MUC1 that is underglycosylated when compared with the version expressed by normal resting cells [10]. The incomplete glycosylation exposes a normally cryptic polypeptide core whose amino acid sequences could be recognized by the immune system as epitopes [11]. The appearance of these epitope domains is largely restricted to cancer cells; therefore, MUC1 is an attractive target for cancer immunodiagnosis [12] and immunotherapy [13]. Most, if not all, MUC1-specific monoclonal antibodies (MAbs) react with protein core epitopes of 3–5 amino acids within the hydrophilic region APDTRPAP of the 20-mer repeat [14]. Two of these antibodies, HMFG1 and HMFG2, used for the present investigation, recognize a tetramer (Pro-Asp-Thr-Arg, PDTR) and a trimer (Asp-Thr-Arg, DTR) sequence, respectively [15,16]. Hydropathicity and secondary structure prediction analysis on the 20-mer repeat unit indicated a hydrophilic region extending through residues PDTRPAP and a high propensity to form β-turn structure located at the PDTR sequence [16]. Indeed, CD and NMR experiments, supported by independent computational non-restrained study on both synthetic PDTRPAPGSTAP and the 20-mer peptides, have identified a type I β-turn in the PDTR region [17,18]. Comparative NMR study on the solution conformation of three APDTXPAPG peptides (where X = R, I or L) clearly demonstrated that the β-turn motif is not necessarily locked by the salt bridge between Arg and Asp [19]. It has been also reported that the anti-MUC1 antibody HMFG-1 recognizes only the native peptide, but fails to bind to other, Leu- or Ile-substituted peptides possessing slightly altered structure or lower population of turn-conformer [19]. A similar observation was reported by Fontenot *et al.* [20] after the NMR analysis of the $(VTSAPDTRPAPGSTAPPAHG)_3$ trimer. In this 60-amino acid residue peptide, they found that the elongated MUC1 structure contains knobs connected by extended spacer sequences. The solvent accessible tip of the knob contains APDTR forming a type II β-turn.

Based on these findings, the aim of this study was to design and synthesize a new, small chimera peptide antigen with preserved MUC1 epitope specificity. In this construct, the PDTR sequence of MUC1 was inserted as a 'guest' sequence in the 'host' structure of α-conotoxin GI, a 13-residue peptide (ECCNPACGRHYSC) isolated from the venom of *Conus geographus* [21]. The α-conotoxin GI structure is stabilized by two disulphide bridges in positions 2–7 and 3–13 and all structural analyses indicate the presence of a β-turn conformation in the 8–12 region [22–24]. A tetramer sequence Arg-His-Tyr-Ser (RHYS) of this part of the molecule has been replaced by a dominant antibody epitope PDTR [16]. Considering that the replacement of RHYS resulted in a loss of an aromatic (Tyr) residue, which is useful for determination of peptide concentration, the *N*-terminal Glu^1 was replaced by a Tyr. Therefore, an α-[Tyr^1]-conotoxin GI was also prepared [25]. In this paper, we report on the preparation, conformational analysis and the built-in epitope-specific antibody binding properties of the new synthetic chimera peptide. Data obtained with two MUC1-specific antibodies clearly show that the tetramer guest epitope PDTR is recognized both in the linear and the cyclic forms of the antigen. At the same time, we have observed that restricted accessibility of a trimer epitope DTR could occur, not only in the target bicyclic form of the chimera but even in its linear, but partially protected, precursor.

MATERIALS

DMSO, methanol, acetonitrile (MeCN), acetic acid (AcOH), methyl-*t*-butyl ether and 2-propanol,

Fmoc-amino acid derivatives and 4-(2′,4′-dimethoxyphenyl-aminomethyl)phenoxy) (Rink)-amide resin (0.43 mmol/g) were purchased from Novabiochem (Laufelfingen, Switzerland) and other chemicals (TFA, TFE, piperidine and HOBt) were purchased from SDS (Peypin, France). Thioanisole, 1,2-ethanedithiol (EDT), phenol, ethylenediaminetetraacetetic acid (EDTA) and guanidinium HCl were purchased from Fluka (Buchs, Switzerland). *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate (HBTU) and HOBt were purchased from Applied Biosystem (Paris, France), while iodine, dithiotreitol (DTT) and 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), silver trifluoromethane sulphonate [AgOTf], iodoacetatic acid and $Tl(tfa)_3$ were purchased from Sigma-Aldrich (St. Quentin Fallavier, France).

METHODS

Synthesis

Synthesis of linear MUC1-α-(Tyr[1])-conotoxin chimera peptides (MCnTx1 and MCnTx2). Synthesis was carried out on an Applied Biosystems 431A Peptide Synthesiser (Perkin Elmer, Foster City, CA, USA) using Fmoc/*t*-butyl strategy and HBTU/HOBt coupling according to Fields *et al.* [26]. Rink-amide resin (0.43 mmol/g) was used as solid support [27] and the amino acid side chain blocking groups were Bu^t ether for Thr and Tyr, Bu^t ester (OBu^t) for Asp, trityl (Trt) for Asn and Pmc for Arg. The thiol group of the Cys residue was protected orthogonally with either the Trt or the Acm group. The peptides were deprotected and cleaved from the resin as *C*-terminal amides by treatment with Reagent K (82.5% TFA, 5% water, 5% phenol, 5% thioanisole and 2.5% ethanedithiol, v/v) for 1 h at room temperature (RT), followed by precipitation with methyl-Bu^t ether at 4°C, solubilization in 10% acetic acid and lyophilization. EDT was replaced by DTT successfully under the cleavage of MCnTx2. The linear peptide MCnTx1 was prepared by carboxymethylation of the free thiol groups in positions 2 and 7. More precisely, 20 mg of cleaved crude peptide ($\sim$ 13 μM) was dissolved in 2.5 ml of 0.2 M Tris–HCl buffer, pH 8.0, containing 6 M Gdn.HCl, 2 mM EDTA and 50.7 mM DTT. After 1 h at RT, iodoacetic acid (824 mM final concentration) was added in darkness under stirring. The reaction was stopped 50 min later by loading the solution onto a Sephadex G-15 (Pharmacia, Uppsala, Sweden) column (2 × 50 cm) and eluted with 10% AcOH. Fractions containing the chimera peptide were lyophilized and further purified on a Vydac C_{18} column (1 × 25 cm, 5 μm particles, 300 Å pore size) (Hesperia, CA, USA). The absence of free sulphydryl groups was checked using the Ellman test [28].

Formation of the monocyclic MUC1-α-(Tyr[1])-conotoxin chimera peptides. Monocyclic peptide MCnTx1(C^2–C^7) was produced from the crude lyophilized MCnTx1 preparation that was first dissolved in water (15 mg/ml) and then diluted at 0.1 mg/ml in 0.02 M NH_4HCO_3 buffer, pH 8.3. The solution was left under constant stirring for 24 h at RT. The oxidation solution was then acidified to pH 3.0 with formic acid and was loaded directly onto a C_8 Aquapore RP300 column (1 × 25 cm) (Brownlee Laboratory, San Jose, CA, USA) at 3 ml/min flow rate. The monocyclic peptide was then eluted using a linear gradient 6–13% MeCN and 0.1% aqueous TFA over 40 min at 4 ml/min flow rate.

Monocyclic peptide MCnTx2(C^3–C^{13}) was prepared from the crude MCnTx2 product by the same treatment but 0.5 equivalent of DTNB to the free thiol groups was added into the oxidation mixture. The reaction was stopped after 2 h by addition of formic acid to pH 3.0.

Formation of the bicyclic MUC1-α-(Tyr[1])-conotoxin chimera peptide. Bicyclic peptide was obtained either from monocyclic peptide MCnTx1(C^2–C^7) (Route A) or from monocyclic peptide MCnTx2(C^3–C^{13}) (Route B).

Route A. Ten micromoles of the monocyclic MCnTx1(C^2-C^7) were dissolved in 25 ml 50% AcOH and added drop-wise into 25 ml of a freshly prepared mixture of iodine in glacial AcOH to a final concentration of 140 μM peptide and 22 μM iodine. The mixture was stirred for 15 min at RT and the reaction was terminated by adding 1.5 ml of saturated ascorbic acid in water. The solvent volume was reduced by rotary evaporation to 3 ml and the crude product was purified by gel filtration using a Sephadex G15 column (1 × 20 cm). The peptide was eluted with 10% AcOH and fractions containing the correctly folded peptide were further purified on a Vydac C_{18} (1 × 25 cm, 10 μm particles, 300 Å pore size) column using a linear gradient elution of 10–16% MeCN in 0.1% aqueous TFA over 40 min at 5 ml/min flow rate.

Route B. Two procedures were applied. (i) First, Acm groups were removed from the purified monocyclic MCnTx2(C^3–C^{13}) peptide (100 μM) with 40 equivalent of AgOTf in 2 ml TFA in the presence of 30 μl anisole at 0°C for 1.5 h. The unprotected peptide was precipitated with methyl-Bu^t ether and

collected by centrifugation. The oxidation was made in 10% DMSO/1 M HCl solution (0.1 mg/ml peptide concentration) overnight at RT. Alternatively, (ii) Acm-protected purified monocyclic MCnTx2(C^3–C^{13}) peptide (100 μM) was treated with 0.6 equivalent of $Tl(tfa)_3$ to the peptide in 2 ml TFA solution containing of 2% anisole at 0°C for 1 h. The reaction mixture in both cases was loaded onto a Sephadex G15 column (1 × 20 cm) and eluted with 10% AcOH solution. The collected fractions were purified on a Vydac C_{18} column (1 × 25 cm, 10 μm particles, 300 Å pore size) and eluted at 5 ml/min flow rate with a linear gradient of 10–16% MeCN in 0.1% aqueous TFA for 40 min.

Analysis

HPLC. Analytical RP-HPLC was carried out on a Spectra-Physics system formed by a P2000 pump, UV 2000 detector and ChromJet recorder (TSP, Les Ulis, France) using a Vydac C_{18} column (0.46 × 15 cm, 5 μm particles, 300 Å pore size) as a stationary phase. A linear gradient 5–25% B for 30 min (eluent A = 0.1% TFA in water and eluent B = 0.09% TFA in MeCN:H_2O [90:10, v/v]) was used as the mobile phase at a flow rate of 1 ml/min, at ambient temperature and with detection at 215 nm. Purification of crude products was carried out either on a C_8 Aquapore RP300 column (1 × 25 cm, 7 μm silica, 300 Å pore size) or on a Vydac C_{18} column (1 × 25 cm, 5 μm silica, 300 Å pore size). The gradient was 9–20% B for 45 min at 5 ml/min using the same eluents.

Amino acid analysis. The amino acid composition of peptides was determined by amino acid analysis using a Beckman (Fullerton, CA, USA) Model 6300 amino acid analyser. Prior to analysis, the samples were hydrolysed in 6 M HCl in sealed and evacuated tubes at 110°C for 24 h.

ES-MS. ES-MS was carried out on a Nermag R10-10 ES apparatus coupled to an Analitica of Branford electrospray source [29]. The quadropole was scanning over the range m/z 300–2000. Hewlett Packard-ChemStation software (Hewlett Packard, Les Ulis, France) was used to drive the spectrometer and to acquire the data.

Conformational analysis by CD-spectroscopy. CD spectra were recorded using a Jobin-Yvon Mark VI dichrograph (Longjumeau, France) equipped with a thermostatically controlled cell-holder and an IBM-PC operating with a CD6 data acquisition and manipulation program. Spectra were registered in the 195–260 nm range using quartz cells of optical path 0.1 cm at 20°C, under constant nitrogen flush. The dichrograph was calibrated with D-(-)-pantoyllactone at 220 nm and with epiandrosterone at 304 nm [30]. Spectra were recorded by accumulating four scans obtained with an integration time of 0.5 s every 0.2 nm. The peptides were dissolved in 0.01 M sodium phosphate buffer, pH 7.2, at a concentration of 2×10^{-5} M. Peptide concentrations were determined by UV spectroscopy at $\lambda = 280$ nm. The molar extinction coefficient was calculated on the basis of the known amino acid content [31]. The data of the CD measurements are presented in molar ellipticity ($[\Theta]_M$ in deg.cm^2/dmol).

Binding Inhibition Assay

MUC1-α-[Tyr^1]-conotoxin chimera peptides were tested for their capacity to inhibit the binding of HMFG1 and HMFG2 antibodies to AK-[CAPDTRPAPG] or BSA-[KAPDTRPAPG] synthetic or native MUC1 mucin target antigen. In the conjugates used as synthetic target antigen constructs, MUC1 10-mer oligopeptides [CAPDTRPAPG or KAPDTRPAPG] are covalently attached to the branched polymeric polypeptide poly[Lys(DL-$Ala_{3.0}$)] (AK) or to BSA carriers [32]. AK-[CAPDTRPAPG] or BSA-[KAPDTRPAPG] conjugates at 3 μg/ml or MUC1 at 1 μg/ml in PBS containing 0.02% NaN_3 was added to 60-well Microtest Plates at 10 μl/well (Nunc, Roskilde, Denmark) and incubated at room temperature for 18 h. The wells were washed four times with a washing buffer of PBS containing 0.1% casein and 0.02% NaN_3. During the final wash cycle, the wells were incubated with washing buffer for at least 1 h to complete the blocking of non-specific adsorption sites. HMFG1 antibody in 3 μg/ml and HMFG2 antibody in 1 μg/ml concentration or washing buffer alone in negative controls, 5 μl/well, was added to the wells in which 5 μl of the test peptide solution had been added (in a concentration range of 6×10^{-7}–7×10^{-4} mol/l). HMFG2 has a higher affinity for the target antigens, thus allowing lower concentration resulting in higher sensitivity in the inhibition assay. After incubation for 1.5 h at RT, the wells were aspirated and washed four times. ^{125}I-labelled rabbit anti-mouse immunoglobulin was added (1–1.6×10^5 cpm/well), incubated for 1 h, aspirated and washed six times with washing buffer. The wells were finally separated and counted individually in an LKB Compugamma Counter (Stockholm, Sweden). In each case, the concentration of added peptide required to inhibit antibody binding by 50% (IC_{50}) was calculated as a measure of antigenic potency [33].

Table 1 Structure and Analytical Data of Synthetic Mucin 1(PDTR)-α-Conotoxin Chimeras

Compounds	Disulfide bonds	M[H⁺]ᵃ Calc.	Found	HPLC analysisᵇ R_t[min]	Amino acid analysis Asp	Pro	Thr	Gly	Ala	Tyr	Arg
Acm Acm H-YCCNPVACG**PDTR**C-NH_2	linear	1543.8	1543.9	18.7	2.13	2.00	0.94	1.00	1.06	0.94	1.01
Acm Acm H-YCCNPVACG**PDTR**C-NH_2	linear	1543.8	1543.6	17.1	2.10	1.98	0.96	1.05	1.04	0.95	1.02
Acm Acm H-YCCNPVACG**PDTR**C-NH_2	1 loop	1541.7	1541.4	16.5	2.05	1.99	0.95	1.06	1.00	0.96	1.04
Acm Acm H-YCCNPVACG**PDTR**C-NH_2	1 loop	1541.7	1541.6	18.3	2.05	1.98	0.94	1.04	1.02	0.93	1.04
H-YCCNPVACG**PDTR**C-NH_2	2 loop	1397.6	1397.5	19.1	2.03	1.97	0.98	1.05	1.04	0.94	1.06

[a] ES-MS
[b] Column: Vydac C18, 300Å, 5μ, 0.46 x 15 cm; eluent A: 0.1% TFA/H_2O, B: 0.1% TFA/AcN-H_2O (90:10, v/v); gradients: 5-25%B in 30 min

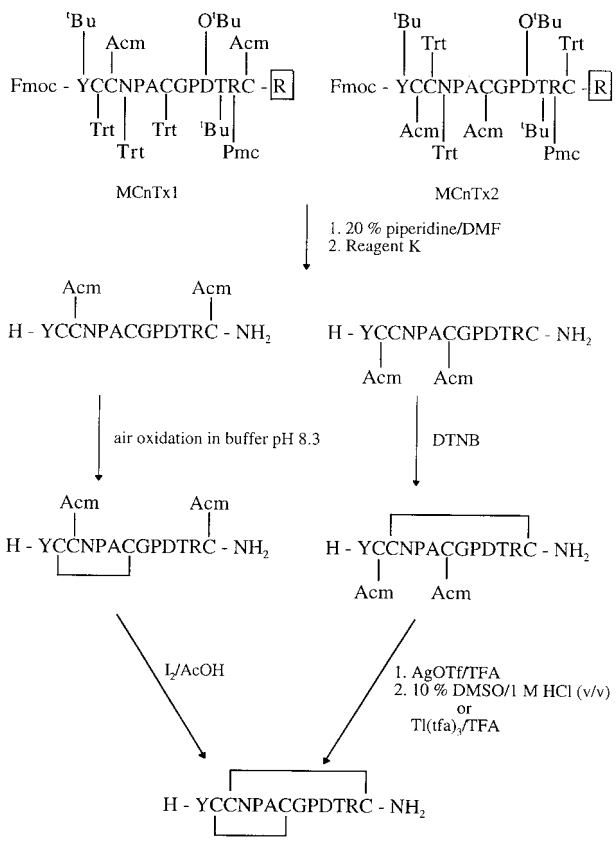

Figure 1 Outline of the synthesis of MUC1-α-[Tyr¹]-conotoxin chimera containing PDTR sequence from mucin 1 glycoprotein on Rink-amide resin.

RESULTS AND DISCUSSION

Synthesis of MUC1-α-(Tyr¹)-conotoxin Chimera Peptides

In this work, we report on the synthesis of a MUC1-α-[Tyr¹]-conotoxin GI chimera peptide containing a Tyr instead of Glu residue in position 1 and a tetramer sequence (PDTR) of MUC1 glycoprotein, as outlined in Figure 1. Characteristics of peptides prepared are summarized in Table 1. Syntheses were carried out on Rink-amide resin by Fmoc/Bu^t strategy. Reagent K cleavage mixture was used for deprotection. The crude linear peptide products, MCnTx1 and MCnTx2, present as a major single peak in RP-HPLC chromatograms (Figure 2) were oxidised without any purification.

The four Cys residues in positions 2, 3, 7 and 13 of the sequence were protected in pairs ortogonally with Trt and Acm groups (Figure 1). Two synthetic strategies were developed. In the first case, thiol groups of Cys residue in positions 2 and 7 were protected by Trt blocking groups, while the two other side chains were blocked by Acm groups (MCnTx1). In the second case, the side chain of Cys in positions 2 and 7 were Acm-protected, while those in positions 3 and 13 had Trt groups. All the protecting groups except of Acm were detached simultaneously with TFA. Both crude products MCnTx1 (Acm protection on positions 3 and 13)

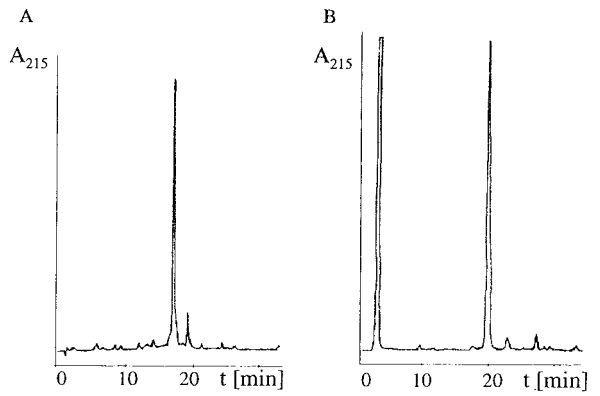

Figure 2 Analytical RP-HPLC profile of the linear MUC1-α-[Tyr^1]-conotoxin chimera preparations: crude peptide (A) MCnTx1 and (B) MCnTx2. Elution was carried out using a linear gradient: 5–25% eluent B for 30 min (eluent A 0.1% TFA in H_2O and eluent B 0.09% TFA in acetonitrile:H_2O (90:10, v/v) at a flow rate 1 ml/min at ambient temperature with detection at $\lambda = 215$ nm. Twenty microlitre volume of the samples (1 mg/ml in eluent A) was injected.

and MCnTx2 (Acm protection on positions 2 and 7) were pure enough to form the first disulphide bridge without any further purification (Figure 2). The monocyclic version of MCnTx1 [MCnTx1(C^2–C^7)] chimeric peptide was prepared by air oxidation in slightly alkaline buffer solution [34]. The HPLC profile of the reaction mixture shows a total conversion after 24 h (Figure 3(A)). After purification of the monocyclic MCnTx1(C^2–C^7) chimeric peptide, the second disulphide bridge (C^3–C^{13}) was introduced with iodine treatment in 50% AcOH solution (Figure 1) [35], as demonstrated by the chromatogram of the reaction product (Figure 4(A)).

Folding of the MCnTx2 linear peptide to the monocyclic MCnTx2(C^3–C^{13}) compound was carried out using DTNB (Ellman's reagent) [28], which allowed faster oxidation and yielded in the monocyclic peptide in less than 2 h. The purity of this product was as good as the monocyclic MCnTx1(C^2–C^7) chimeric peptide (Figure 3(B)). Two procedures were tried for the second loop formation. In the first case, the Acm groups were cleaved by silver triflate in TFA and the gel-filtered monocyclic peptide chimera was oxidized in 10% DMSO/1 M HCl solution [36]. This oxidation procedure needed a longer period of time (12 h) and we recorded the presence of several side products (Figure 4(B)). According to the ES-MS and the amino acid analysis, these peaks correspond to either disulphide bond isomers or truncated peptide

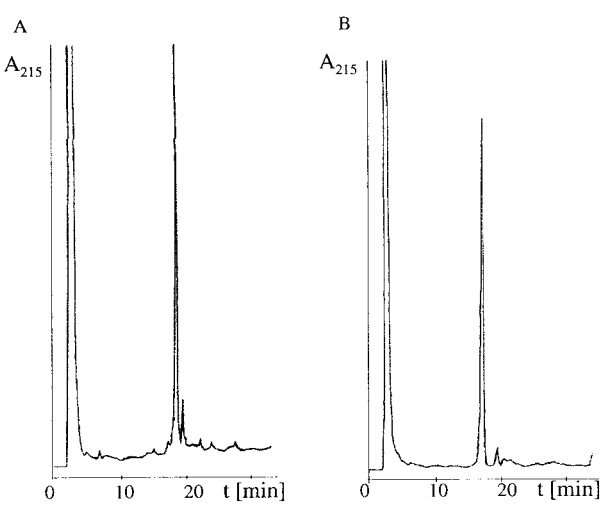

Figure 3 Analytical RP-HPLC profile of the monocyclic MUC1-α-[Tyr^1]-conotoxin chimera preparations after gel filtration: (A) peptide MCnTx(C^2–C^7) prepared by air oxidation and (B) peptide MCnTx(C^3–C^{13}) oxidised by DTNB. Elution was carried out as in Figure 2.

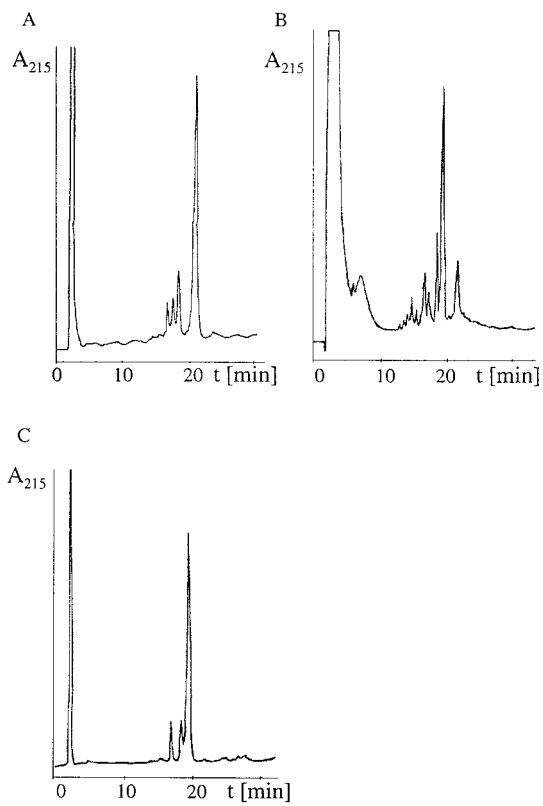

Figure 4 Analytical RP-HPLC profile of the bicyclic MUC1-α-[Tyr^1]-conotoxin chimera preparations: (A) crude peptide obtained from MCnTx(C^2–C^7) by iodine oxidation, (B) crude peptide obtained from MCnTx(C^3–C^{13}) after 10% DMSO/1 M HCl treatment or (C) Tl(tfa)$_3$ oxidation procedure. Elution was carried out as in Figure 2.

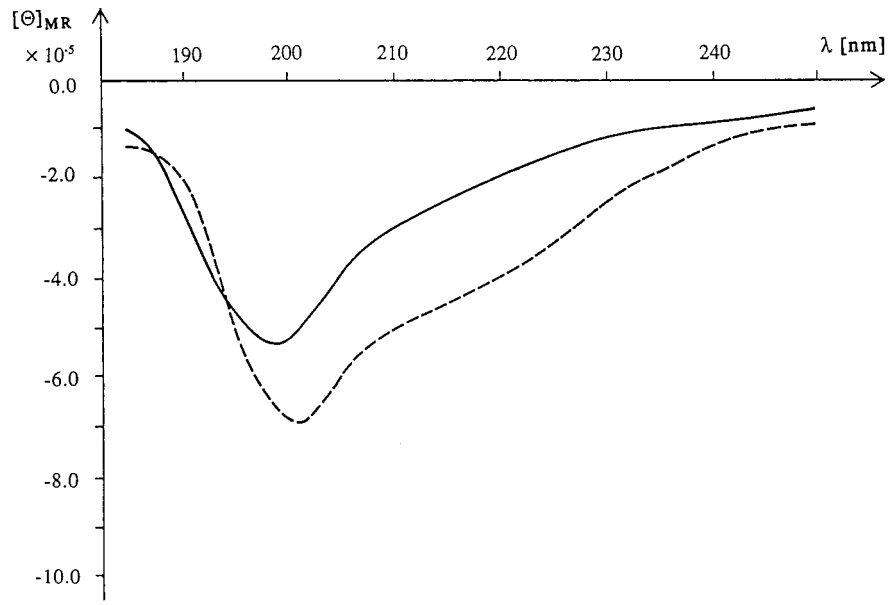

Figure 5 CD spectra of MUC1-α-[Tyr^1]-conotoxin chimera containing PDTR epitope sequence from mucin 1 glycoprotein. The spectra were recorded with peptides (0.5 mg/ml) in 0.01 M PBS, pH 7.2, at RT. Results are expressed as mean ellipticity values $[\Theta]_M$. Linear peptide MCnTx1 (—) and bicyclic MUC1-α-[Tyr^1]-conotoxin chimera peptide (– – –)

with the loss of a Tyr residue in position 1. Similar observations were made earlier during the synthesis of herpes simplex virus (HSV)-α-conotoxin GI chimera peptides [25]. In order to improve the efficacy of the second loop formation, we applied $Tl(tfa)_3$ as the oxidation agent in TFA in the next set of experiments. In this procedure, removal of Acm groups and disulphide bond formation occur at the same time [37]. This folding method resulted in excellent yield and only a small amount of disulphide isomers as side products was detected (Figure 4(C)).

Solution conformation

CD spectra of MUC1-α-[Tyr^1]-conotoxin chimera peptides containing the PDTR epitope sequence were recorded in 0.01 M PBS, pH 7.2. The linear (MCnTx1) and the bicyclic MUC1-α-[Tyr^1]-conotoxin chimera peptides with two disulphide bonds show partially ordered conformation with turn character (Figure 5). The presence of disulphide bridges promoted the ordered structure formation, as demonstrated by the shift of the negative maximum toward the higher wavelength (from 198 nm to 203 nm) and by the increased $[\Theta]_{220}/[\Theta]_{203}$ values. Namely in the case of the linear peptide $[\Theta]_{220}/[\Theta]_{198} = 0.35$, while the spectrum of the bicyclic results in a markedly higher value ($[\Theta]_{220}/[\Theta]_{203} = 0.57$) (Figure 5). The addition of TFE to the solution mixture resulted in more ordered conformation in both peptide forms (data not shown), and under both conditions the bicyclic MUC1-α-[Tyr^1]-conotoxin chimera peptide displayed spectral features similar to those of the α-[Tyr^1]-conotoxin bicyclic peptide. It is interesting to note that almost similar tendencies in conformational changes were observed with HSV-α-conotoxin GI chimera peptides [25]. These preliminary findings, based on the characteristics of CD spectra, might indicate that the bicyclic α-[Tyr^1]-conotoxin and its MUC1-α-[Tyr^1]-conotoxin analogue also share similar secondary structures, even if the exact type of turn of loop 8–11 cannot be assessed by the present analysis. This would suggest that the structural constrains induced by the two disulphide bonds in the α-conotoxin may be essential to induce a reverse turn in the 'guest' sequence PDTR in a high proportion of the conformers.

MAb Recognition of MUC1-α-(Tyr^1)-Conotoxin Chimera Peptides

The MUC1-α-[Tyr^1]-conotoxin chimera peptides were tested for their antibody binding properties in a competitive RIA experiment with MAbs HMFG1 and HMFG2 recognizing the PDTR and DTR epitopes, respectively. We utilized three target antigens. Besides the native MUC1 antigen, two synthetic peptide epitope conjugates containing

protein (BSA) or synthetic branched polypeptide AK [32] carrier and APDTRPAPG epitope domain elongated by either a Cys or a Lys residue at its *N*-terminal.

First, we studied the inhibition of MAbs HMFG1 and HMFG2 binding to target antigens by the linear and the bicyclic MUC1-α-[Tyr^1]-conotoxin chimera peptides. As shown in Figure 6, both peptides reacted with MAb HMFG1 but failed to inhibit the binding of MAb HMFG2 to the AK-[CAPDTRPAPG] conjugate antigen. Essentially, the same differences were observed between the two antibodies using the other synthetic construct BSA-[KAPDTRPAPG] or the native MUC1 mucin antigen (data not shown). Based on these findings, we excluded MAb HMFG2 from further investigation.

In the next series of experiments, we tested the binding of the linear, the monocyclic and the bicyclic MUC1-α-[Tyr^1]-conotoxin chimera peptides to MAb HMFG1 antibody using BSA-[KAPDTRPAPG] or native MUC1 target antigen. As a positive control, we also included the APDTRPAPG nonapeptide containing the PDTR sequence. As summarized in Figure 7, the linear peptide MCnTx1 inhibited the antibody binding to both target antigens applied. This inhibition occurred at relatively low concentration ($IC_{50} = 66$ μmol/l) in the case of the BSA-[KAPDTRPAPG] target antigen (Figure 7(A)). Similarly, the positive control peptide was reactive with the MAb. The bicyclic form of the chimera peptide bound to MAb HMFG1, only at a higher concentration, which could be characterized by an extrapolated IC_{50} value of > 2000 μmol/l. No inhibition of binding was observed with the monocyclic chimera peptide.

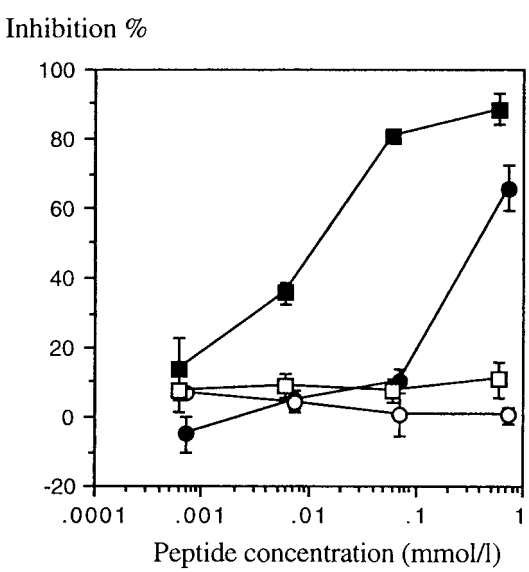

Figure 6 Inhibition of the binding of monoclonal antibodies to AK-[CAPDTRPAPG] target antigen with MUC1-α-[Tyr^1]-conotoxin chimera peptides in competition RIA. MAb HMFG1 with linear peptide MCnTx1 (■) or with bicyclic peptide (●). MAb HMFG2 with linear peptide MCnTx1 (□) or with bicyclic peptide (○).

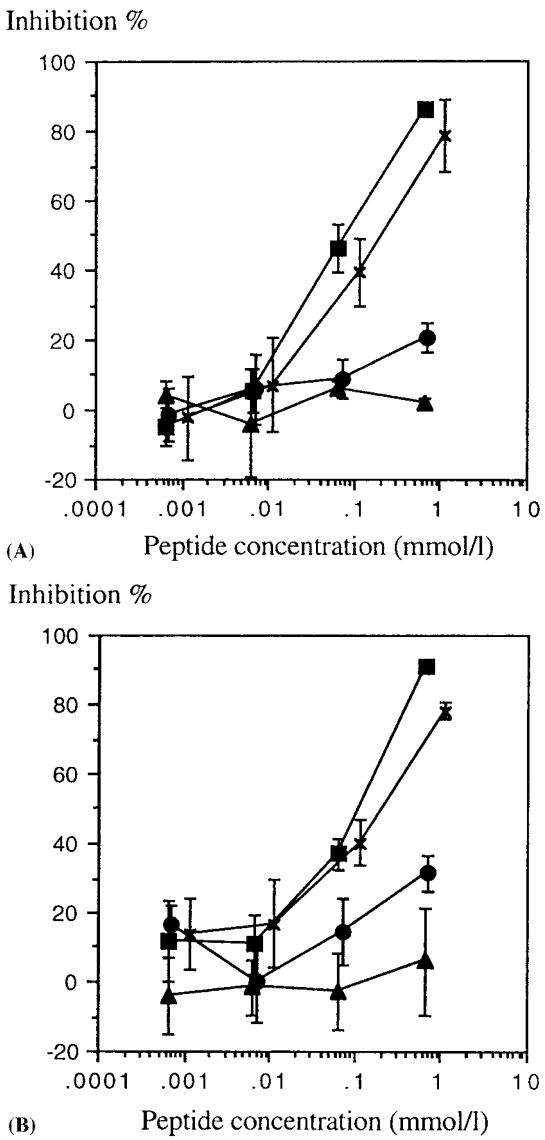

Figure 7 Inhibition of the binding of HMFG1 MAb to (A) BSA-[KAPDTRPAPG] or (B) target antigen with MUC1-α-[Tyr^1]-conotoxin chimera peptides in competition RIA: linear peptide MCnTx1 (■), monocyclic peptide (▲), bicyclic peptide (●) and control peptide APDTRPAPG (×).

Similar differences could be documented between the linear and the bicyclic peptides with native MUC1 target antigens (Figure 7(B)) The linear MUC1-α-[Tyr^1]-conotoxin chimera peptide was more efficient in inhibiting the MAb binding to MUC1 mucin target antigen ($IC_{50} = 115$ μmol/l) than the bicyclic variant, showing activity at about 1 mmol/l peptide concentration (extrapolated $IC_{50} > 2000$ μmol/l). Taken together, the observed differences were not dependent on either the synthetic or the native nature of the target antigen used in these experiments.

Binding data summarized above clearly show that MAb HMFG1 specific for PDTR tetramer sequence recognized the bicyclic MUC1-α-[Tyr^1]-conotoxin chimeric peptide. This indicates that the inserted epitope from the MUC1 immunodominant domain preserved its ability to bind the MUC1 glycoprotein-specific antibody. At the same time, it should be noted that the binding of the bycyclic chimera is less pronounced as compared with the linear APDTR-PAPG control peptide. The approximately 8-fold difference might reflect slight conformational differences caused by the different flanking regions surrounding the PDTR epitope. Consequently, we can presume that the β-turn forced on the PDTR sequence within the bicyclic chimera is not identical to that of the native mucin. It is also likely that the structure of the bicyclic peptide is too rigid to adapt to the antibody, while the flexible linear peptide is able to form the correct secondary structure induced upon binding to MAb HMFG1.

Only one chimera peptide containing MUC1 sequence has been reported in the literature so far. However, this construct could be considered as a 'reversed' chimera in comparison with the peptide described in this paper. Fontenot *et al.* [38] prepared chimera peptides based on the 'knob-like' structure of MUC1 with protruding motifs containing the APDTR pentamer found in the (VTSAPDTR-PAPGSTAPPAHG)$_3$ trimer. In these constructs, MUC1 acted as the 'host' and the epitope sequences from the third variable region (V3) of the HIV surface glycoprotein gp120 functioned as 'guest' segments. In the 60- and 120-mer chimeric peptides IHIGP-GRAF, HIGPGRA or GPGRAF from the V3 region replaced the sequences of the β-turn forming part of MUC1 (VTSAPDTRP, TSAPDTR or APDTRP), respectively. The authors found that not only the length of the built-in V3 sequence (6- versus 9-mer) but also the length of chimera (60- versus 120-mer) influenced markedly the recognition of the antigen by polyclonal antisera. It has been pointed out that the local structure within the 20-mer repeats of the 60- or 120-mer might be altered by short- (flanking regions) or long-range (global folding) interactions. These differences in the 3D structure are likely to alter the binding properties of these compounds. In the light of these results, there might be some advantages to developing small, structurally constrained chimeric constructs over the use of large ones with more complex tertiary or supermolecular assembly structures disturbing the antibody–antigen interaction.

During the development of the MUC1 epitope peptide-based immunotherapy against breast carcinoma, Sandrin *et al.* [39] observed that there is an interesting cross-reaction between the terminal disaccharide epitope formed by α(1,3)galactosyl transferase and MUC1 glycoprotein. Recently, it has also been demonstrated that the anti-Galα(1,3)Gal antibodies strongly reacted with peptide APDTR-PAPG, indicating that this part of MUC1 is responsible for this undesirable cross-reaction, which prevents the efficient induction of cytotoxic T lymphocyte (CTL) responses in carcinoma patients [40]. The findings reported in this paper might initiate the replacement of the linear MUC1 peptide with a bicyclic chimera variant, which possesses the specificity of the MUC1 epitope but — as a result of its altered steric structure — will not be recognized by the anti-Galα(1,3)Gal antibodies present in high quantities in carcinoma patients.

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