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SOLID-PHASE SYNTHESIS AND IMMUNOREACTIVITY OF PENTA-*O*-(*N*-ACETYL-α-D-GALACTOSAMINYL)-MUC1 EICOSAPEPTIDE, A GLYCOSYLATED COUNTER PART OF THE HIGHLY IMMUNOGENIC TANDEM REPEAT SEQUENCE OF CARCINOMA-ASSOCIATED MUCIN

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Abstract: Penta-*O*-glycosylated MUC1 peptide H–Val-Thr*-Ser*-Ala-Pro-Asp-Thr*-Arg-Pro-Ala-Pro-Gly-Ser*-Thr*-Ala-Pro-Pro-Ala-His-Gly-OH (*α-D-GalNAc*p*) (**2**) has been synthesized by a solid-phase method on Sasrin resin using Fmoc-glycoaminoacids **3** and **4** as building blocks. The monoclonal antibodies SM3 and HMPV, specific to the MUC1 peptide **1**, also react strongly with **2**.

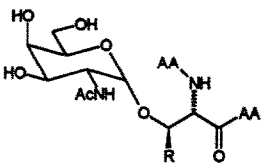
Mucins are highly *O*-glycosylated (~75% of total weight), high molecular weight glycoproteins expressed in endodermal epithelial cells, particularly those showing glandular secretory activity.[1] The core polypeptide moiety of mucins has been characterized by the presence of repeating Ser- or Thr-rich peptides consisting of 20 amino acid residues, as originally reported by Pigman and associates,[2] and confirmed by cloning of mucin genes. The human mammary mucin is encoded by the MUC1 gene and contains an extracellular domain consisting of a tandem repeat sequence of the MUC1 eicosapeptide 1[3] (Fig. 1). *O*-Glycosylation of Ser and Thr residues contributes to polymorphism in the mucin molecule.

H-Val-Thr*-Ser*-Ala-Pro-Asp-Thr*-Arg-Pro-Ala-Pro-Gly-Ser*-Thr*-Ala-Pro-Pro-Ala-His-Gly-OH

MUC1 eicosapeptide **1**

Ser* = Ser, Thr* = Thr

glycosylated MUC1 eicosapeptide **2**

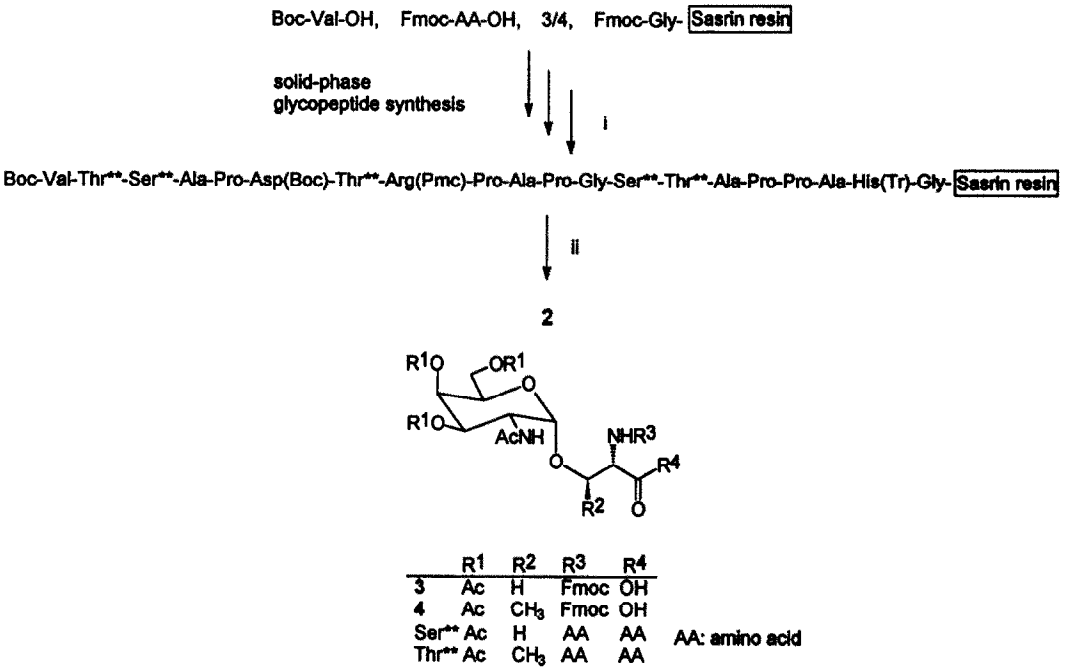


	R
Ser*	H
Thr*	CH_3

AA: amino acid

Fig. 1

Several monoclonal antibodies (mAbs), developed against either breast tumors or deglycosylated human milk fat globule proteins, have been shown to react strongly against 1.[4-6] Since these mAbs have identified the increased expression of this core peptide on breast carcinomas, it has been suggested that formerly hidden mucin core peptides are exposed due to aberrant glycosylation by cancer cells.[1] It is also well documented that these carcinomas accumulate incomplete glycosylation products known as tumor-associated carbohydrate antigens (TACAs), including Tn (GalNAcα1→*O*-Ser/Thr) and sialosyl Tn (NeuAcα2→6GalNAcα1→*O*-Ser/Thr) antigens.[7] It is conceivable that the glycosylation of Ser/Thr residues would influence the mAb binding to the peptide core by altering peptide conformation[8,9] or by masking the epitope. It is, therefore, interesting to determine the effect of glycosylation on the MUC1 immunoreactivity. We have synthesized the penta-*O*-(*N*-acetyl-α-D-galactosaminyl)-MUC1 peptide **2** and determined its reactivity against anti-MUC1 mAbs SM3[5] and HMPV.[6]



	R1	R2	R3	R4	
3	Ac	H	Fmoc	OH	
4	Ac	CH_3	Fmoc	OH	
Ser**	Ac	H	AA	AA	AA: amino acid
Thr**	Ac	CH_3	AA	AA	

Scheme I. *Reagents and conditions*: i, (1) De-*N*-Fmoc: 1:1 piperidine-DMF, 20 min, (2) Activation: Fmoc-AA-OH, 3/4, or Boc-Val-OH, HOBt, DMF, (*i*-Pr)$_2$N=C=N(*i*-Pr)$_2$, 0°C– rt, 30 min, (3) Coupling: 1 h; ii, (1) 1:1:0.4 TFA-CH_2Cl_2-anisole, 1 h, (2) 0.08 M NaOH in aq. MeOH, 0°C, 1 h, (3) HPLC on C4, 100% H_2O containing 0.1% TFA – 100% CH_3CN containing 0.1% TFA, 5 mL/min.

The glycopeptide **2** was synthesized on a Sasrin resin (Bachem Bioscience Inc., Philadelphia, PA, USA) by a solid-phase method[10] (Scheme I). Starting with Fmoc-Gly linked to the resin, Fmoc-amino acids (except for Val which was used as Boc-Val-OH) and Fmoc-glycoamino acids **3**/**4**[11] were successively coupled in DMF by a standard 1-hydroxybenzotriazole (HOBt) protocol.[12] The side-chain functions of His, Arg, and Asp were protected, respectively, by Tr,[13] 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc),[14] and *t*-Bu groups. For coupling of **3**/**4**, the excess of glycoamino acid used was recovered after an aqueous work-up, followed by flash column chromatography on silica gel (9:1 EtOAc–AcOH), in a 70–80% recovery yield without any racemization. The glycopeptide was cleaved from the resin by treatment with 50% TFA in CH_2Cl_2, which also removed Boc, Tr, Pmc, *t*-Bu groups. Saponification and subsequent purification by reversed-phase HPLC yielded **2**[15] (Fig. 2).

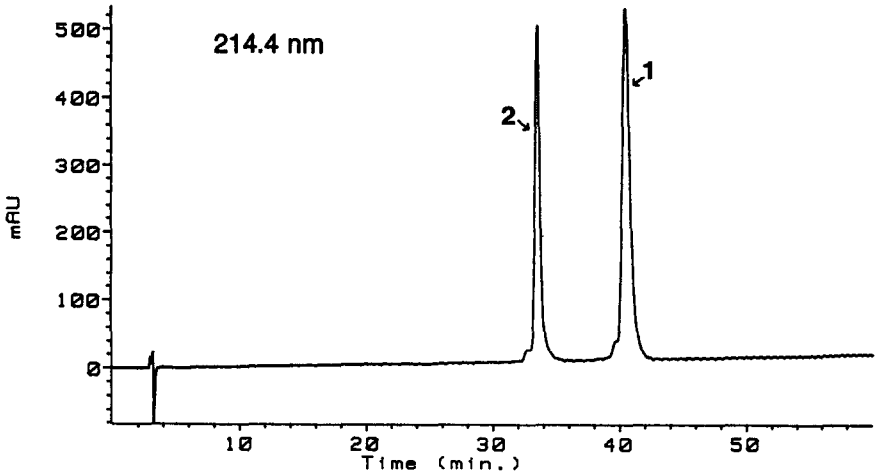


Fig. 2. HPLC profile of peptide **1** and glycopeptide **2**. Condition: Alltech Macrosphere RP300 C18, 5 μ, 250 x 4.6 mm; 100% H_2O (0.1% TFA) → 20% CH_3CN (0.1% TFA) in 60 min.

The peptide **1**[16] and glycopeptide **2** were conjugated with keyhole lympet hemocyanin (KLH) using glutaraldehyde[17] to increase their binding to a plastic plate, and their reactivities against mAbs SM3 and HMPV were examined in an enzyme-linked immunosorbant assay (ELISA) (Fig. 3). Surprisingly, both mAbs bind equally well to **1** and **2**. Furthermore, binding of SM3 and HMPV to breast cancer mucin, purified from patient pleural effusion, was inhibited by both **1** and **2** to a similar extent (Fig. 4). Since SM3 and HMPV do not recognize mucin from normal tissues,[5,6] our results raise the question of what degrees of glycosylation mask the peptide epitopes[18] differentiating between normal and cancer tissues.

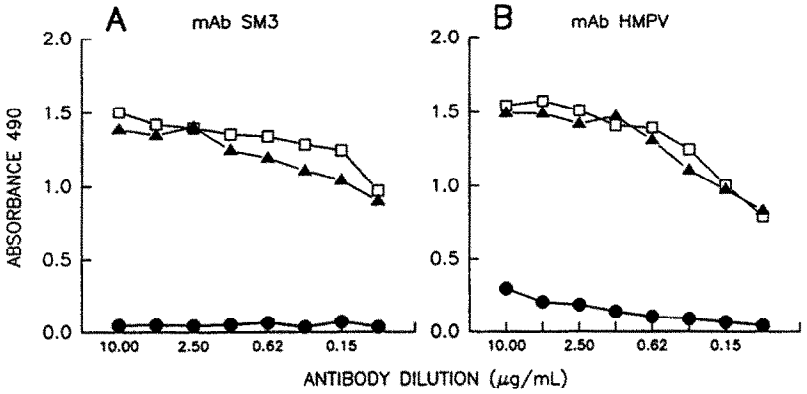


Fig. 3. Reactivity of mAb SM3 (A) and HMPV (B) against KLH (●), peptide 1-KLH conjugate (□), and glycopeptide 2-KLH conjugate (▲). The antigens were coated on a microtiter plate and the activity of mAbs was determined by ELISA.

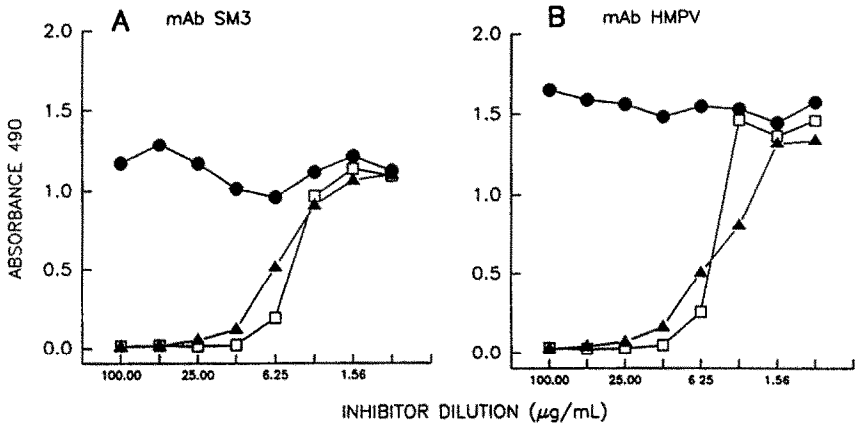


Fig. 4. Inhibition of SM3 (A) and HMPV (B) reactivity to human breast cancer mucin by peptide 1 (□), glycopeptide 2 (▲), and no inhibitor control (●). Breast cancer mucin was coated to a microtiter plate and the inhibition of reactivity was measured by ELISA.

The mAb SM3 has been shown to inhibit lysis of tumor cells by the cytotoxic T-lymphocytes (CTLs) from patients with breast and pancreatic cancers.[19] Therefore, it is possibile that these CTLs may recognize glycosylated peptide sequences. This possibility is of interest in connection with the prevailing view that only peptides are capable of inducing CTL activity.[20,21] Further studies are currently under way.

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