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GLYCOSYLATION OF THE ACTIVE SEQUENCE SER-ILE-LYS-VAL-ALA-VAL FROM THE α1 CHAIN OF LAMININ REDUCES TUMOR CELL ATTACHMENT ACTIVITY

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Abstract: N-terminal serine O-glycopeptides of SIKVAV-, a sequence located on the long arm of the α1 chain of laminin, were synthesized to study the effect of glycosylation on the adhesive properties of this biologically relevant peptide. The data show that covalently linked carbohydrates decrease the cell-attachment activity in a structurally dependent manner.

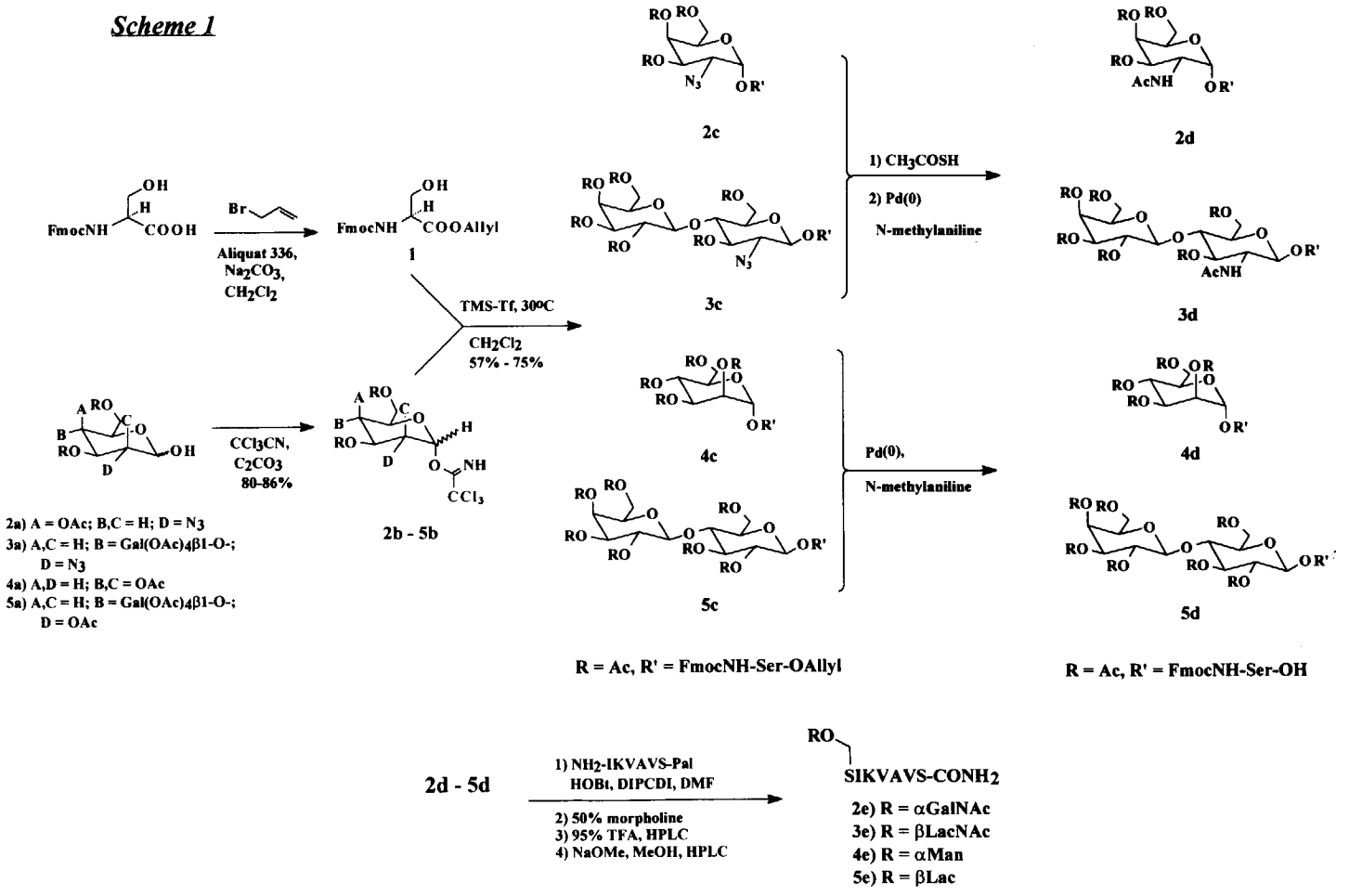
Interest in the pharmacological activity of covalently linked oligosaccharide chains present on the cell surface currently rivals that of the biomolecules to which they are attached (i.e. proteins and lipids).[1] Carbohydrate oligomers, which can be both N- and O- linked to proteins, have been shown to impart certain physical traits to the polypeptide such as solubility, stability and enhanced rates of secretion.[2] In addition, cell-surface sugars have been deemed essential for many biochemically relevant events such as embryogenesis,[3] cell adhesion,[4] lymphocyte homing,[5] tumorigenesis,[6] and metastatic dissemination and progression.[7] Consequently, methods for the analysis/structure elucidation[8] and synthesis[9] of glycan chains of cellular glycoconjugates have been vigorously pursued in recent years.

The aquisition of metastatic potential of a particular neoplastic phenotype is almost always associated with aberrant glycosylation of the tumor cell-surface glycoproteins.[10] These aberrations have been implicated as being important in directing the cell through a cascade of events leading to the formation of a metastatic colony at a distant site from the primary tumor.[11] A crucial primary event which facilitates intravasation into the microvasculature is the attachment of tumor cells to the basement membrane surrounding the blood vessels. Laminin is a large (~ 800,000 kDa) multi-domain, basement membrane glycoprotein which is believed to play a critical role in the attachment of tumor cells to this extracellular matrix. As high as 20-25% of laminin may be comprised of asparagine linked oligosaccharides, and these sugar chains have been shown to be essential for the manifestation of certain biological functions.[12] A stretch of amino acids at the C-terminal region of the long arm of the laminin α1 chain, which contains the residues serine-isoleucine-lysine-valine-alanine-valine

(SIKVAV-), has been shown to promote cell attachment, neurite outgrowth and migration, as well as collagenase IV production and angiogenesis.[13] In an effort to study the effect of glycosylation on the biological properties of this important segment of the laminin α1 chain, we prepared a series of glycosylated peptides from this region using solid phase Fmoc based peptide synthesis.

Since we decided to focus our initial study on cell attachment effects, we prepared glycopeptides of the shortest sequence known to adhere to neoplastic cells (i.e., SIKVAV-). Thus, we chose the N-terminal serine of the SIKVAV- segment as the site for initial glycosylation studies (there are no natural N- or O-linked glycosylation sites in this short sequence). The synthesis of four glycosylated analogues of the heptamer SIKVAVS (where the C-terminal serine was employed for ease of peptide elongation on the solid support) is outlined in Scheme 1. We chose α-mannose (Man), α-galactosamine (GalNAc), β-lactose (Lac), and β-lactosamine (LacNAc) as the carbohydrates for initial evaluations since they represent common motifs found in N- (Man, Lac, LacNAc) and O-linked (GalNAc) glycan chains of glycoproteins and mucins. The 2-acetamido sugars were derived from azidonitration of the acetylated glycals of galactose and lactose.[14] Carbohydrate-modified serine building blocks were prepared by Schmidt glycosylation[15] of appropriate anomeric trichloroimidates with the known N-Fmoc, O-allyl serine derivative **1** prepared from phase transfer allylation[16] of commercially available Fmoc-serine-OH. Glycosylations were carried out in the presence of TMS-triflate and 4Å molecular sieves at -30°C with yields of purified products generally in the 70-75% range. Acetates were employed as temporary hydroxyl protection for their ease of removal without consequence to the final glycosylated peptide. The anomeric selectivity for glycosylations with 2-azido galactose and 2-azido lactose trichloroimidate derivatives **2b** and **3b** has been documented to proceed with the desired α- and β-stereochemistries, respectively.[15] The free carboxylates of compounds **2c-5c** were liberated by allyl transfer to palladium(0)[16] in near quantitative yields. Peptide coupling to the N-terminal isoleucine of the hexamer IKVAVS-resin (prepared by standard Fmoc-based peptide synthesis on 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid [PAL] resin) in the presence of hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIPCDI) was complete in 2-24 h with coupling efficiencies of 50-70%. After base treatment to remove the Fmoc group, the resulting protected glycopeptides were cleaved from the solid support by treatment with 95% TFA and the acetylated products were purified by HPLC.[17] Deprotection with NaOMe/MeOH (powdered NaOMe, 2-3 mol equivalents, HPLC grade MeOH, 25°C, 10-15 min) afforded after reverse phase HPLC, the desired glycopeptides **2e-5e** in 50-60% overall yield of purified product.[18]

Compounds **2e-5e** were tested for their ability to promote PC12 pheochromocytoma tumor cell attachment. Figure 1 shows that the lactose **5e** and galactosamine **2e** containing glycopeptides promoted cell attachment as well as did the control peptide whereas binding was reduced by the addition of either mannose


Scheme 1
FmocNH
COOH
Br
Aliquat 336,
Na2CO3,
CH2Cl2
FmocNH
COOAllyl
1
TMS-Tf, 30°C
CH2Cl2
57% - 75%
CCl3CN,
C2CO3
80-86%
2b - 5b
2a) A = OAc; B,C = H; D = N3
3a) A,C = H; B = Gal(OAc)4β1-O-;
D = N3
4a) A,D = H; B,C = OAc
5a) A,C = H; B = Gal(OAc)4β1-O-;
D = OAc
2c
3c
4c
5c
1) CH3COSH
2) Pd(0)
N-methylaniline
Pd(0),
N-methylaniline
2d
3d
4d
5d
R = Ac, R' = FmocNH-Ser-OAllyl
R = Ac, R' = FmocNH-Ser-OH
2d - 5d
1) NH2-IKVAVS-Pal
HOBt, DIPCDI, DMF
2) 50% morpholine
3) 95% TFA, HPLC
4) NaOMe, MeOH, HPLC
SIKVAVS-CONH2
2e) R = αGalNAc
3e) R = βLacNAc
4e) R = αMan
5e) R = βLac

(**4e**) or lactosamine (**3e**) to the control sequence. Coating efficiency of compounds **2e-5e** and the SIKVAVS control peptide were studied by a fluorescence directed assay as described previously.[19] All compounds attached to the wells almost equally (ca 0.5%) indicating that different attachment activities are not due to unequal adherence to plastic. Inhibition of laminin binding to PC12 cells by the analogues revealed the two compounds **2e** and **5e** which actively promoted tumor cell attachment also inhibited the adhesion of laminin to the cells but this effect was variable (not shown). These peptides will be further evaluated for other activities such as cell migration and neurite outgrowth in due course.

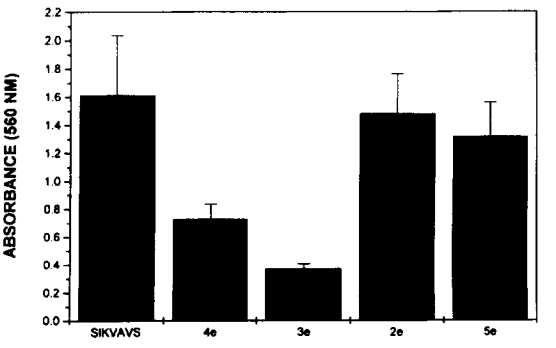


Figure 1. Attachment of PC12 cells to glycopeptides. SIKVAVS is the unglycosylated control. Attachment was observed by coating 96-well plates with 50 μg of peptide, incubating sequentially with a suspension of PC12 cells and crystal violet, and measuring the optical density of the attached cells relative to control. A dose response was observed for the glycopeptides at 50, 25, 12.5 and 6.25 μg per well

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17. Conditions for HPLC: Vydac C18 preparative column (20 mm ID), solvent A: 0.05% TFA/H_2O; B: 0.05% TFA CH_3CN, gradient 80:20 A:B -20:80 A:B over 30 min, flow rate, 9 ml/min; 220 nm detection.
18. The structures of previously unknown intermediates and glycopeptides **2d-5d**, **2e-5e** were confirmed by 500 MHz NMR and FAB MS. The authors thank Dr. Peter P. Roller, LMC, NCI, NIH for the mass spectral data.
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