

## Glycodendriproteins: A Synthetic Glycoprotein Mimic Enzyme with Branched Sugar-Display Potently Inhibits Bacterial Aggregation

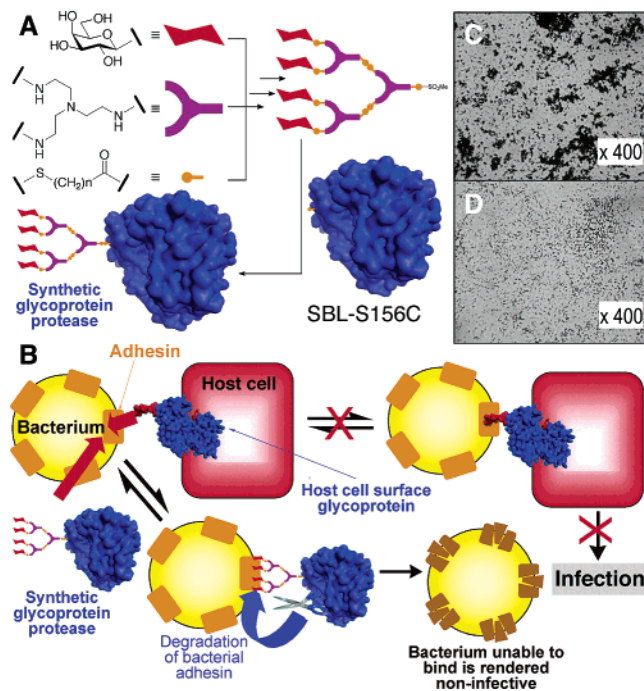
Phillip M. Rendle,<sup>†,‡</sup> Andreas Seger,<sup>†,‡</sup> Joao Rodrigues,<sup>†,∇</sup> Neil J. Oldham,<sup>†</sup> Richard R. Bott,<sup>‡</sup> J. Bryan Jones,<sup>§</sup> Marjorie M. Cowan,<sup>\*,||</sup> and Benjamin G. Davis<sup>\*,†</sup>

Department of Chemistry, University of Oxford, Mansfield Road, Oxford OX1 3TA, UK, Genencor International, 925 Page Mill Road, Palo Alto, California 94304, Department of Chemistry, University of Toronto, Ontario M5S 5H6, Canada, and Department of Microbiology, Miami University, Oxford, Ohio 45056

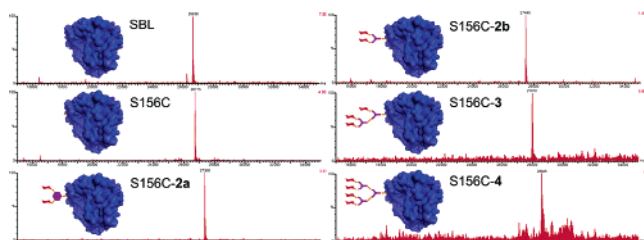
Received December 13, 2003; E-mail: Ben.Davis@chem.ox.ac.uk; cowanmm@muohio.edu

The continuing ability of bacteria to resist current antibiotic treatments highlights the need for alternative strategies for inhibiting their pathogenicity. Branched, complex carbohydrate structures on glycoproteins on host cell surfaces provide a binding point for many pathogens, including bacteria.<sup>1–3</sup> Monovalent carbohydrate ligands are only poorly recognized<sup>4</sup> by carbohydrate-binding proteins (lectins), and therefore small molecules are only effective in blocking bacterial adhesive events in high, therapeutically unrealistic concentrations (IC<sub>50</sub> ~mM (vide infra)). When more than one saccharide of the right type and orientation are clustered, there is an increase in affinity and specificity.<sup>5,6</sup> Elegant examples<sup>3,7,8</sup> have shown that exploitation of lectin binding does not require natural, multi-antennary structures as long as energetically efficient methods for carbohydrate presentation may be found.<sup>5,9</sup> We confirm here that glycodendrimers<sup>7,10</sup> can mimic the branched carbohydrates on glycoproteins. Moreover, when attached to a protein at a predetermined site, a new class of glycoconjugates, “glycodendriproteins”, designed to mimic glycoproteins is created. Such synthetic glycoproteins created from protein-degrading enzymes potentially reduce the binding ability of pathogenic bacteria (Figure 1).

The chemical construction of glycoproteins offers certain advantages over their isolation.<sup>11</sup> Isolation or recombinant expression typically produces mixtures of differently glycosylated proteins, termed glycoforms,<sup>12</sup> that display crucially different properties.<sup>13</sup> Several techniques offer sources of controllable, well-defined, homogeneous glycoproteins (pure glycoforms),<sup>14–19</sup> yet, to date, few<sup>20,21</sup> have allowed the incorporation of the branched, multivalent carbohydrates needed for high affinity.<sup>22</sup> Glycoprotein enzymes with one to four carbohydrate-tipped antennae were constructed using reagents **1–4** (Figure 1A and Scheme 1) activated with sulfhydryl-specific methanethiosulfonate (MTS). Conjugation with a single cysteine group in the protein-degrading proteinase, subtilisin, from *Bacillus lentus* (SBL, EC 3.4.21.62) created hybrid glycoproteins capable not only of binding but also of degradation (Figure 1B). SBL displays broad selectivity and functional similarity to regulatory proteinases<sup>23</sup> and contains no natural cysteines; therefore, reagents **1–4** react only with cysteine introduced by mutagenesis. Protease localization strategies allow selective degradation of protein targets provided that a single<sup>24</sup> suitable homing ligand is used.<sup>25</sup> However, until now, this strategy was limited to monovalent ligands and was poorly effective for lectins. One mono-antennary **1**<sup>15</sup> and four multi-antennary reagents **2–4** were constructed (Scheme 1)<sup>22</sup> containing either conformationally flexible TREN core **6** to create



**Figure 1.** (A) Glycodendriprotein construction. (B) Putative anti-infective mechanism, and visual comparison of *A. naeslundii*–*S. oralis* aggregation assays: control (C) and treated with 15 μg/mL S156C-2b (D).



**Figure 2.** ESMS of SBL, S156C, and glycodendriproteins S156C-2a, 2b, 3, 4.

**2b, 3, 4**, or more rigid mesitylene core **5** to create **2a**. Since our target pathogen *Actinomyces naeslundii* binds to β-D-galactose (Gal),<sup>26,27</sup> Gal units were attached using 1-thio-Gal without the need for carbohydrate protection. Mutagenesis of Ser156 to create SBL-Ser156Cys (S156C) was based on previous surveys of optimal targeting ligand positioning.<sup>25</sup> Quantitative modification of the introduced cysteine (Figure 1) with **1–4** was established by ESMS (Figure 2), electrophoresis, and thiol titration. All confirmed the identity and high purity of the resulting synthetic glycoproteins.<sup>22</sup>

These Gal-presenting synthetic glycoproteins were tested against surface-immobilized model Gal-binding lectin, peanut agglutinin (PNA), in enzyme-linked lectin assay (ELLA)<sup>28,22</sup> to mimic the

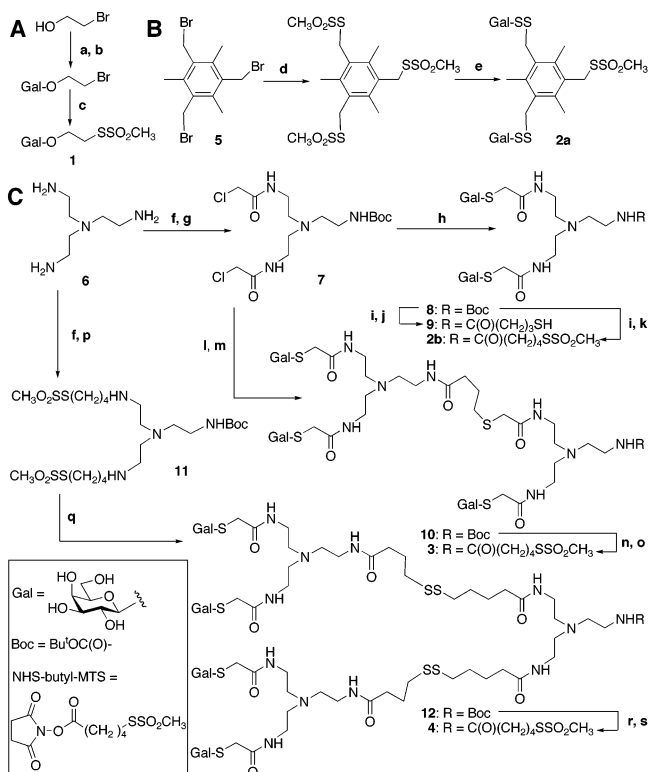
<sup>†</sup> University of Oxford.  
<sup>‡</sup> Genencor International.  
<sup>§</sup> University of Toronto.

<sup>||</sup> Miami University.

<sup>∇</sup> Current address: Carbohydrate Chemistry Team, Industrial Research, Ltd., PO Box 31310, Lower Hutt, NZ.

<sup>\*</sup> Current address: Johnson Matthey Catalysts, Cambridge CB4 0FP, UK.

<sup>\*</sup> Current address: Department Chemistry, York YO10 5DD, UK.

**Scheme 1.** Synthesis of Glycodendrimer Glycosylating Reagents 1–4<sup>a</sup>

<sup>a</sup> Reagents and conditions: **A**: a) Gal(Ac)<sub>5</sub>, BF<sub>3</sub>·Et<sub>2</sub>O, DCM, 76%; b) MeONa, MeOH, 83%; c) NaSSO<sub>2</sub>CH<sub>3</sub>, DMF, 78%; see ref 15. **B**: d) NaSSO<sub>2</sub>CH<sub>3</sub>, DMF, 56%; e) 2 equiv Gal-S<sup>-</sup>Na<sup>+</sup>, DMF, 0 °C 55%. **C**: f) Boc<sub>2</sub>O, DCM, -78 °C, 68%; g) (ClCH<sub>2</sub>CO)<sub>2</sub>O, DCM, 97%; h) 2 equiv Gal-S<sup>-</sup>Na<sup>+</sup>, DMF, 88%; i) CF<sub>3</sub>COOH, DCM, 91%; j) thiobutylolactone, dithiothreitol, NaHCO<sub>3</sub>, water, EtOH, 69%; k) NHS-butyl-MTS, DMF, 87% over 2 steps from **8**; l) 1 equiv Gal-S<sup>-</sup>Na<sup>+</sup>, DMF, 48%; m) **9**, DMF, 78%; n) CF<sub>3</sub>COOH, DCM, 94%; o) NHS-butyl-MTS, DMF, 77%; p) NHS-butyl-MTS, DMF, 87%; q) **9**, DMF, 81%; r) CF<sub>3</sub>COOH, DCM; s) NHS-butyl-MTS, DMF, 67% over 2 steps from **12**.

display of surface lectins on bacterial surfaces. This revealed increasing affinity with increasing Gal-antennae ( $K_D$  for S156C-1, **2a**, **2b**, **3**, **4**  $\approx 1.8 \times 10^{-3}$ ,  $1.1 \times 10^{-3}$ ,  $1.5 \times 10^{-3}$ ,  $3.4 \times 10^{-4}$ ,  $1.4 \times 10^{-7}$  M, respectively). Only a low-level ( $K_D \approx 10^{-2}$  M) binding of S156C-4 to the control mannose-binding lectin from snowdrop<sup>29</sup> was observed.

With this model Gal-binding lectin interaction established, we next evaluated the ability to inhibit the function of a Gal-binding pathogen. Gram-positive *A. naeslundii* aggressively colonizes oral cavities,<sup>27</sup> surgical prostheses,<sup>30</sup> and internal cavities.<sup>31</sup> We tested the ability of our glycoproteins to inhibit the co-aggregation of *A. naeslundii* with co-pathogen *Streptococcus oralis* (Figure 1C,D).<sup>22</sup> *A. naeslundii* uses the pili fimA adhesin, which binds Gal-tipped structures,<sup>26</sup> to bind structures on the surface of *S. oralis* during this crucial phase in the colonization process.<sup>25,32</sup> The inhibitory potency of S156C-1–4, designed to degrade the *A. naeslundii* Gal-binding adhesin using 1–4 as homing ligands, depended strongly on carbohydrate structure.<sup>25</sup> Optimal inhibitory potency was observed for S156C-2b that has a bi-antennary carbohydrate structure.<sup>33</sup> The limited inhibitory potency of S156C-2a demonstrated that bi-antennary display alone is not enough. Dose response<sup>22</sup> revealed that S156C-2b is a nanomolar inhibitor of *A. naeslundii* co-aggregation: IC<sub>50</sub> = 20 nM, >10<sup>6</sup> more potent than small-molecule adhesin inhibitor lactose (IC<sub>50</sub> = 33 mM). Three key properties of hybrid glycoprotein S156C-2b are essential for optimal inhibition: (i) its multi-antennary carbohydrate display, (ii)

its protein degrading activity, and (iii) Gal presentation. Protein lacking carbohydrate (SBL-WT) was only moderate (IC<sub>50</sub> = 2.4  $\mu$ M) in its inhibition and glycoprotein lacking protein-degrading activity (S156C-2b treated with irreversible inhibitor PMSF<sup>22</sup>) was ineffective (IC<sub>50</sub> > 5  $\mu$ M). Replacement of Gal in S156C-2b by glucose (Glc) gave a control glycoprotein S156C-2b(Glc) that showed only similar levels of inhibition to SBL-WT. The nanomolar level of co-aggregation inhibition displayed by S156C-2b is, to the best of our knowledge, the most potent to date. Furthermore, the glycodendrimer strategy allows ready carbohydrate retooling for an alternative lectin or pathogen.

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**Supporting Information Available:** Glycodendrimer reagent and glycodendrimer syntheses and characterization, binding assays, dose response curves, and further results, figures, and discussion (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- A. naeslundii* fimA may be functionally active as a dimer,<sup>26</sup> PNA as a tetramer. Bi-antennary complex glycoproteins are postulated to be natural fimA ligands.<sup>27</sup>

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