Module 1. Chemoenzymatic synthesis of glycans

Objective:

This module will deal with the preparation of glycan ligands for targeting Siglec expressing cells. The hands on component will involve the synthesis and purification of (A) 9-NH_2-Neu5Acα2-3LacNAc-ethyl azide and (B) NSA-Neu5Acα2-3LacNAc-ethyl azide which is a ligand used for targeting murine Siglec-F (NSA = 2-naphthalene sulfonyl amide).

Schedule:

1. Setup reaction 1 and reaction 2 (Part A)
2. Setup reaction 3 (Part B)
3. Monitor reactions 1-3
4. Purify reaction 1 and reaction 3

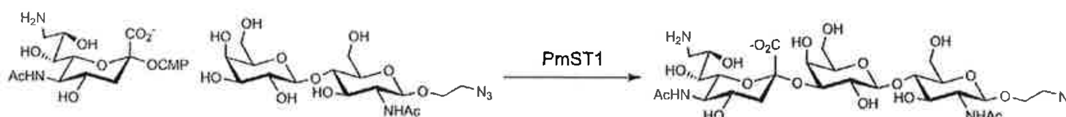
Concepts:

- Enzymatic, chemical and chemo-enzymatic synthesis of glycans
- Regio- and stereo-selectivity
- Retrosynthesis of glycans
- Glycosyltransferases
- Glycosyl donor and acceptor species
- Reaction steps: Setup, monitoring and work-up
- Limiting reagents
- Chromatography: Thin Layer Chromatography (TLC), C-18 reverse phase, gel filtration (size exclusion), ion-exchange.
- Qualitative analysis of product (NMR and MS)

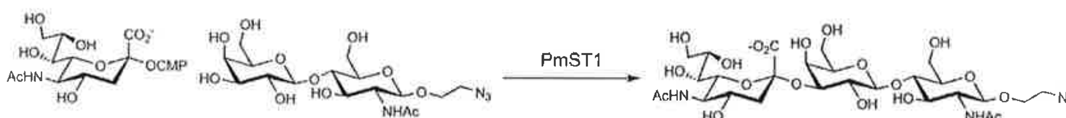
Part A: Sialylation of LacNAc ethyl azide

In this section the acceptor sugar, LacNAc-ethyl azide will be reacted with the nucleotide donors, CMP 9-NH_2-Neu5Ac (Reaction 1) and CMP-Neu5Ac (Reaction 2). The reactions are catalyzed by *Pasteurella multocida* α2-3-sialyltransferase (PmST1) (Ref: Yu, H. et al., *J. Amer. Chem. Soc.* **2005**, *127*, 17618.)

Reaction 1:



Reaction 2:



Reagents, Materials and Equipment:

- LacNAc-ethyl azide (MW = 452.41 g/mol)
- Stock solution CMP 9-NH_2-Neu5Ac (1.5 eq; MW = 657.43 g/mol; [C] = *will be provided*)
- Stock solution CMP Neu5Ac (1.5 eq; MW = 658.42 g/mol; [C] = *will be provided*)
- PmST1 (Activity = 4.5 U/mL)
- Reaction buffer: Tris-HCl (100 mM, pH 8.5) with $MgCl_2$ (20 mM)
- Product (standard for TLC)
- TLC eluents: EtOAc-MeOH-AcOH-H_2O (6:3:3:2); *i*PrOH-H_2O-NH_4OH (6:3:2)
- General: TLC plates, Eppendorf, pipettes and tips, TLC chamber and eluent, litmus paper
- Equipment: rotary evaporator, reaction incubator-shaker, pH meter, vortex, desiccator
- NMR and MS spectra of product and starting materials
- Lab coat, safety glasses, gloves

Procedure:

Setup:

1. Samples of LacNAc ethyl azide (0.5 mg) in 1.7 mL Eppendorf tubes will be provided.
2. Label the Eppendorfs for reaction 1 and reaction 2.
3. Add reaction buffer to each Eppendorf (~10 mM acceptor concentration).
4. Vortex the sample to ensure solid is completely dissolved. Spin the sample down to ensure that all of the solution is collected at the bottom of the Eppendorf.
5. Check the solution pH and carefully adjust to ~pH 8 with aq. NaOH (1 M) if necessary.
6. *Demonstration*. Thin Layer Chromatography (**Figure 1**).

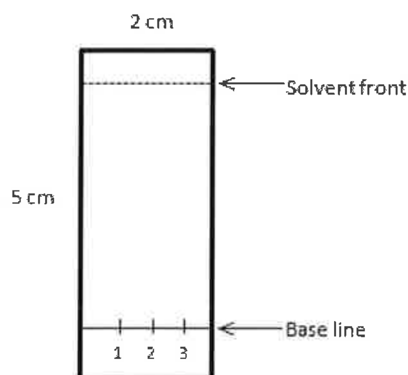


Figure 1. TLC plate for monitoring reactions. Samples are spotted on the hash marks along the baseline. The plates are then eluted with the desired solvent until the solvent front reaches ~0.5 cm from the top of the plate.

7. Using the TLC plate cutter prepare 2x5 cm TLC plates, or a size as desired.
8. In pencil, carefully so as not to damage the silica surface, draw a baseline ~0.75 cm from the bottom along the short edge of the plate.
9. Along the baseline make three short marks in pencil to indicate where to spot the samples.
10. Using a micropipetter spot ~0.5 μL of samples on the TLC plate.
11. On the baseline, spot the LacNAc standard on marks 1 (left) and 2 (center). On mark 2 (center) and 3 (right) spot the reaction mixture. The center spot is the co-spot. This TLC represents the reaction at time T=0 before adding the donor and enzyme.
12. Place the TLC plate in a desiccator under vacuum for ~10 minutes to remove the H_2O.

13. Setup the TLC chamber by pouring eluent into the glass jar. (TLC eluent = EtOAc-MeOH-AcOH-H_2O, 6:3:3:2). Note: The solvent level should be below the baseline drawn on the TLC plate.
14. Place the desiccated TLC plate in the TLC chamber. The solvent can be observed travelling up the plate.
15. Develop the plate until the solvent front is ~0.5 cm from the top of the plate. Remove the plate from the chamber and mark the position of the solvent front with pencil.
16. Place the TLC plate on a hot plate and allow the solvent to evaporate.
17. The TLC plate can be viewed under a UV-lamp to detect UV active material (i.e. donor sugars). Circle any UV active spots in pencil. Note: The LacNAc ethyl azide should not be UV active.
18. Dip the plate in a 10% H_2SO_4 ethanol staining solution, remove the excess solution using a paper towel then char the plate on a hot plate. *Caution: The hot plate should not be too hot to avoid over charring the plate.*
19. Analyze the stock solutions of the donor CMP 9-NH_2-Neu5Ac and CMP-Neu5Ac by TLC (eluents: *i*PrOH-H_2O-NH_4OH 6:3:2; EtOAC-MeOH-AcOH-H_2O 6:3:3:2) and make note of the Retention factor (R_f) using both eluents.
20. *Calculation*. Calculate the quantity of donor CMP 9-NH_2-Neu5Ac (1.5 eq.) and CMP-Neu5Ac (1.5 eq) required for each reaction.
21. Add CMP 9-NH_2-Neu5Ac (1.5 eq.) stock solution to the **reaction 1** Eppendorf containing LacNAc ethyl azide. Add CMP-Neu5Ac (1.5 eq) stock solution to the **reaction 2** Eppendorf containing LacNAc ethyl azide.
22. Check the solution pH and carefully adjust to ~pH 8 with aq. NaOH (1 M) if necessary. Note: Spot ~0.25 μL of the reaction solution onto litmus paper.
23. *Calculation*. Determine the quantity of enzyme (PmST1) required to complete the reaction in approximately two hours. The enzyme unit (U) is a unit for the amount of a particular enzyme. One U is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per minute under specified conditions (i.e. temperature, pH, substrate concentrations). *Rule of thumb: The enzyme activity (U) is determined under specified conditions and as the reaction proceeds and products are formed the activity of the enzyme will decrease, thus when determining the quantity of enzyme to add to the reaction use 10x the quantity that is calculated.*

Reaction:

24. Using a pipetor add the calculated volume of PmST1 from the stock solution to **reaction 1** and **reaction 2**.
25. Place the reaction eppendorf in the incubator and gently mix the solution at 37 °C.
26. Monitor the reaction by TLC at 15-30 minute intervals (eluent: EtOAC-MeOH-AcOH-H_2O 6:3:3:2). Perform the TLC as discussed above starting in step 7. Use LacNAc-ethyl azide as a co-spot.
27. When the starting material, LacNAc-ethyl azide, is consumed (i.e. no longer visible by TLC) the reaction is complete.

Work-up and product purification:

28. *Demonstration*: Ion-exchange chromatography.
29. Setup a pipette column (as demonstrated) containing Dowex PO_4- resin (~0.5 cm). Wash the resin with ~3 column volumes of dH_2O.
30. Transfer **reaction 1** solution to the top of the pipette column. Allow solution to enter the column then elute with dH_2O. Collect 0.5 ml fractions (~6) in Eppendorf tubes.
31. Monitor each of the fractions by 1-dimensional TLC. As shown in **Figure 2**, carefully draw a grid in pencil on the surface of the TLC plate. Using a micropipetter spot 0.5 μl of each fraction in a numbered grid square. Observe the plate under UV lamp followed by charring with H_2SO_4/EtOH staining solution. Make note of the fractions which contain material.

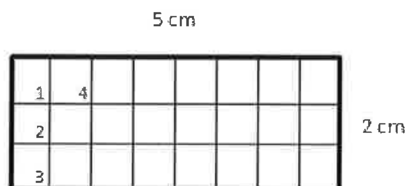


Figure 2. 1-D TLC plate for locating products within a group of column fractions. The number 1-4 indicate the fraction number.

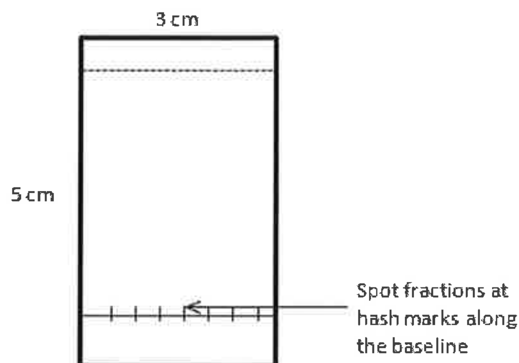


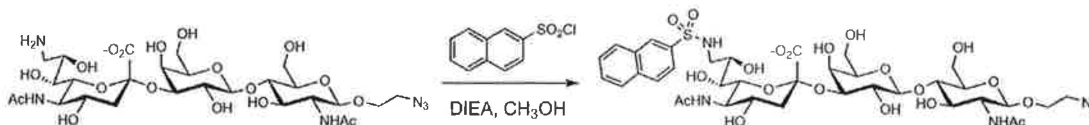
Figure 3. TLC plate for spotting multiple fractions to analyze purity of column fractions.

32. Analyze by TLC the fractions from step 30 which were UV active or charred. Prepare a TLC plate as depicted in **Figure 3**. Spot the fractions on the baseline marks. Desiccate the plate then develop with eluent (EtOAc-MeOH-AcOH-H_2O, 6:3:3:2).
33. Combine fractions which contain product and transfer to a tared clean glass vial.
34. Freeze the aqueous sample using dry ice then lyophilize.
35. NMR of the starting material (LacNAc) and product will be provided.
36. *Demonstration*. Purification of glycans by gel filtration.

Part B: Chemical synthesis of sulfonamide analogs

In this section, 9-NH_2-Neu5Acα2-3LacNAc-ethyl azide will be reacted with 2-naphthalene sulfonyl chloride (Reaction 3). The product will be purified by C-18 reverse phase chromatography.

Reaction 3:



Reagents, Materials and Equipment:

- 9-NH_2-Neu5Acα2-3LacNAc-ethyl azide (MW = 764.67 g/mol)
- 2-Naphthalene sulfonyl chloride (MW = 226.67 g/mol)
- Methanol
- Diisopropyl ethylamine (DIEA)
- Product (standard for TLC)
- TLC eluents: EtOAc-MeOH-AcOH-H_2O (6:3:3:2)
- General: TLC plates, pipettes and tips, TLC chamber and eluent, C-18 Sep-pak cartridge (1 cc), methanol
- Equipment: rotary evaporator, reaction incubator-shaker, pH meter, vortex, desiccator
- NMR and MS spectra of product and starting materials
- Lab coat, safety glasses, gloves

Setup:

1. A sample of 9-NH_2-Neu5Acα2-3LacNAc-ethyl azide (0.5 mg) will be provided.
2. Add methanol (0.5 mL) to the reaction vial. The starting material may not completely dissolve.
3. Calculate the quantity of DIEA (5 eq) and 2-naphthalene sulfonyl chloride (2 eq) required for the reaction
4. Add DIEA (5 eq.) to the reaction mixture followed by 2-naphthalene sulfonyl chloride (2 eq).
5. Check the solution pH. The pH should be between 8-9. Add additional DIEA (1 eq) if necessary. Note: Spot ~0.25 μL of reaction mixture onto litmus paper.

6. Monitor the reaction by TLC after 15 minutes (eluent: EtOAC-MeOH-AcOH-H_2O 6:3:3:2). Perform the TLC as discussed above in Part A step 7. Use 9-NH_2-Neu5Acα2-3LacNAc-ethyl azide as a co-spot.
7. When the starting 9-NH_2-Neu5Acα2-3LacNAc-ethyl azide is consumed (i.e. no longer visible by TLC) the reaction is complete.

Work-up and product purification:

8. Before working up the reaction check the solution pH. The pH should be between 8-9. Add additional DIEA (1 eq) if necessary.
9. Place the reaction in a desiccator to concentrate the solution and remove the methanol.
10. Add dH_2O (0.5 mL, pH 10) to the desiccated reaction mixture. Note: The aqueous solution (pH 10) will ensure that excess 2-naphthalene sulfonyl chloride is hydrolyzed.
11. Prepare the C-18 sep-pak cartridge (1 cc). Wash the column with methanol (1 ml) followed by ddH_2O (3x1 ml). The product contains a hydrophobic handle (2-naphthalene) that allows purification by C-18 reverse phase chromatography.
12. Setup numbered Eppendorfs (without caps) to collect fractions eluted from the column.
13. Apply the aqueous reaction solution to the C-18 sep-pak column using a pipetor. Allow the solution to enter the column before proceeding.
14. Slowly elute the column with ddH_2O (2 mL) collecting 1.0 mL fractions.
15. Monitor each of the fractions by 1-dimensional TLC. As shown in Figure 2, carefully draw a grid in pencil on the surface of the TLC plate. Using a micropipetter spot 0.5 μl of each fraction in a numbered grid square. Observe the plate under UV lamp followed by charring with H_2SO_4/EtOH staining solution. Make note of the fractions which contain material.
16. Elute the column with 20% aq. methanol (3 ml) followed by 30% aq. methanol. Collect 1.0 ml fractions. Monitor each of the fractions by TLC as above.
17. Combine fractions which contain product and transfer to a tarred clean glass vial.
18. Place vial on rotary-evaporator to remove methanol.
19. Lyophilize the remaining aqueous solution.
20. NMR of the product will be provided.