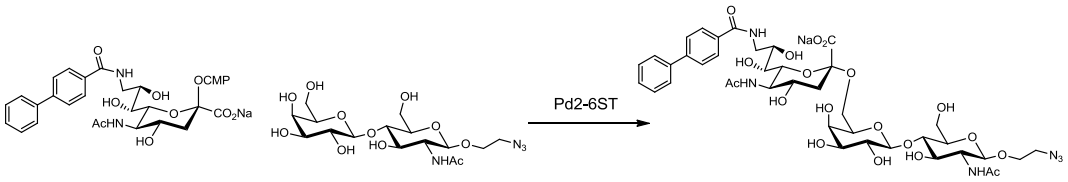
Module 1: Chemoenzymatic synthesis of glycans

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 9-*N*-biphenyl-Neu5Acα2-6LacNAc-ethyl azide (2 mg scale/group) which is a ligand used for targeting human Siglec-2 (CD22).

Chemistry:



The synthesis involves the reaction of the acceptor sugar, LacNAc-ethyl azide with the nucleotide donor, 9-BPC-Neu5Ac-CMP. The reaction is catalyzed by *Photobacterium damsela* α2,6-sialyltransferase.

Concepts:

- Enzymatic, chemical and chemo-enzymatic synthesis of glycans
- Regio- and stereo-selectivity
- Retrosynthesis of glycans
- Glycosyltransferases
- Donor and Acceptor species
- Reaction steps: Setup, monitoring and work-up
- Limiting reagents
- Thin Layer Chromatography (TLC)
- Work-up and purification of the reaction
- Qualitative analysis of product (NMR and MS)

Reagents, Materials and Equipment:

- LacNAc-ethyl azide (1 mg; MW = 452.41 g/mol)
- Stock solution 9-BPC-Neu5Ac-CMP (1.2 eq; MW = 837.63 g/mol) ([C] = *will be provided*)

- Pdα2,6ST (Activity = *will be provided*)
- 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, C-18 Sep-pak, methanol
- Equipment: rotary evaporator, reaction incubator-shaker, pH meter, vortex, desiccator
- NMR and MS spectra of product and starting materials
- Lab coat, Glasses, Gloves

Procedure:

Reaction setup:

1. Accurately weigh out 1 mg of LacNAc ethyl azide and transfer to a 1.7 ml eppendorf.
2. Add reaction buffer to the eppendorf (~10 mM acceptor concentration).
3. 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.
4. Check the solution pH and carefully adjust to ~pH 8 with aq. NaOH if necessary.
5. *Demonstration*. Thin Layer Chromatography (Figure 1).

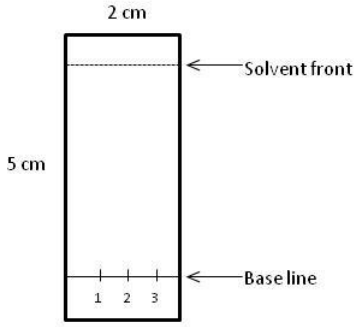


Figure 1.

6. Using the TLC plate cutter prepare 2x5 cm TLC plates, or a size as desired.
7. In pencil, carefully so as not to disturb the silica surface, draw a baseline ~0.75 cm from the bottom along the short edge of the plate.
8. Along the baseline make three short marks in pencil to indicate where to spot the samples.

9. Using a micropipetter spot ~0.5 μL of samples on the TLC plate.
10. 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.
11. Place the TLC plate in a desiccator under vacuum for ~10 minutes to remove the H_2O.
12. Setup the TLC chamber by pouring ~10 mL of eluent into the glass jar. (TLC eluent = EtOAc-MeOH-AcOH-H_2O, 6:3:3:2).
13. Place the dried TLC plate in the TLC chamber ensuring that the solvent level is not above the baseline. The solvent can be observed travelling up the plate.
14. 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.
15. Place the TLC plate on a hot plate and allow the solvent to evaporate.
16. 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.
17. 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.*
18. Analyze the stock solution of the donor 9-BPC-Neu5Ac-CMP 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.
19. *Calculation*. Calculate the quantity of donor 9-BPC-Neu5Ac-CMP (1.2 eq.) required for the reaction and volume of stock solution to add to reaction.
20. Add 9-BPC-Neu5Ac-CMP (1.2 eq.) stock solution to eppendorf containing LacNAc ethyl azide.
21. Check the solution pH and carefully adjust to ~pH 8 with aq. NaOH if necessary.
22. *Calculation*. Determine the quantity of enzyme (Pd2-6ST) required to complete the reaction in one hour. 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:

23. Using a pipetor add the calculated volume of Pd2-6ST from the stock solution to the reaction mix.
24. Place the reaction eppendorf in the incubator and gently mix the solution at 37 °C.
25. Monitor the reaction by TLC at 15 minute intervals (eluent: EtOAC-MeOH-AcOH-H_2O 6:3:3:2). Perform the TLC as discussed above starting in step 5. Use stock solutions of either LacNAc-ethyl azide or product for co-spots.

Reaction work-up and product purification:

26. When the starting LacNAc-ethyl azide is consumed the reaction is complete.
27. Transfer the reaction to a centrifugal filter device and centrifuge (15 minutes @13,200 rpm) to remove the protein. Proceed to the next step while centrifuging.
28. Prepare the C-18 sep-pak. Wash the column with methanol (5 ml) followed by ddH_2O (5 ml). The product contains a hydrophobic handle (BPC) that allows purification by C-18 reverse phase chromatography.
29. Setup numbered eppendorfs (without caps) to collect fractions eluted from the column.
30. Apply the filtrate to the C-18 sep-pak column using a pipetor. Allow the solution to enter the column before proceeding.
31. Slowly elute the column with ddH_2O (5 ml) collecting 0.5 ml fractions.
32. 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.

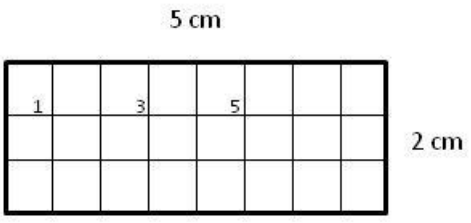


Figure 2.

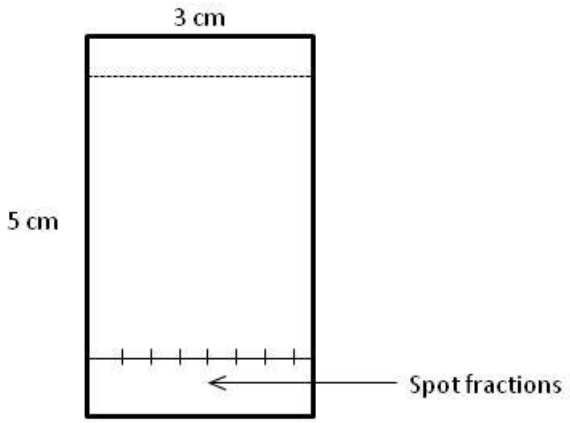


Figure 3.

33. Monitor the fractions from step 31 and 32 which were UV active or charred by TLC. 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). Note: The fractions from the aqueous wash should not contain product.
34. Elute the column with 20% aq. methanol (5 ml) followed by 30% aq. methanol (5 ml). Collect 0.5 ml fractions. Monitor each of the fractions by TLC as above.
35. Combine fractions which contain product and transfer to a tarred clean glass vial.
36. Place vial on rotary-evaporator to remove methanol.
37. Lyophilize the remaining aqueous solution.
38. NMR of the starting material (LacNAc) and product will be provided.
39. *Demonstration.* Purification of glycans by gel filtration.