Protein X-ray crystallography and glycobiology

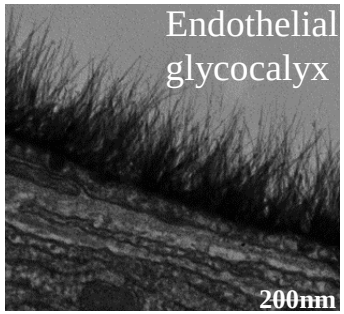
Dr Annabelle Varrot

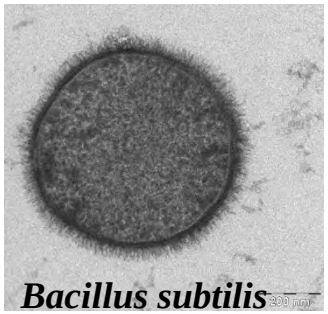
« Structural Glycosciences » Summer School

IBS, Grenoble 7th June 2023

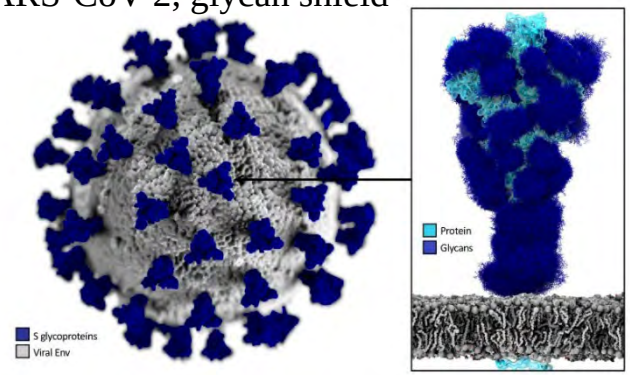
"Sweet side" of cells

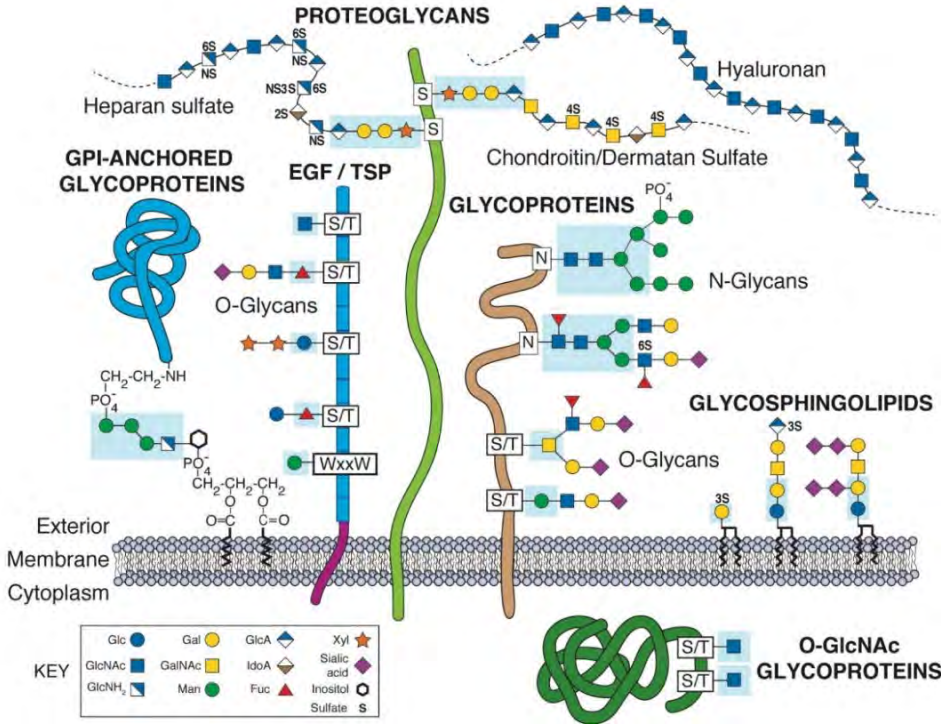
- Sugars: 3rd alphabet of life: Glycome → glycocode
- Every cell is covered by **glycoconjugates**





SARS-CoV-2, glycan shield

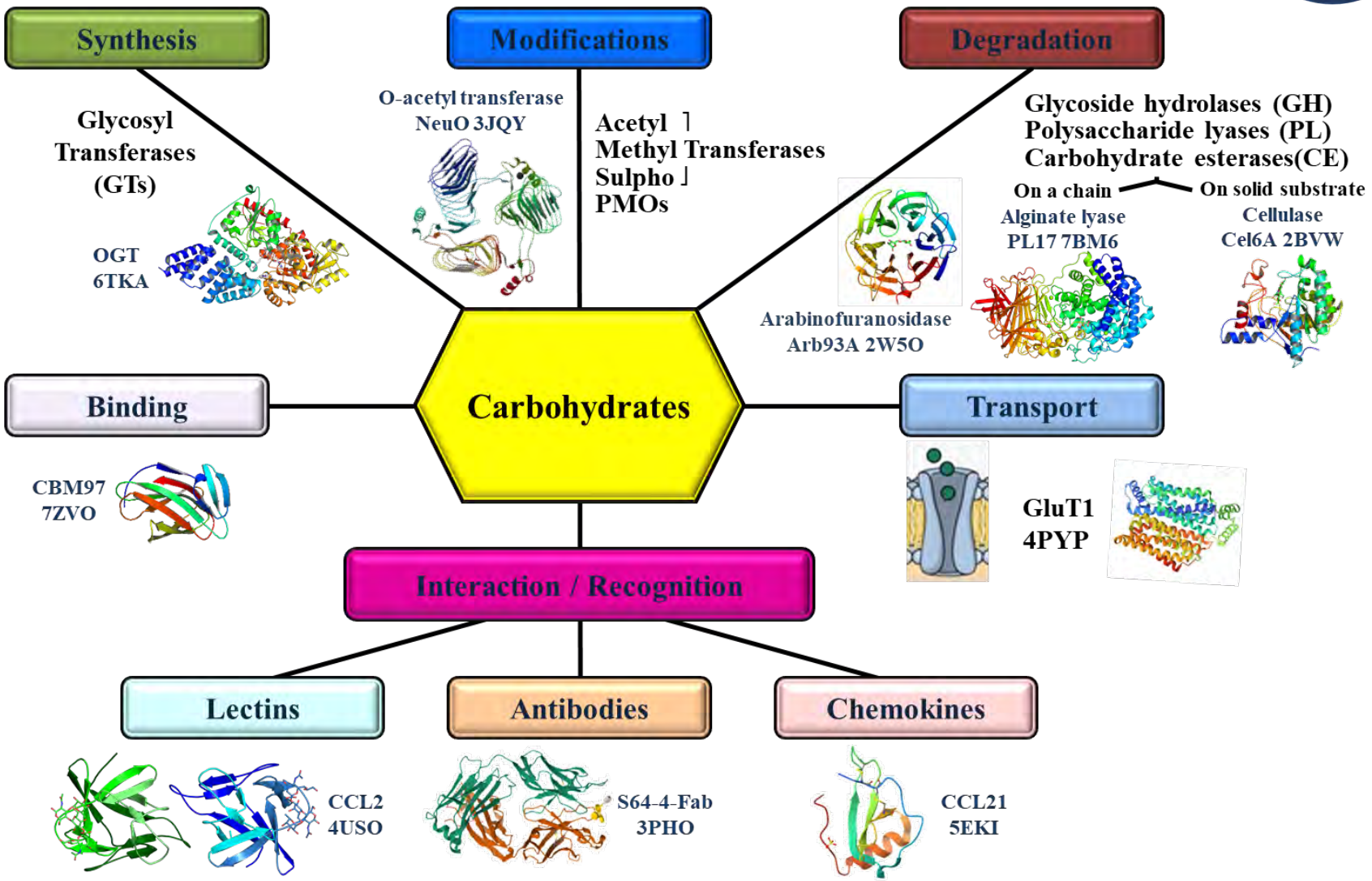




- Essentials in cell identity, fate, recognition & signaling

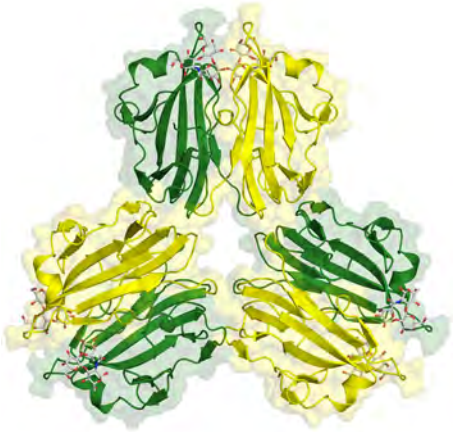
Essential in Glycobiology, The Scripps Research Institute,
Casalino *et al.*, ACS Central Sci (2020), 6: 1722.

Protein-carbohydrate interactions



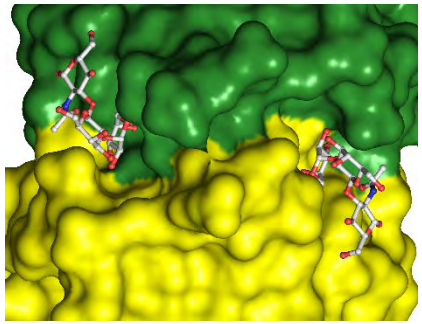
S Perez & O Makshakova, Chem. Rev., 2022, 122,15914

Which info by X-ray crystallography?

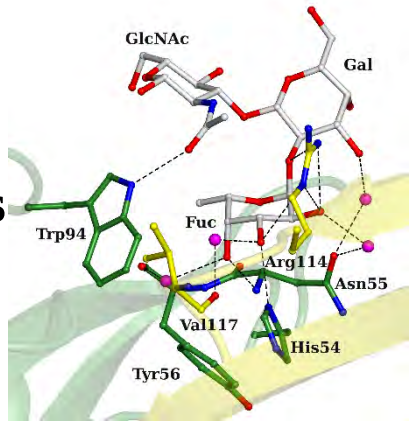


Fold/assembly

Binding site architecture

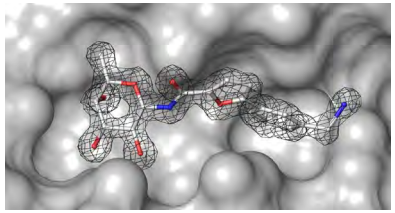


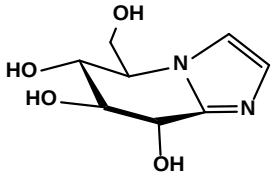
Interactions



Structure

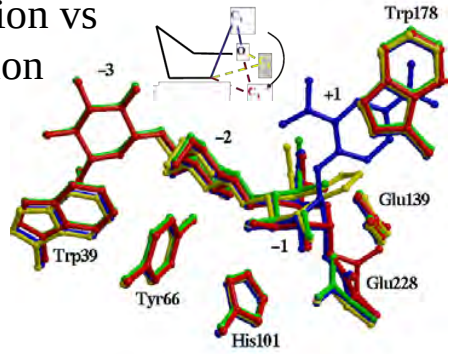
Drug design



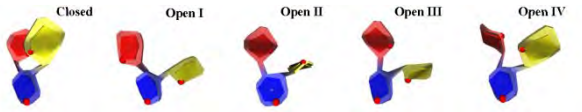


Enzyme mechanism

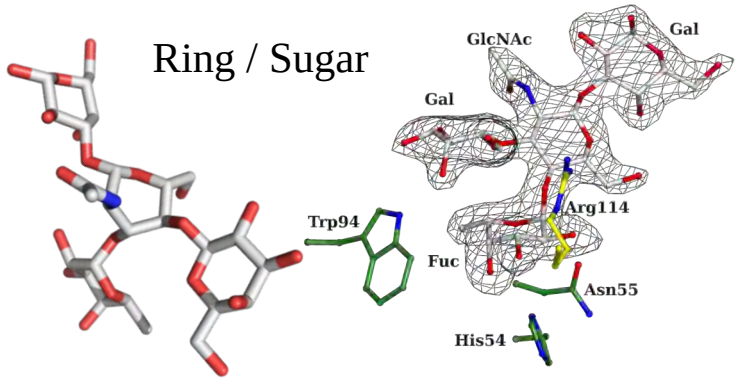
Retention vs Inversion



Conformation

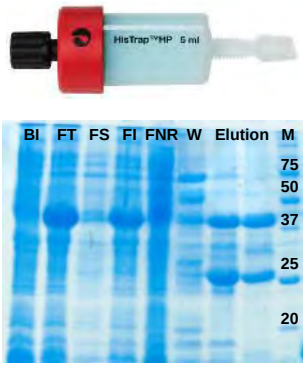


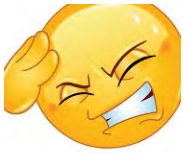
Ring / Sugar



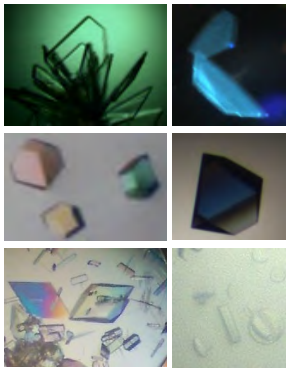
Protein X-ray crystallography

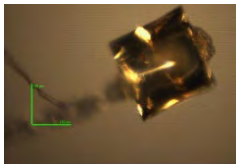
Expression
Purification



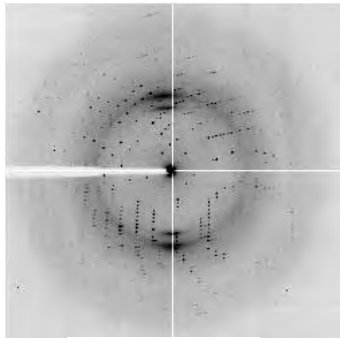


Crystallization





Freezing
& X-ray
diffraction



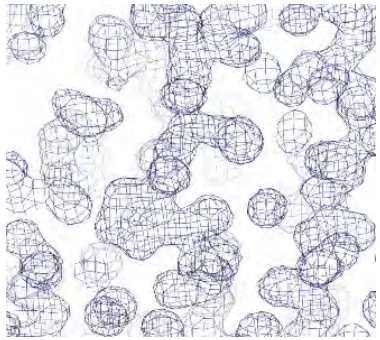
➢Advantages

- Can go to atomic resolution
- Atomic details obtained

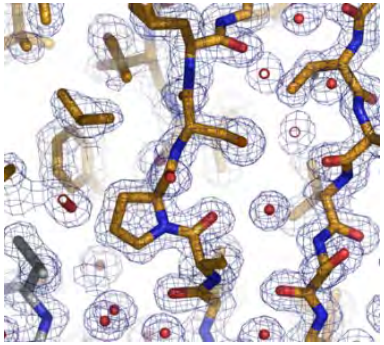
➢Disadvantages

- Molecules in solid-state environment
- Require crystals
- Requires order to diffract

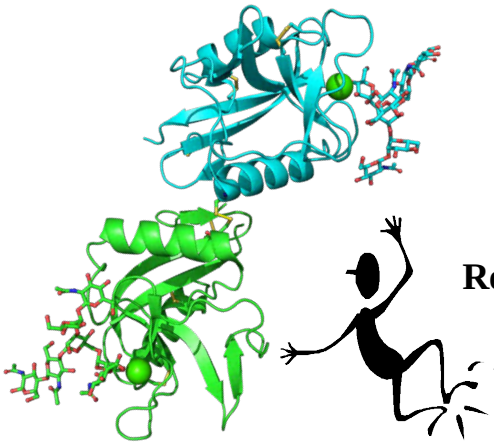
Phasing



Modelling

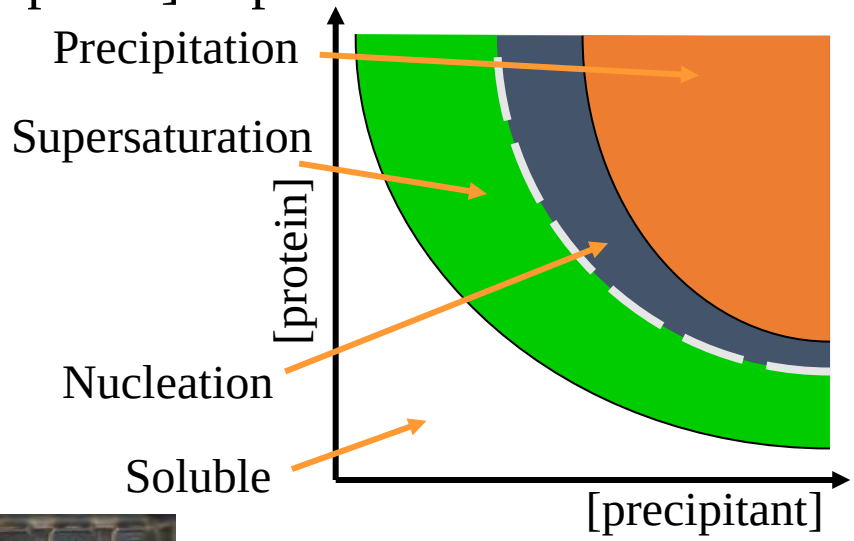


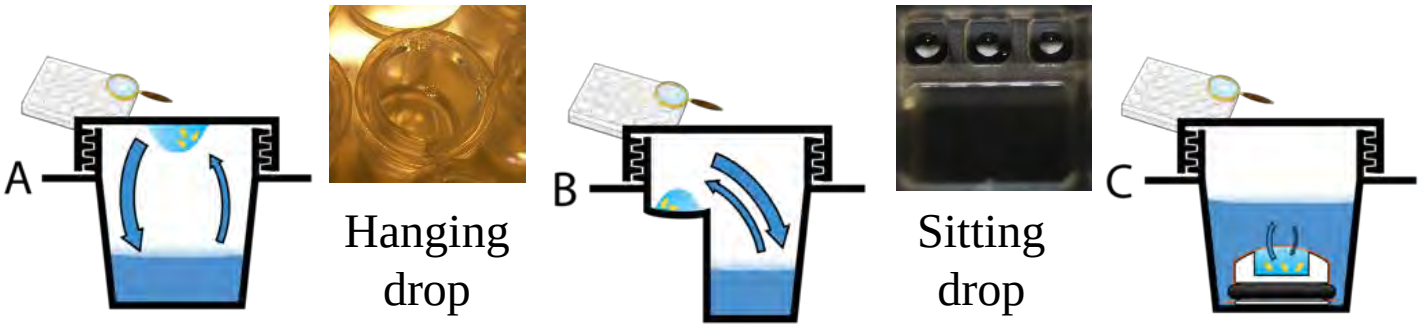
Refinement



Protein crystallogenesis

- Need pure and monodisperse protein
- Empirical
  - pH, buffer, temperature, [salt], [precipitant] dependant
- Precipitants
  - High salt concentration
  - Alcools or volatil compounds
  - Organic polymers
- Manual or robotized
- Vapor diffusion mostly used





Hanging drop

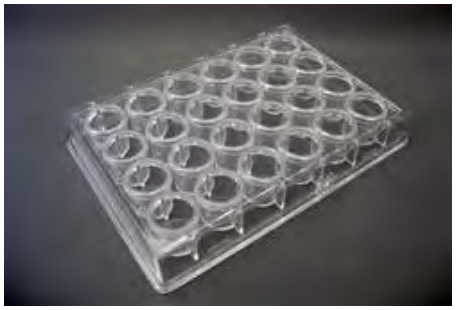
Sitting drop

microdialysis

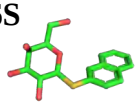
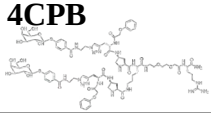
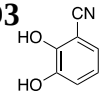
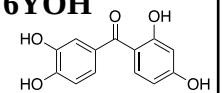
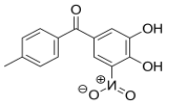
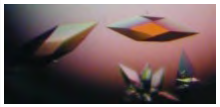
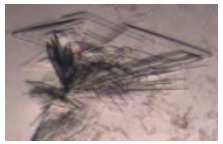
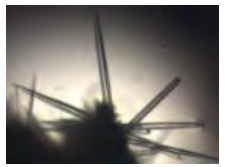
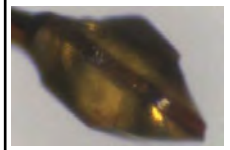
How to obtain crystals of protein complexes with ligand

Cocrystallization

- Put the ligand in reservoir
- Preincubate with the protein
  - Limit protein dilution: >1/10
  - Set duration and temperature
  - Easily reproducible & require little ligand



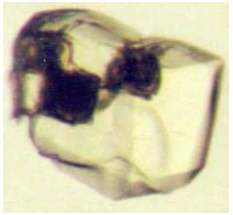
- Can have crystal and spacegroup when change ligand: LecA

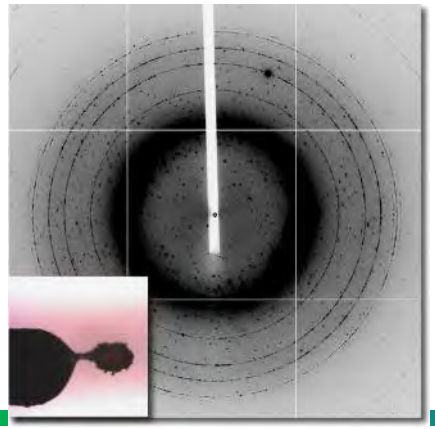
Galα1-2Galβ 2WYF	**Galα1-3Galβ1-4Glc / 2VXJ**	**Galα1-6Glc / 4AL9**	**DEG144 / 4A6S** 	**Bivalent / 4CPB** 	**Cathecol 6YO3** 	**Cathecol 6YOH** 	**Tolcapone** 
20% PEG6K 1 M LiCl 0.1 M NaAc 4	10% PEG5Kmme 25 mM KSCN 0.1 M NaAc 4.6	20% PEG2Kmme 0.2 M KBr 0.1 M NaAc 4.6	0.8 M Li_2SO4 0.1 M NaAc 4.5	10 % PEG8K 10% PEG1K 0. 2 M $MgCl_2$ 0.1 M Tris 8.5	20% PEG6K 1 M LiCl 0.1 M NaAc 4.5 1% DMSO	20% PEG6K 1 M LiCl 0.1 M NaAc 4.5 1% DMSO	24% PEG2Kmme 0.1M KSCN 0.1M NaAc 4.5
2.4 Å, $P2_1$	1.9 Å, P1 79.2 86.5 119.1 93.9 98.2 90.1	1.75 Å, P1 50.1 58.1 75.9 101.1 92.9 101	2.15 Å, $P3_221$	2.15 Å, $P2_12_12_1$	1.84 Å, I2	1.84 Å, $P2_122_1$	1.3 Å, $C222_1$
							

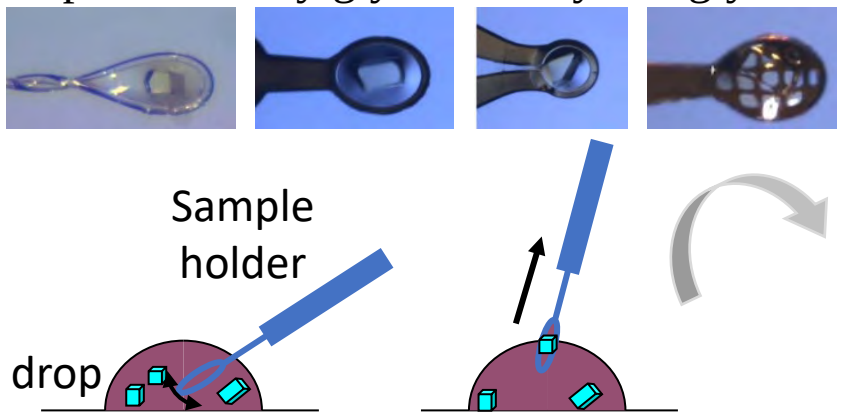
Blanchard, JMB, 2008; Nurisso, JBC, 2010; Rodrigue, Org Biomol Chem, 2013;
Blanchard, Protein, 2014; Novoa, Angewandte, 2014; Kuhaudomlarp, Angew Chem, 2021

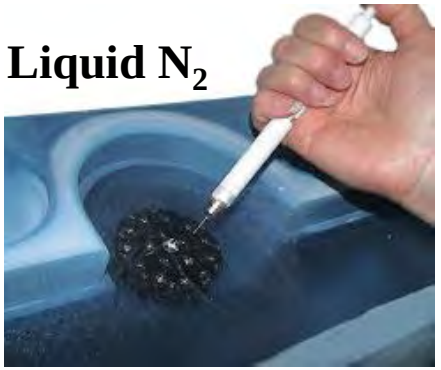
Soaking

- Dry soaking
  - Let 0.1-1µL ligand solution to dry before making drop
- Add the ligand to the reservoir solution
  - Add concentrated solution or a bit of powder
  - Transfer the crystal in solution with ligand
    - Try different concentrations and soaking time
- Soaking whilst freezing
  - Freezing limits radiation damages
  - Add ligand to the cryoprotectant solution
    - Less chance of replacement by glycerol, ethylene glycol and MPD









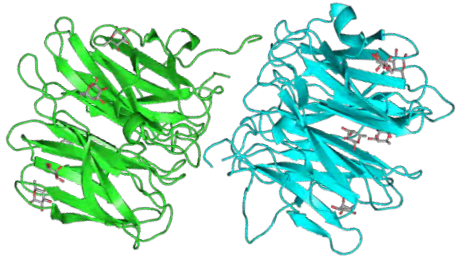
Kuhaudomlarp, Angew Chem, 2021; Mazzota, Molecules, 2023

Phasing: molecular replacement

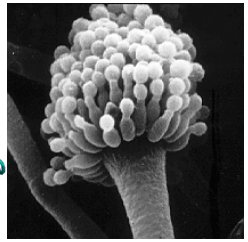
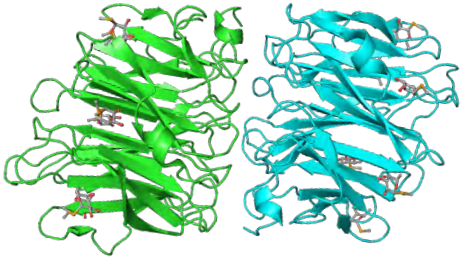
- Have a good model from homologous protein
  - Sequence identity > 25%
  - Can use alphafold if in Uniprot or fold known
  - Good conservation secondary structure: difficult for β-sheets

AAL/1OFZ/Hg
Aleuria auratia

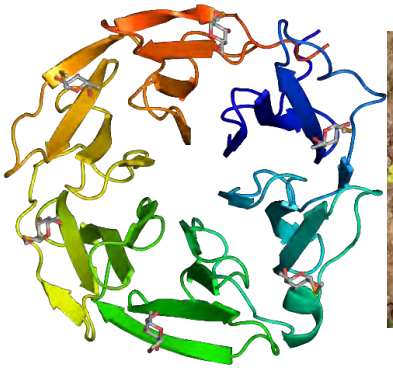


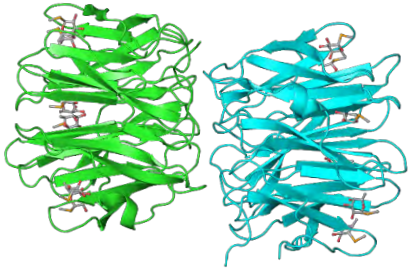


FleA/4AGI/SFU (35%)
Aspergillus fumigatus

TrfbL1/SFU (32/43%)
Terfezia boudieri

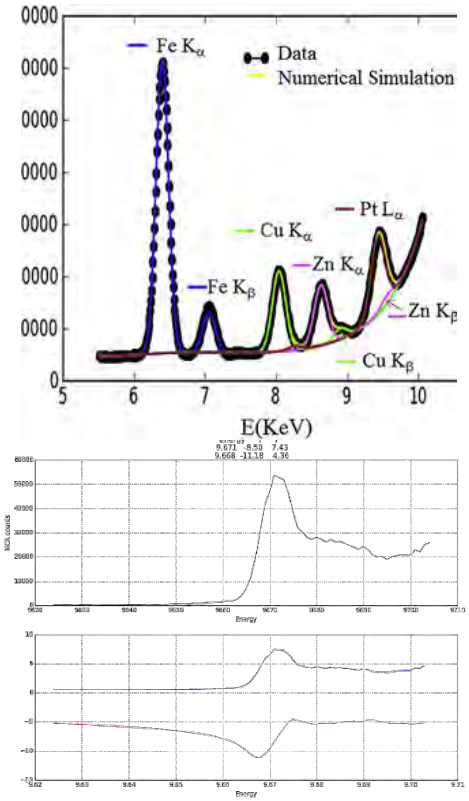




Wimmerova, JBC, 2003; Houser, PloS One, 2013, Basheer, 2023, in prep;
https://alphafold.ebi.ac.uk/

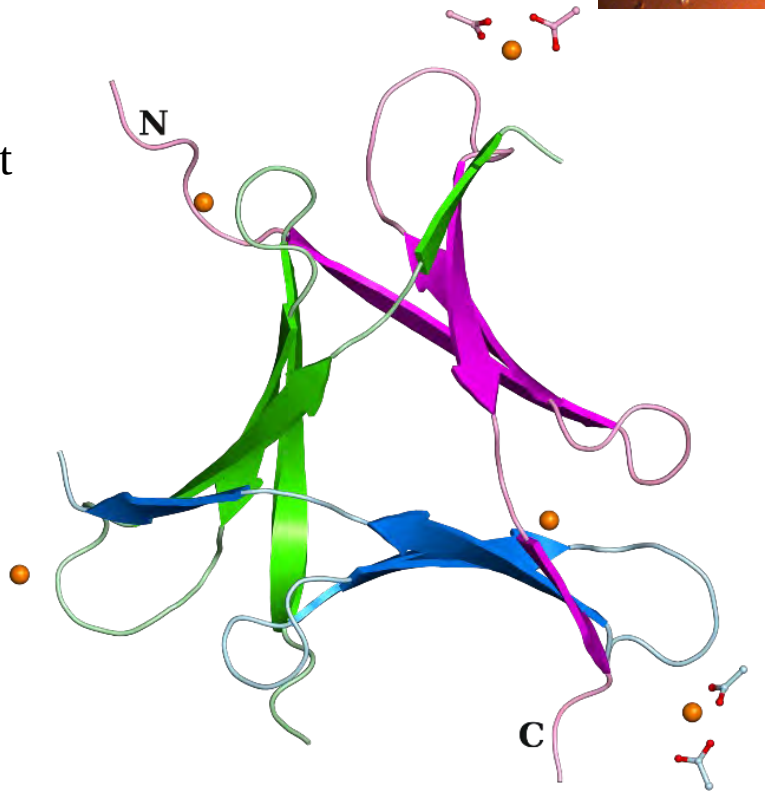
Phasing: isomorphous or anomalous

- Look in your crystallization conditions
  - PhoSL from *Pholiota squarrosa*
    - 300 mM Zinc acetate, 0.1 M Imidazole-HCl pH 6-7



X-ray fluorescent
XRF scan

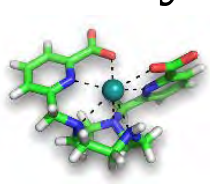
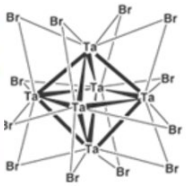
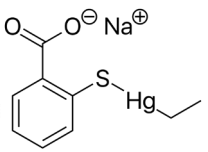
Absorption
scan



Cabanettes, Angew Chem, 2018

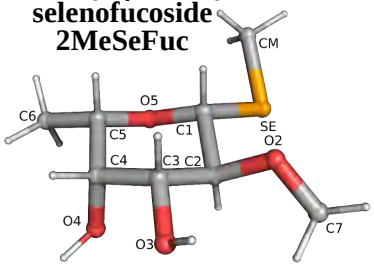
Phasing: isomorphous or anomalous-2

- Modified your protein with selenomet or selenocys
- Use heavy metals, clusters of lanthanides
- Use ligand with heavy atoms (Se, Br, S, F)

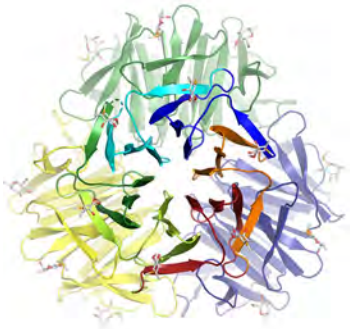




Lb-Tec2

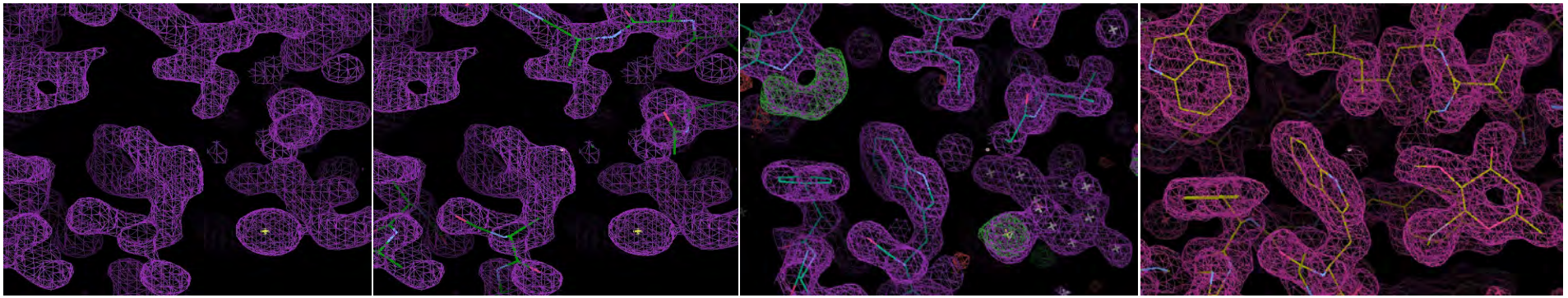
2-O-Methyl-β-methyl-L-selenofucoside 2MeSeFuc




Alex Titz
HIPS
Saarbrücken
Germany
R Sommer



Se peaks | **PolyAla building** | **Sequence assigned** | **Final**



Saboia Aragao, Proteins, 2008; Rocha, Plant Cell, 2016; Sommer, Structure, 2018;Notova, Comm Biol, 2022, https://crystallophore.fr/; Tiralongo, 2023, in prep

Carbohydrate refinement

- Find proper 3-letter code
  - Check ligand database http://ligand-expo.rcsb.org/ld-search.html

RCSB PDB
PROTEIN DATA BANK
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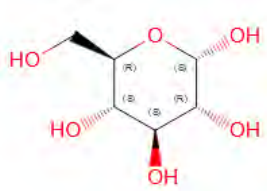
Ligand Expo Search Result Summary

Query: glucopyranose
Query type: Molecular name (exact sub-string)
Result count: 252



GLC

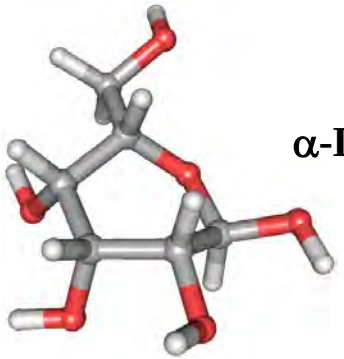
alpha-D-glucopyranose

Find entries where: GLC
- ☑ is present as a standalone ligand in 301 entries
- ☑ as a non-polymer is covalently linked to polymer or other heterogen groups 61 entries
- ☑ is present in a branched oligosaccharide 1,638 entries

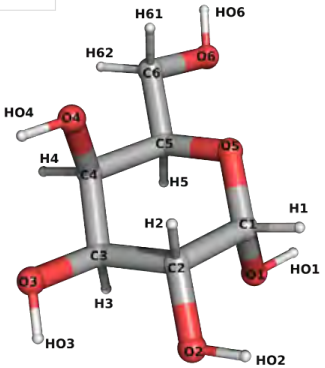
Chemical Component Summary	
Name	alpha-D-glucopyranose
Synonyms	alpha-D-glucose; D-glucose; glucose
Identifiers	(2S,3R,4S,5S,6R)-6-(hydroxymethyl)oxane-2,3,4,5-tetrol
Formula	$C_6 H_{12} O_6$
Molecular Weight	180.16
Type	D-SACCHARIDE, ALPHA LINKING
Isomeric SMILES	C([C@@H]1[C@H]([C@@H]([C@H]([C@H](O1)O)O)O)O)O
InChI	InChI=1S/C6H12O6/c7-1-2-3(8)4(9)5(10)6(11)12-2/h2-11H,1H2/t2-,3-,4+,5-,6+/m1/s1
InChIKey	WQZGKKKJIJFFOK-DVKNGEFBSA-N

Chemical Details	
Formal Charge	0
Atom Count	24
Chiral Atom Count	5
Bond Count	24
Aromatic Bond Count	0

- 1 code per anomer
  - α-D-Glucose: GLC
  - β-D-Glucose: BGC
  - β-L-Glucose: Z8T
- Check library
  - No distorsion

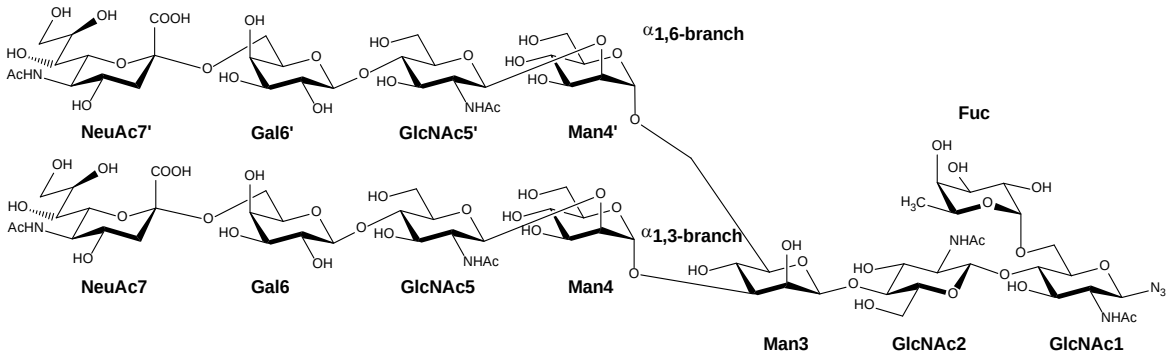


GLA
α-D-galactose

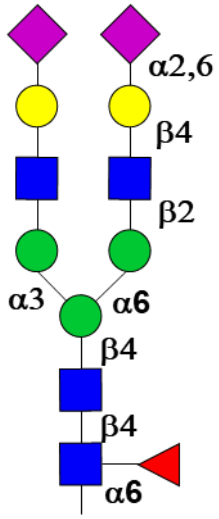


Oligosaccharide refinement

- Define correct linkage description
  - Programs do not know how to deal with L-sugars



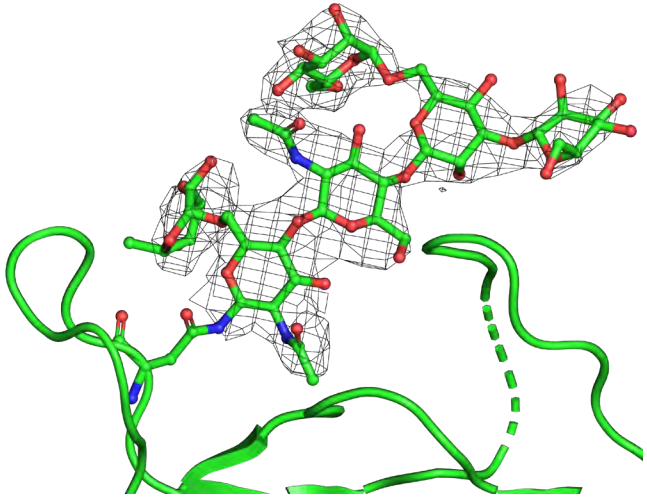
6FX3



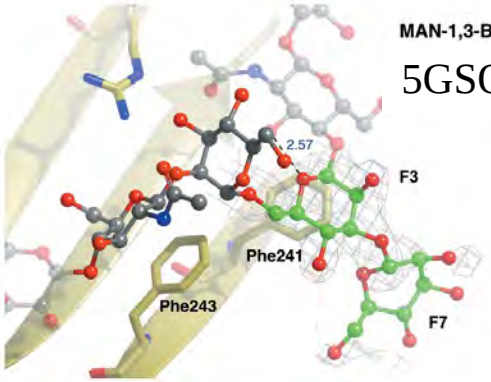

```
LINKR        O6   C4W A 102        C1   FUC A 101        BETA1-6
LINKR        O4   C4W A 102        C1   NAG A 103        BETA1-4
LINKR        O4   NAG A 103        C1   BMA A 104        BETA1-4
LINKR        O6   BMA A 104        C1   MAN A 105        ALPHA1-6
LINKR        O2   MAN A 105        C1   NAG A 106        BETA1-2
LINKR        O4   NAG A 106        C1   GAL A 107        BETA1-4
LINKR        C2   SIA A 108        O6   GAL A 107        SIA-GAL
LINKR        O3   BMA A 104        C1   MAN A 109        ALPHA1-3
LINKR        O2   MAN A 109        C1   NAG A 110        BETA1-2
```

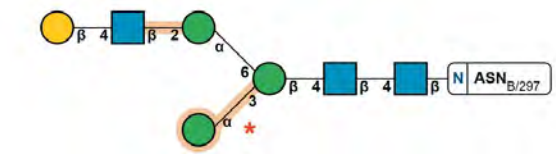
Carbohydrate validation

- Do not overfit at low resolution
- Check nomenclature, ring conformation, density fit
  - Privateer
  - PDB-REDO



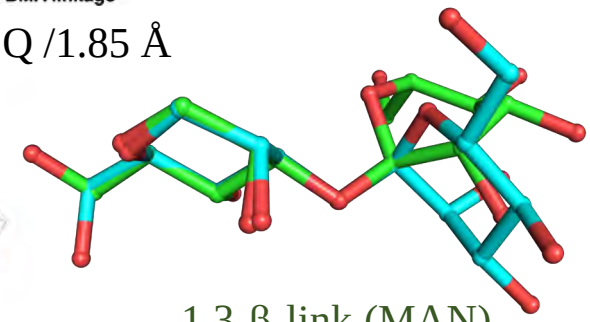
7ALK /3 Å





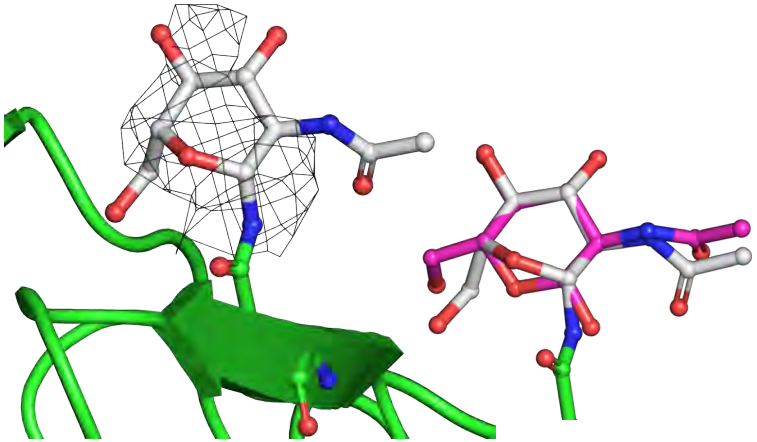
MAN-1,3-BMA linkage

5GSQ /1.85 Å



-1,3-β-link (MAN)

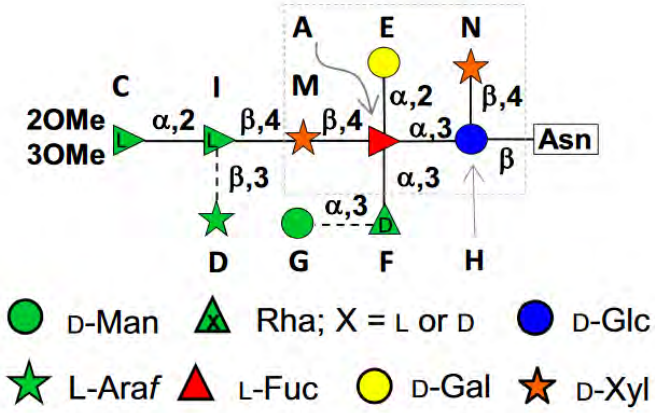
-1,3-α-link (BMA)



Agirre, Nat Struct Mol Biol, 2015; Dialpuri, Acta Crys D, 2023

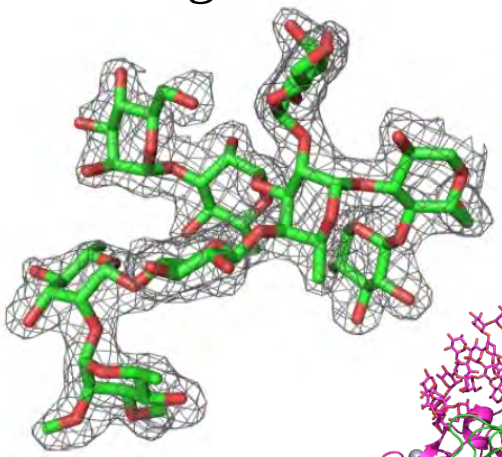
Tricky for non structural glycobiologists

- Major capsid protein (Vp54) of chlorovirus PBCV-1
  - X-ray 2002: try to fit classical N-glycans
  - Sugar NMR 2013: highly complex N-glycosylation
  - X-ray revised in 2017 + modelling



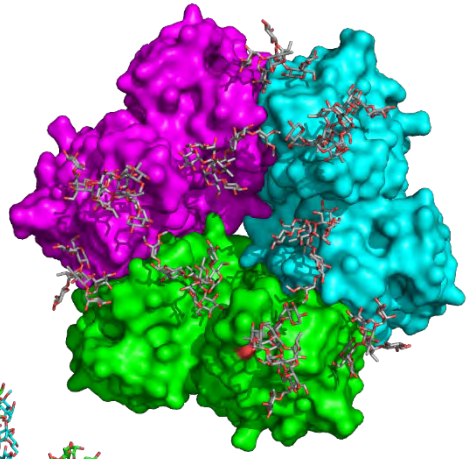
H = BGC
N/M = XYP
F = XXR
I = RM4

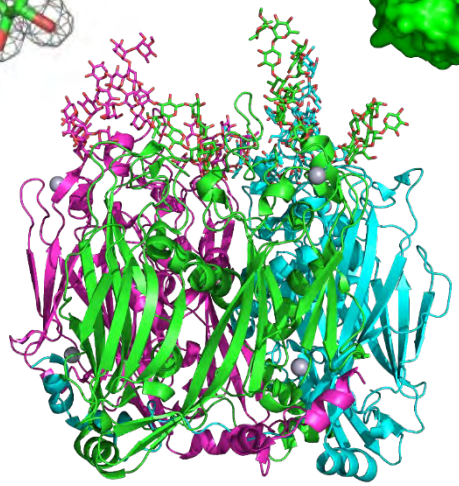
A = FUC
E = GLA
G = MAN
C = 7CV



4 glycosylation sites

5TIP

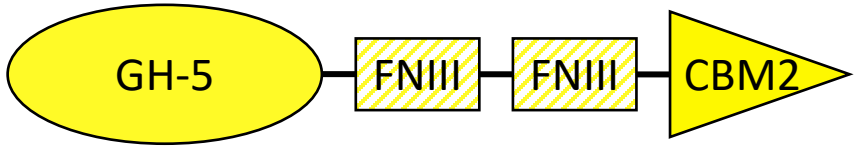


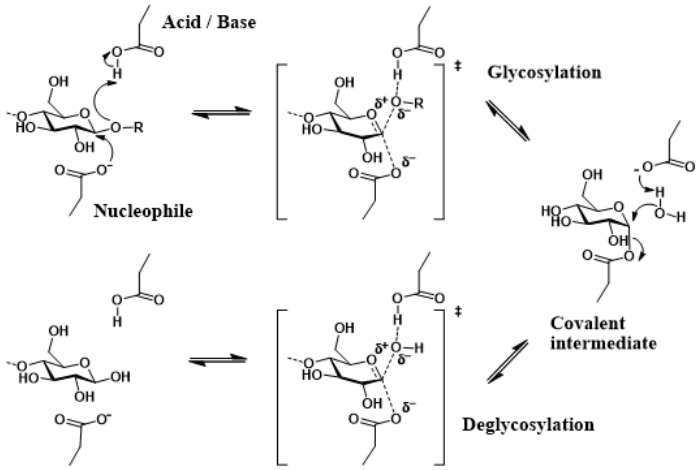


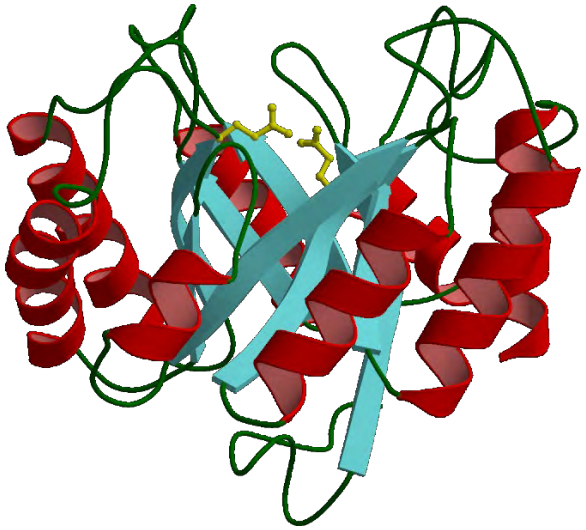
De Castro, PNAS, 2018

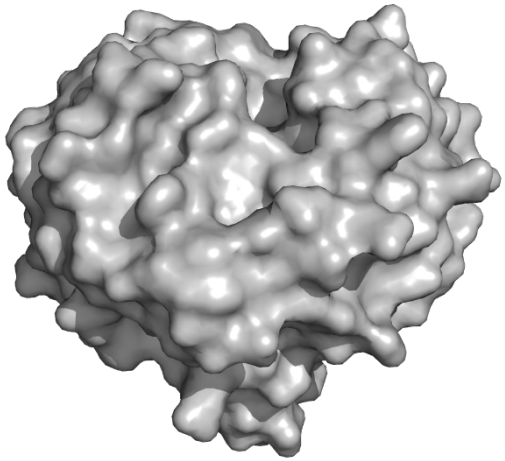
Insights in the retaining mechanism of GHs

- Cellulase Cel5A from *Bacillus agaradhaerens*
  - Modular endoglucanase
  - Active pH range 5.0-13.0
  - Glu139 (acid/base) and Glu228 (nuc)





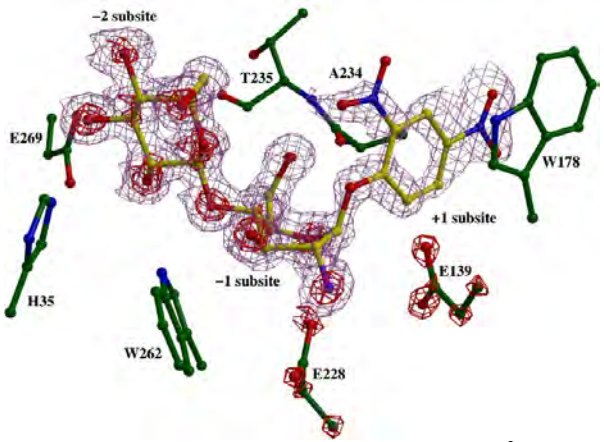




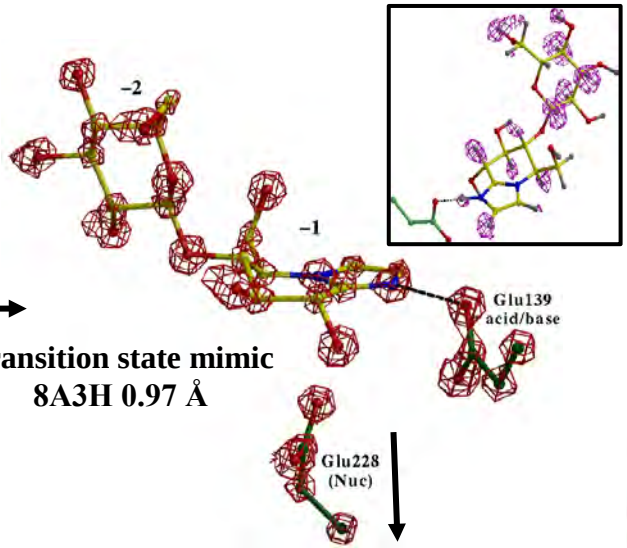
- Trapping of each step by X-ray crystallography
  - Use specific ligand, mutated protein, inactive pH

Davies, Biochemistry, 1998; Varrot, JMB, 2000; Varrot, Acta Cryst D, 2003

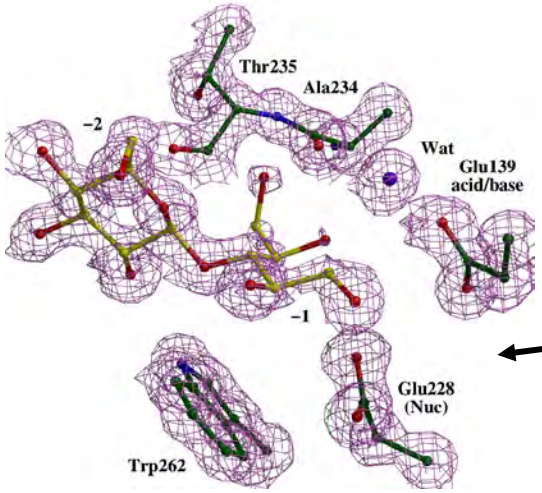
Snapshots at atomic resolution



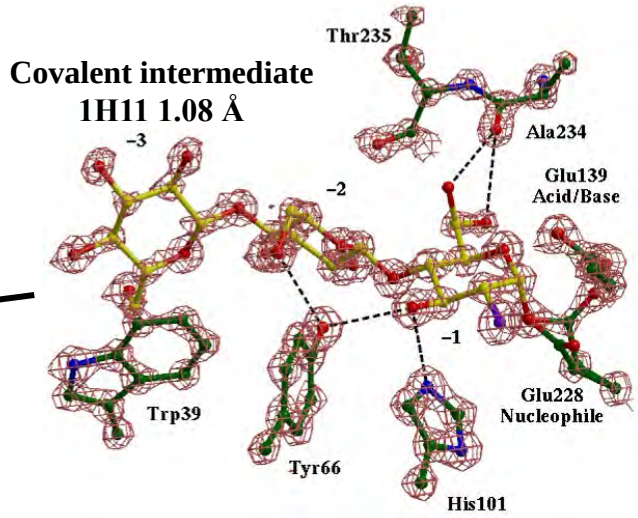
Mickaelis complex 1H2J 1.15 Å



Transition state mimic
8A3H 0.97 Å

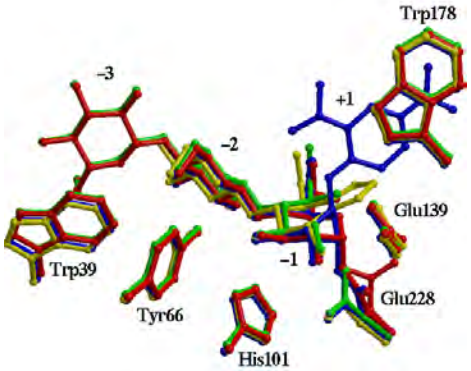


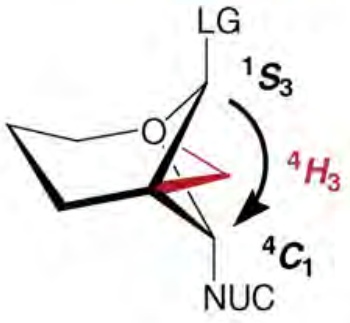
Product complex 1HF6 1.15 Å



Covalent intermediate
1H11 1.08 Å

- Static Cel5A
- Nucleophilic migration

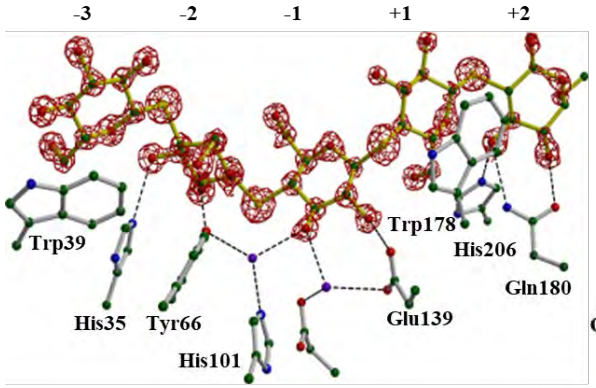




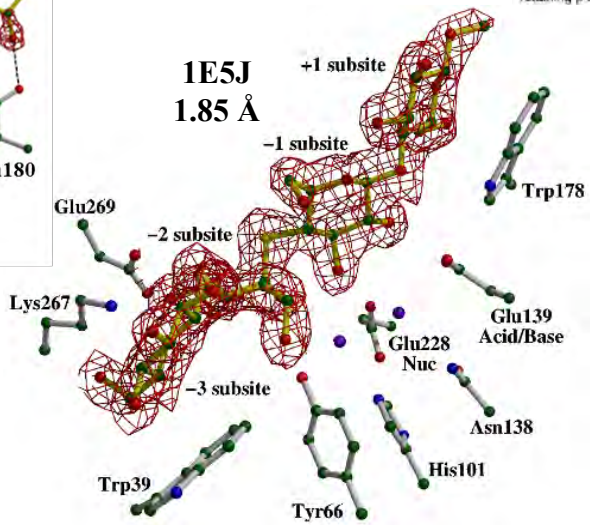
Varrot, Acta Cryst D, 2003

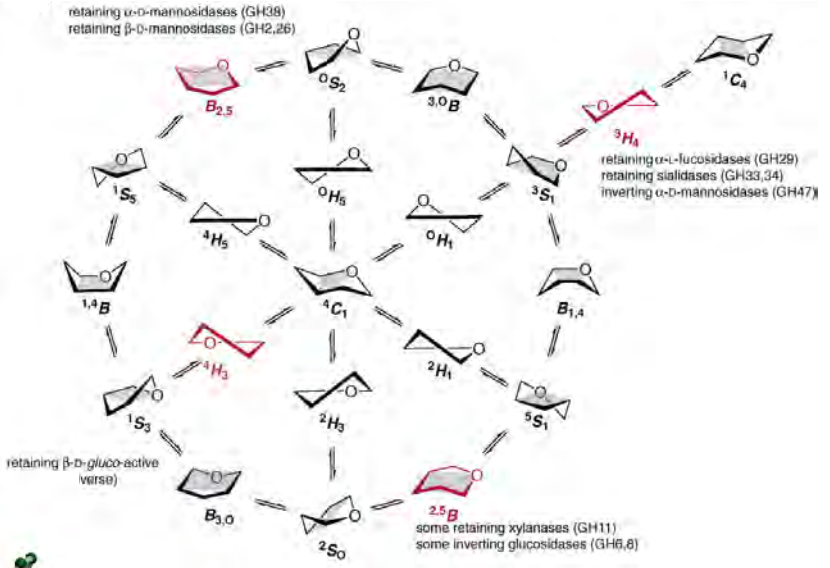
GHs inhibitors design

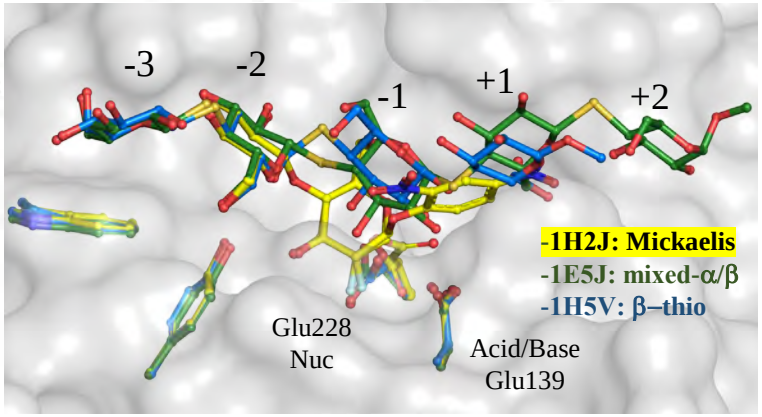
- Interconversion of sugar ring conformations
  - Highlights transition state
  - Essential for inhibitor design
- Serendipity
  - New inhibitors class for glucosidases



1H5V
1.1 Å



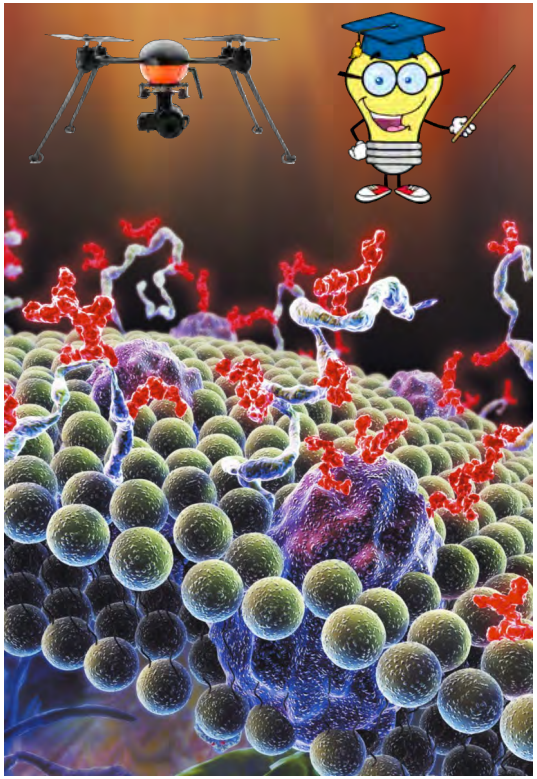




Davies, Biochem Soc Trans, 2003, Varrot Acta Cryst D, 2001; Varrot, ChemBiochem 2001

Lectins

- Ubiquitous carbohydrate binding proteins
- Specific and reversible sugar binding without modification
- Decipher the glycocode
- Implicated in many cellular processes
  - From warning their kin to poisoning their enemies
- Multivalency compensates for low affinity
  - Give ability to agglutinate cells
- New database: https://unilectin.unige.ch/



Cermav cnrs Glyco@Alps UGA Université Grenoble Alpes SIB Swiss Institute of Bioinformatics UNIVERSITÉ DE GENÈVE

Unified exploration platform for manually curated and predicted lectins

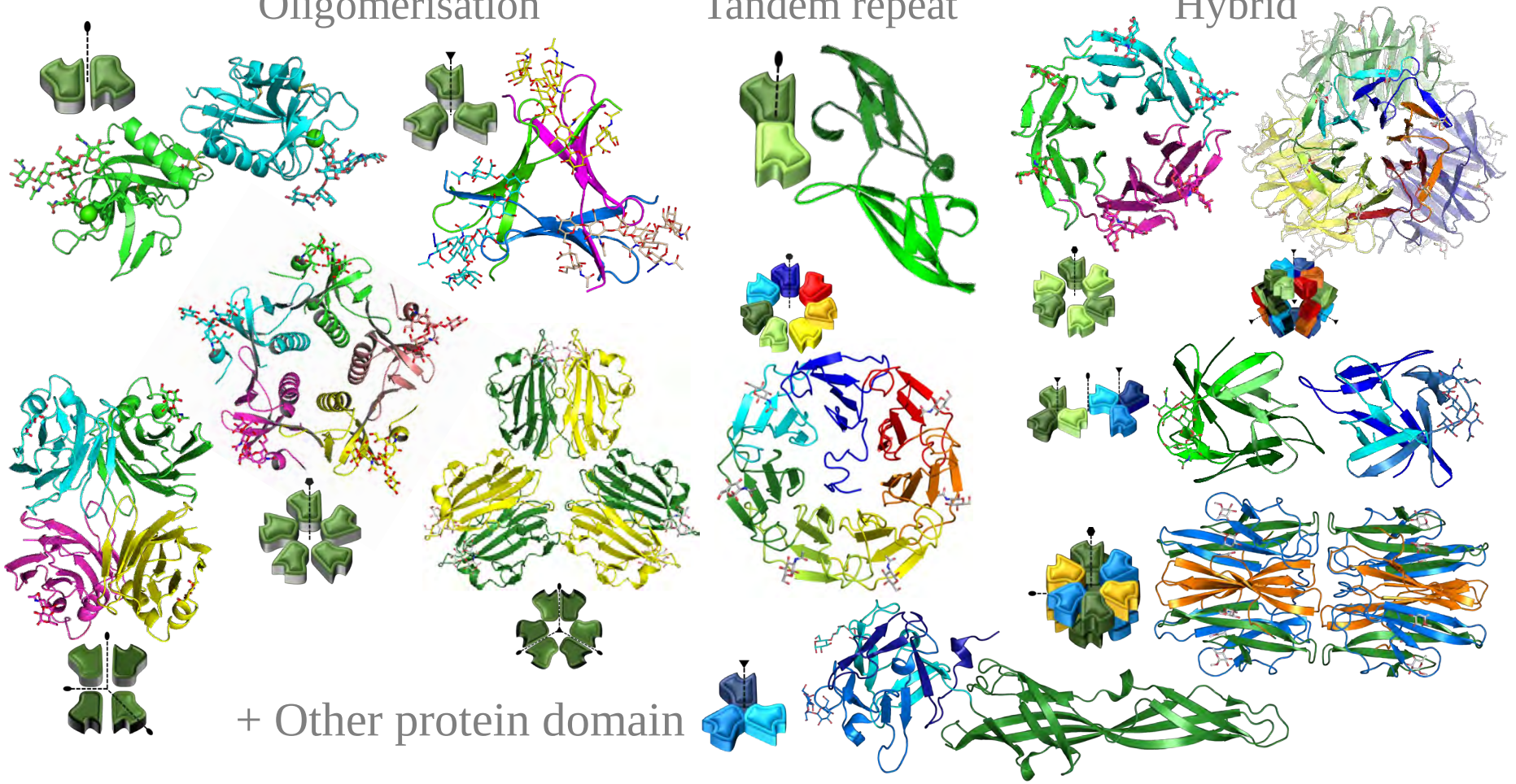
Lectin structure

- High diversity of fold and quaternary structure

Oligomerisation

Tandem repeat

Hybrid

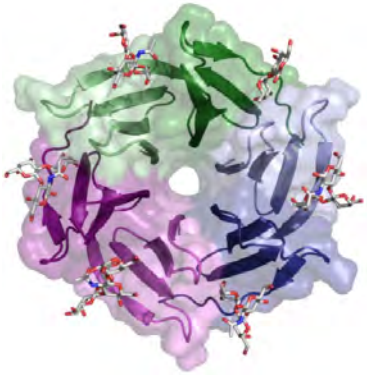


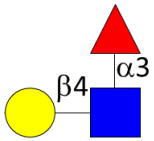
+ Other protein domain

Notova, Cur Op Struct Biol, 2020

Rare conformation

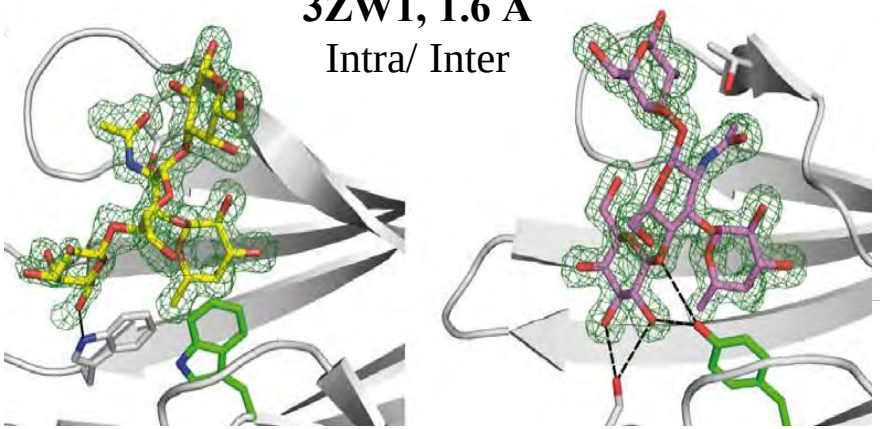
- 1 structure of lectin complex with ring distorsion
- BambL/RSL: Hidden conformation of Lewis X

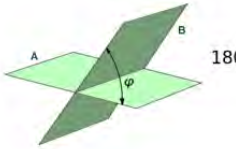




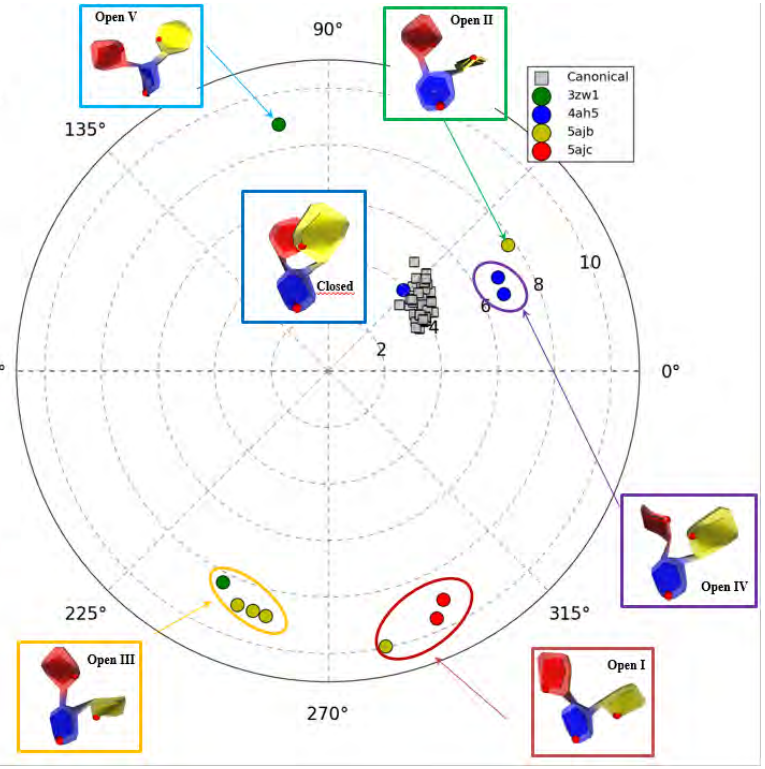
Lewis x

3ZW1, 1.6 Å
Intra/ Inter





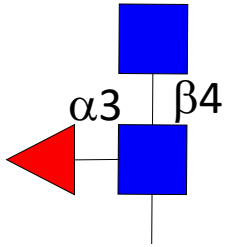
PDB analysis Polar coordinates

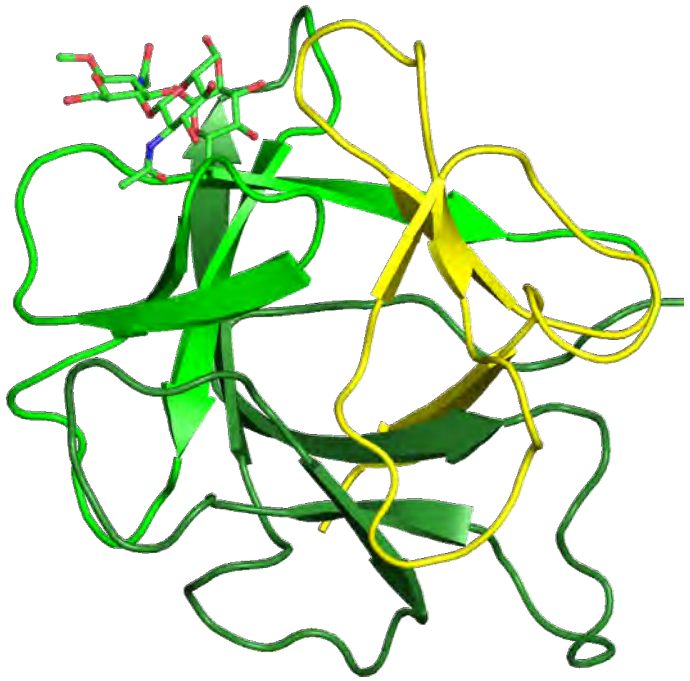


Topin, Plos One; 2013, Topin, ACS chem Biol, 2016; Lepsik, Eur J Med Chem, 2019

Multivalency and function

- CCL2 from *Coprinopsis cinerea*
  - Involved in innate immunity and defense
  - Toxic for nematodes and flies
    - Recognised 3-core fucose of midgut N-glycans



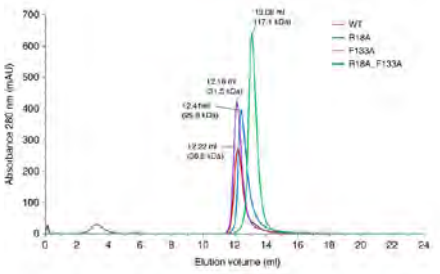


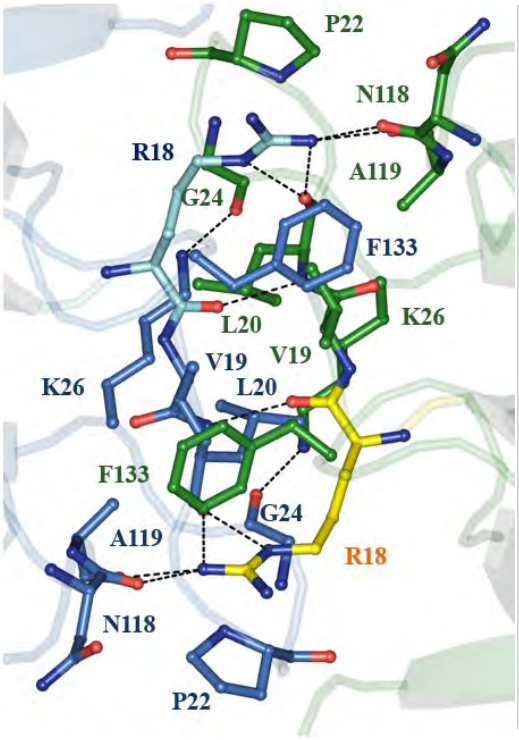
  - Monomer on Superdex75
  - RMN structure: β-trefoil fold
    - Only subsite β functional
  - Could not explain toxicity
    - Monomer and monovalent

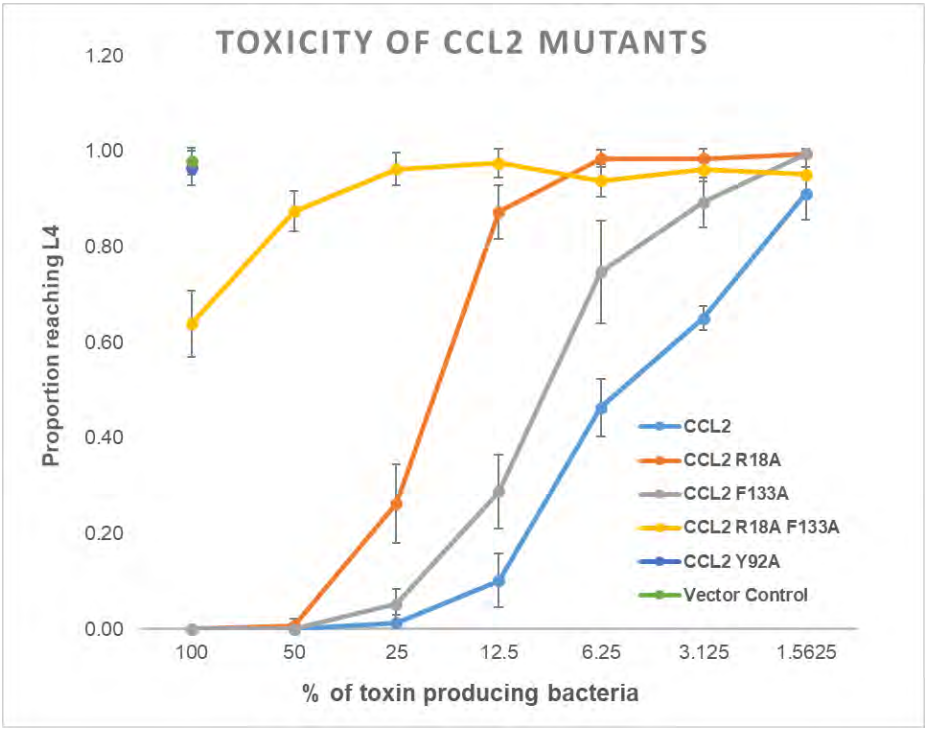
Schubert, Pmos Path, 2012; Bleuler-Martinez, Glycobiology, 2017

Multivalency and function-2

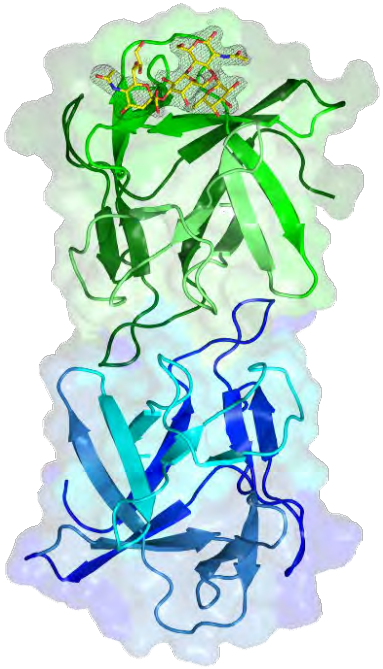
- CCL2 is a dimer
  - Confirmed by SEC on Enrich70, DLS, Native gel
  - Toxicity dependent on its dimerization
    - Disruption of dimer interface requires double mutation







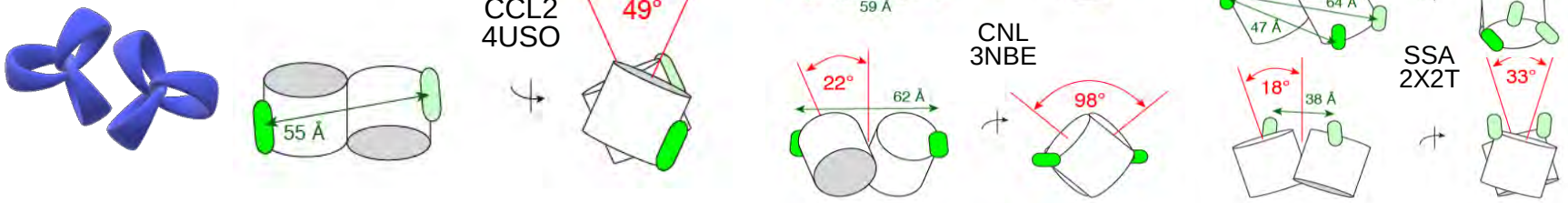
+sLe^x
4USO 1.95Å



Bleuler-Martinez, Glycobiology, 2017

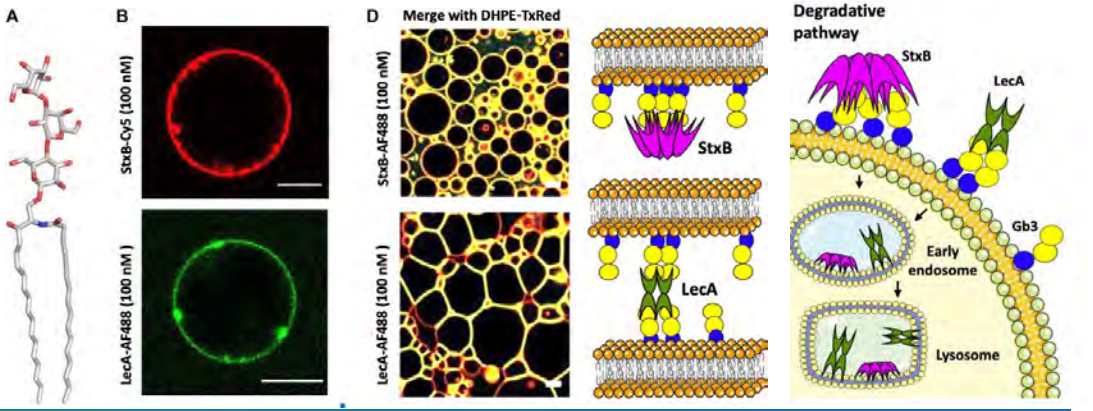
Multivalency and function-2

- Spatial distribution of binding sites impact function and recognition mode of β-trefoil lectins
  - CCL2: 1 binding surface on the side



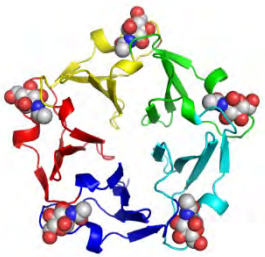
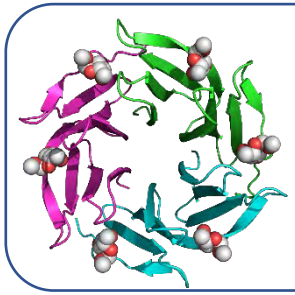
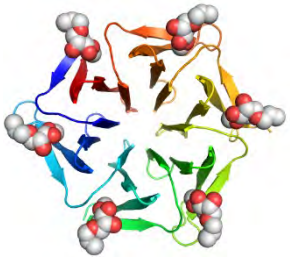
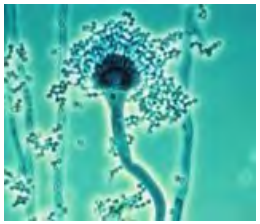
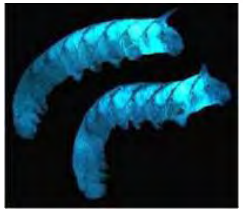
  - Others: 1/2 binding surfaces on the top

- Reorganisation of membrane glycoconjugates
  - Clustering of glycolipids
  - Crosslinking
  - Change in membrane dynamics
  - Endocytosis

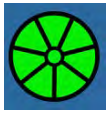


Bleuler-Martinez, Glycobiology, 2017, Siukstaite, Front Mol Biosci, 2021

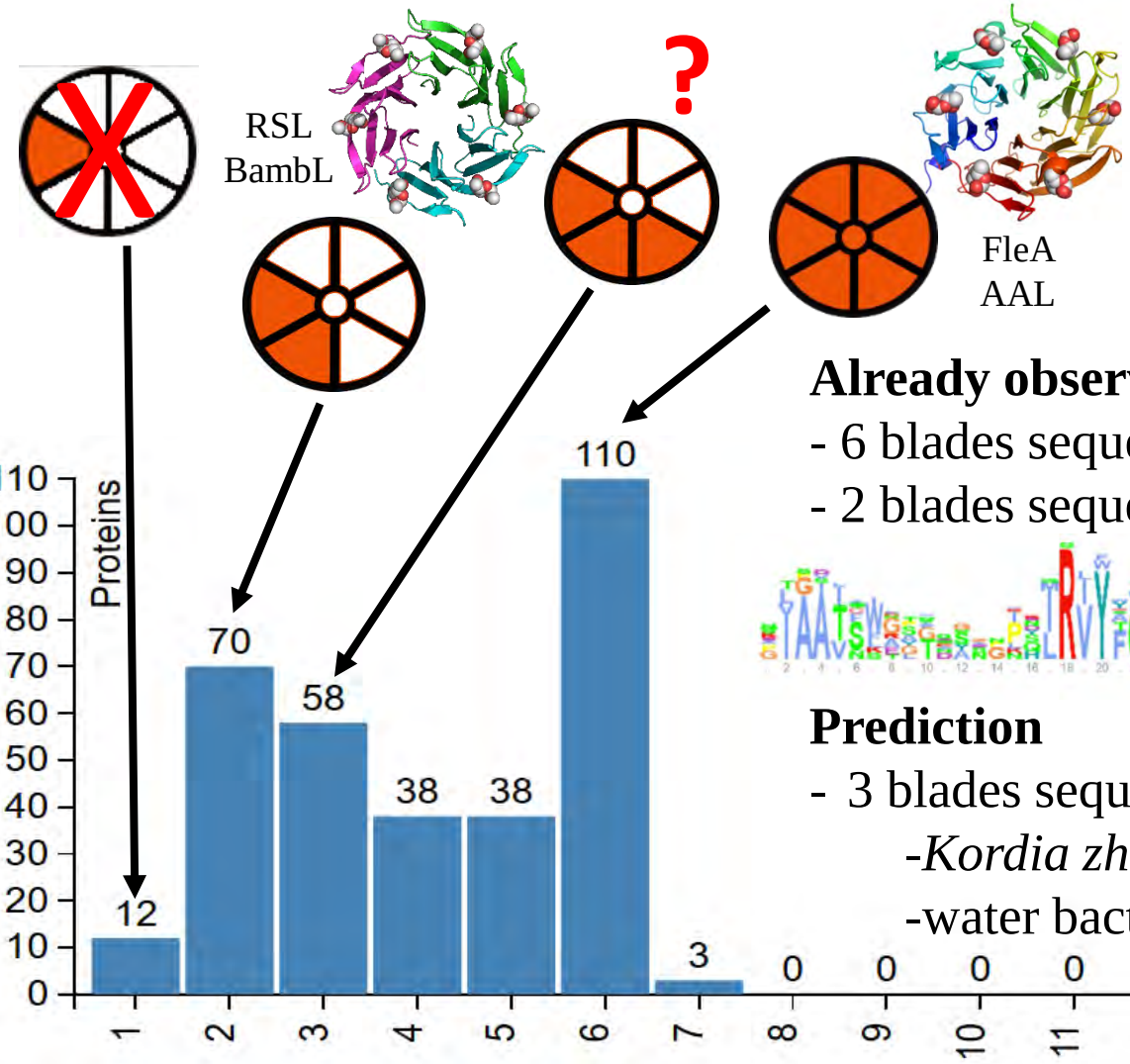
Lectins with β-propeller fold

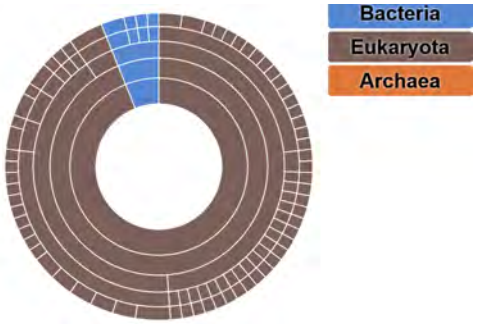
Tachylectin 2 GlcNAc	RSL fucose	FleA fucose	Tectonin/Lb-Tec2 Me-sugar	PLL fucose	PVL GlcNAc
					
					
Tachipleus tridentatus	*Ralstonia solanacearum*	*Aspergillus fumigatus*	*Laccaria bicolor*	*Photorhabdus luminescens*	*Pstatyrella velutina*
Beisel *et al.* *EMBO J.* 1999	Kostlanova *et al.* *J. Biol. Chem* 2005	Houser J, Plos One, 2013, 8:e83077	Sommer *et al.* *Structure* 2018	Kumar *et al.* *J. Biol. Chem* 2016	Cioci *et al.* *J. Mol. Biol.* 2006

- Define blade signature for prediction: PropLec in Unilectin

PropLec5A FLF	PropLec6A RVY	PropLec6B GVN	PropLec7A EVF	PropLec7B GFG	PropLec7C
					

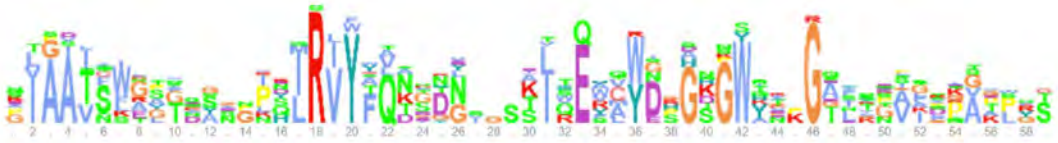
Distribution of predicted blade in PropLec6A





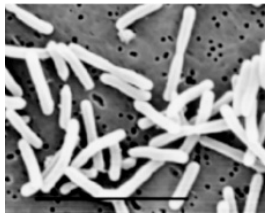
Already observed

- 6 blades sequences in fungi
- 2 blades sequences (fold : 3 x 2 blades)



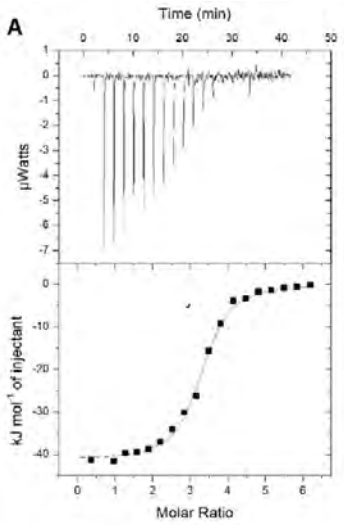
Prediction

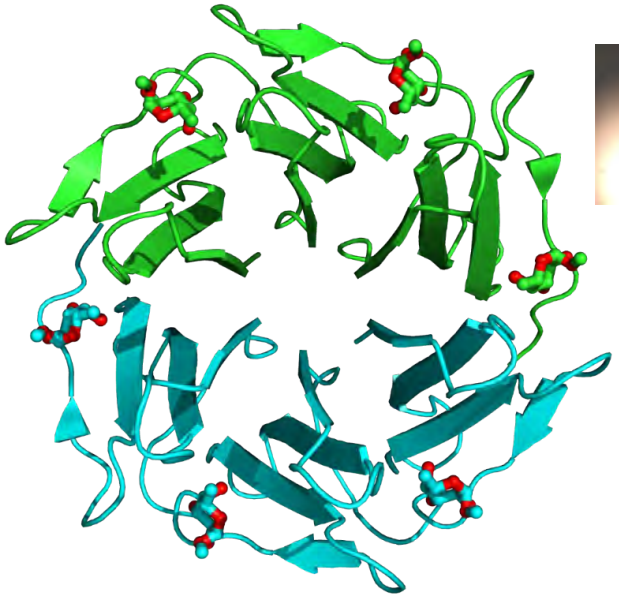
- 3 blades sequences (fold : 2 x 3 blades)
  - *Kordia zhanghouengsis*
  - water bacteria that eats algi



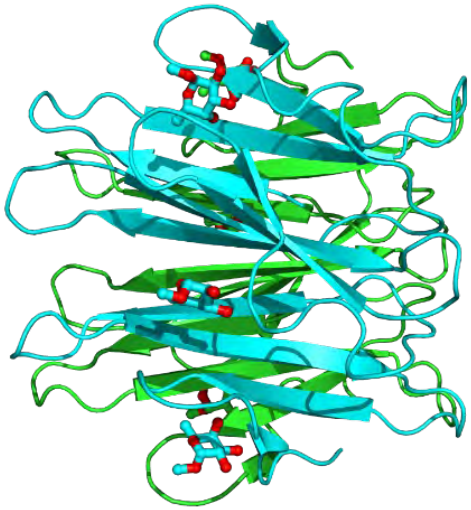
Recombinant KozL

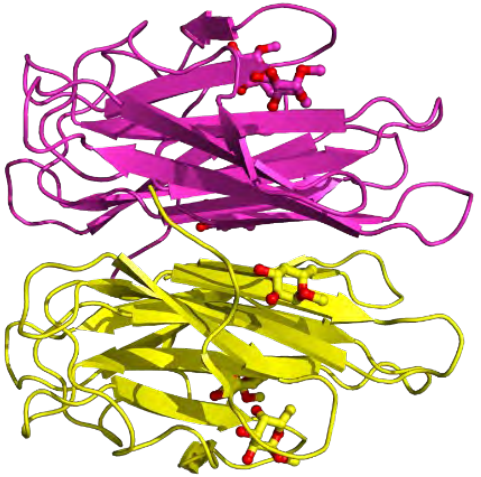
- Overexpressed in *Escherichia coli*
  - Purified on mannose agarose: 35 mg/L
  - ITC: 3 fucose binding sites
  - Tetramer by AUC
- Structure solved by SAD using αSeMeFuc
  - **Prediction corroborated:** 2 x 3 blades





6HTN
1.55 Å

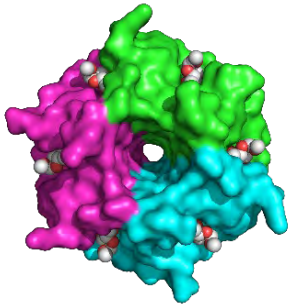




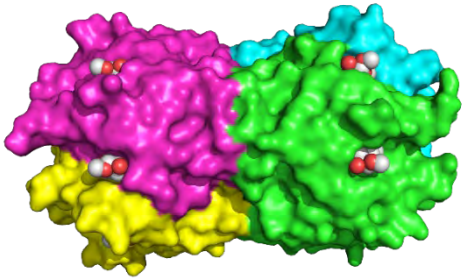
Bonnardel, Structure, 2019

PropLec6: many arrangements

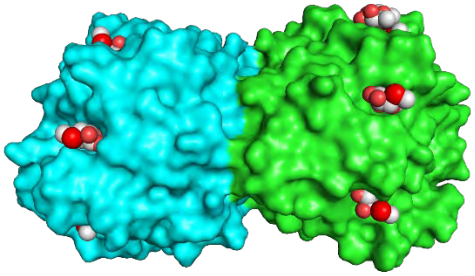
BambL/RSL
6 Fuc



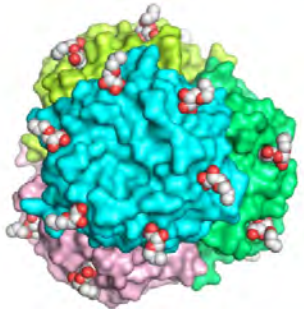
KhozL
12 Fuc



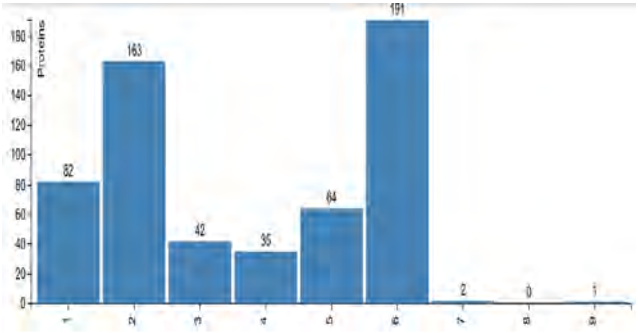
FleA/ SapL1
12 Fuc



Lb-Tect2
24 Me-sug



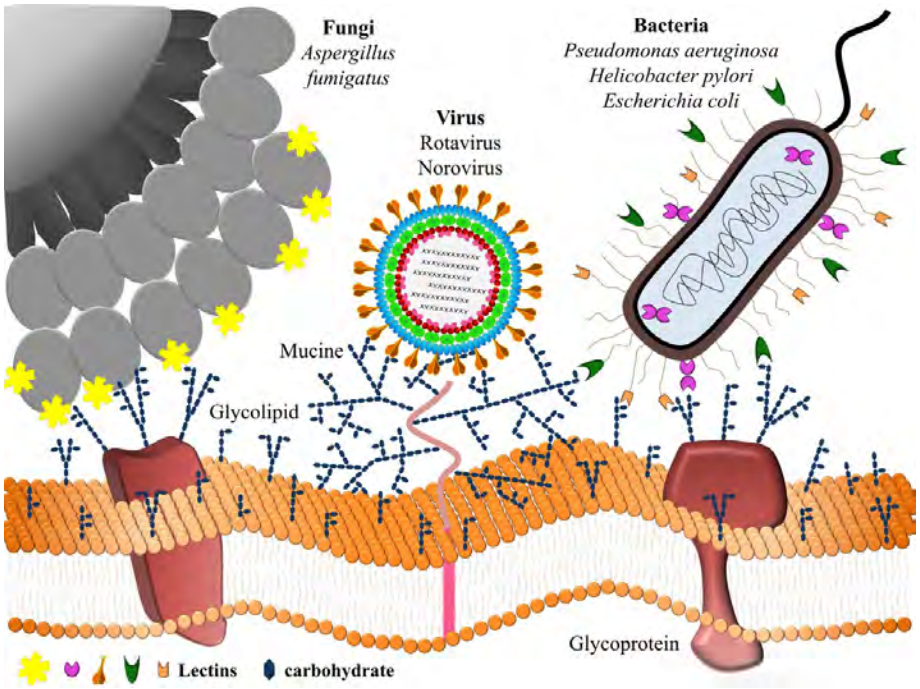
- Is 1 blade occuring?
  - Fold: 6x1 blade

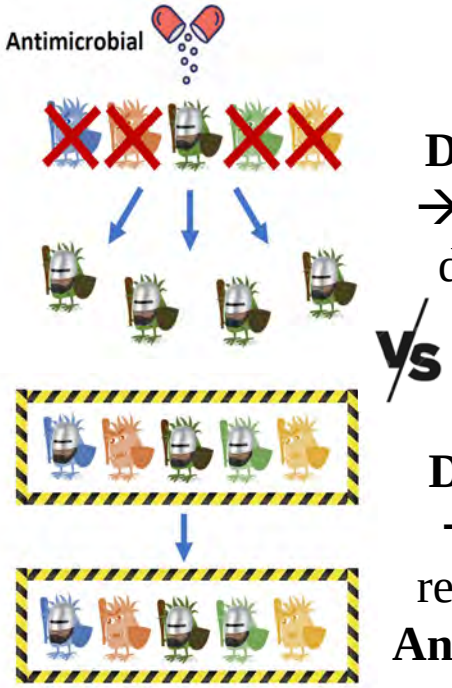


Audfray, JBC, 2012, Houser, Plos One, 2013; Sommer, Structure, 2018; Bonnardel, Structure, 2019, Martinez-Alarçon, Scie Rep, 2021

Lectins as therapeutical targets

- Lectins used host glycoconjugates as entry points
  - Mediate host recognition and adhesion
  - Blocking lectins → prevent infections





Classic:
Destroy pathogens
→ selective pressure driving **resistance**

vs

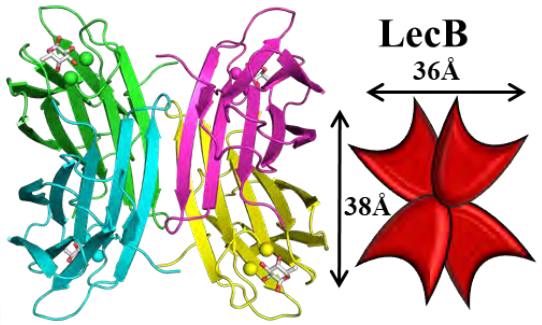
Alternative:
Disable pathogens
→ **Resistance** not required for survival
Antiadhesion therapy

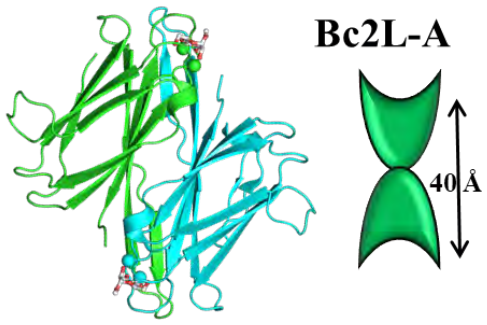
→Development of glycocompounds as antimicrobials

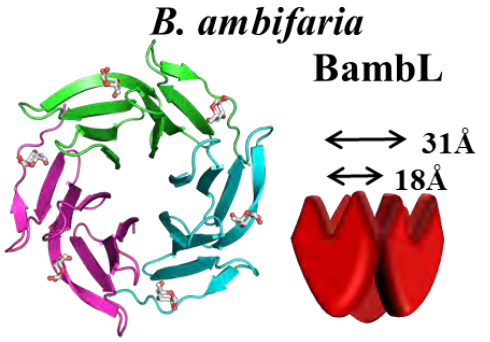
Ofek et al, FEMS Immunology & Medical Microbiology, 2003, 33: 181; Heggelund J, Curr Op Struct Biol, 2017, 44:190

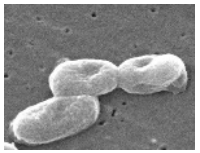
Lectin targets

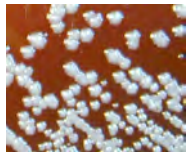
➢Opportunistic pathogens → bronchopulmonary infections







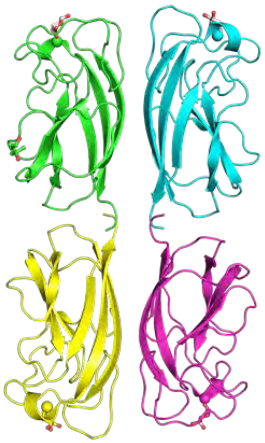


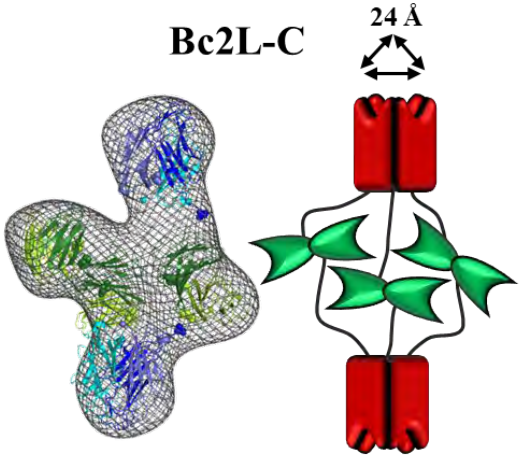


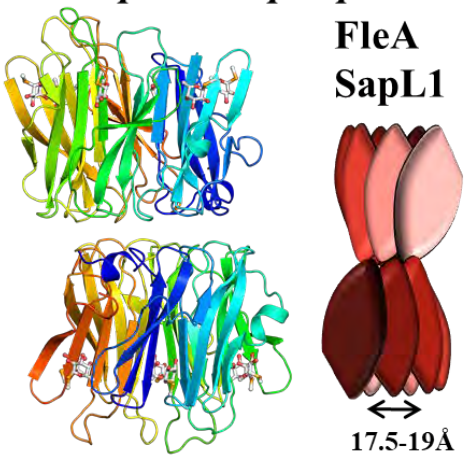
Pseudomonas aeruginosa

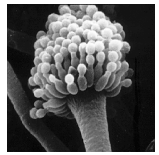
Burkholderia cenocepacia

Aspergillus fumigatus
Scedosporium apiospermum

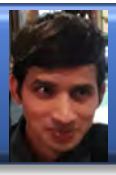






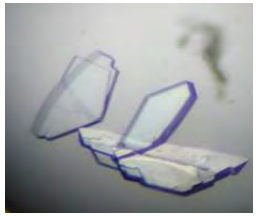


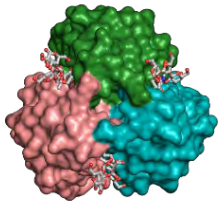
Bc2lC-nt

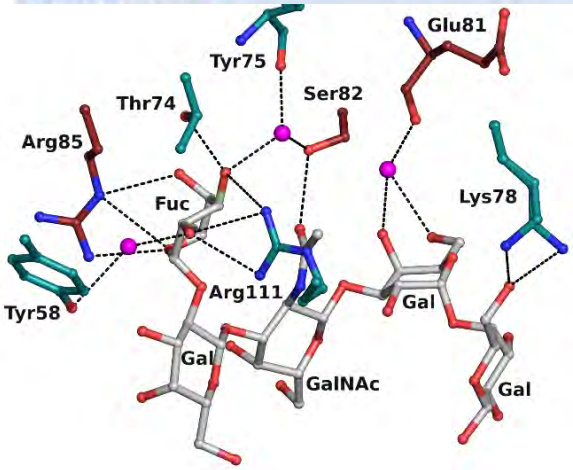



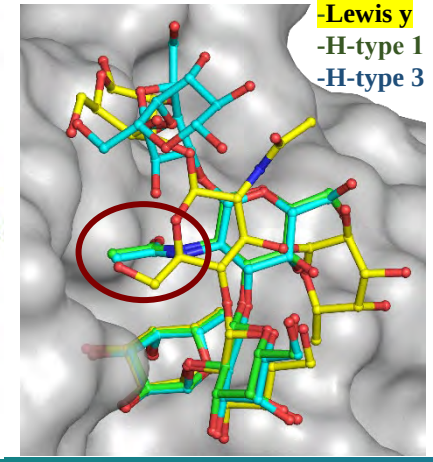
- New construct 1-132 in pCold-TEV: good yield & stable
- Complex with oligos by cocrys and with inhibitors by soaking

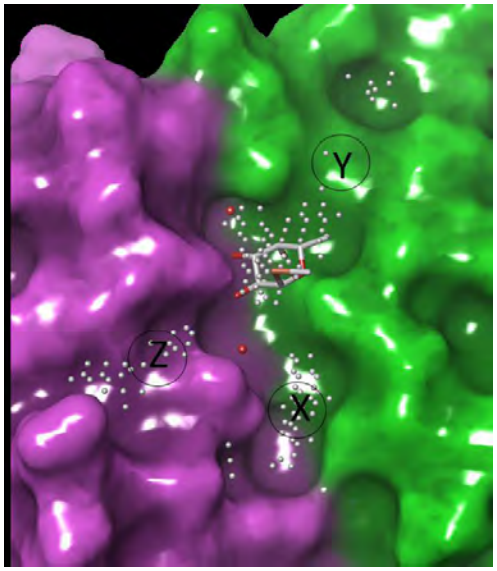
Ligand	k_D (µM)	PDB	Res (Å)
L-Fuc	2430	2WQ4	1.42
L-Gal	2000		
Disac	2500		
H-type 1 tri	25.4		
H-type 1 tetra	56.6	6TID	1.6
Lewis Y penta	53.2	7OLU	1.6
H-type 3-GloboH hexa	26.7	6TIG	1.9

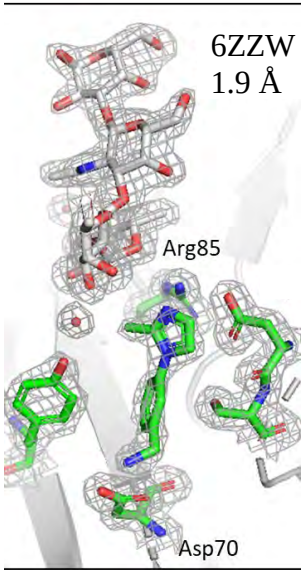








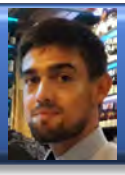




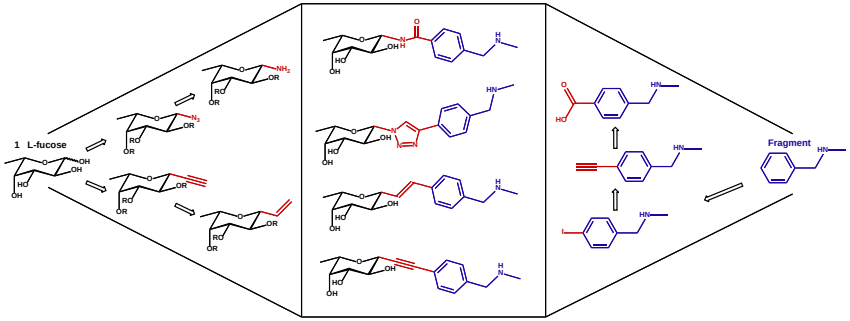
- 3 druggable sites predicted with SiteMap in Maestro

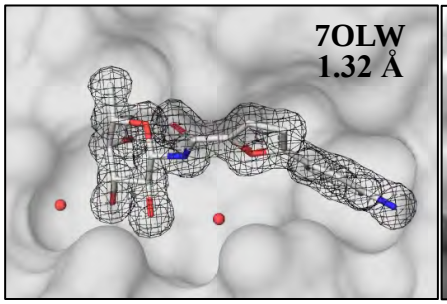
Sulak *et al*, PloS path, 2011, Bermeo *et al*, Molecules, 2020, 25:248; Lal *et al*, Chem. Eur. J. 2021, 27, 10341

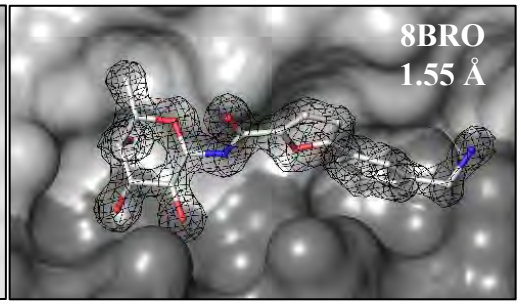
Bc2LC-nt inhibitors



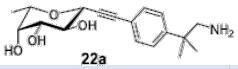
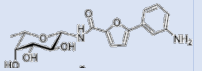
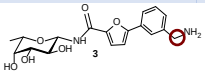
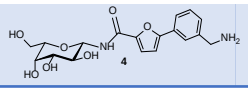
➢ Design, synthesis & evaluation of fucosides derivatives

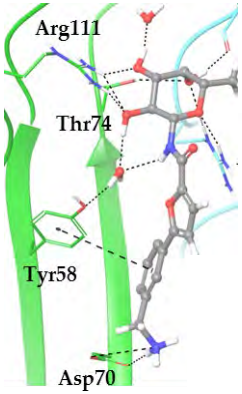




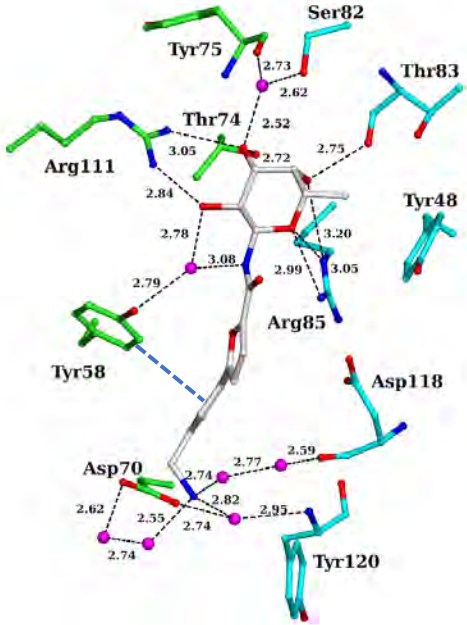


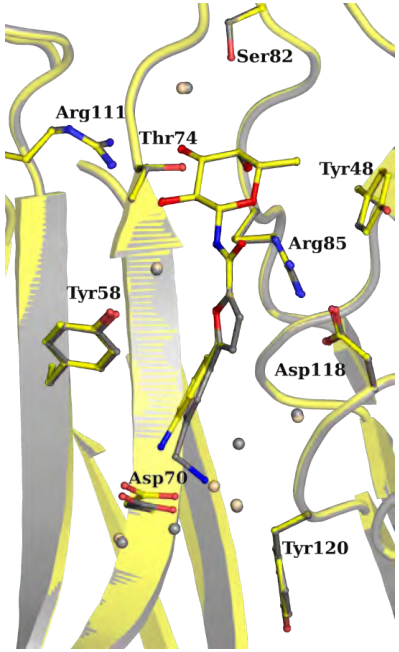
- Transform aniline in aminomethylene
  - One order of magnitude gain
  - Terminal amine does indirect Hbonds

Ligand	k_D (µM)	PDB	Res (Å)
L-Fuc 	2700		
22a 	280	7OLU	1.6
8c 	nd	7OLW	1.32
3 	159	8BRO	1.55
4 	390		



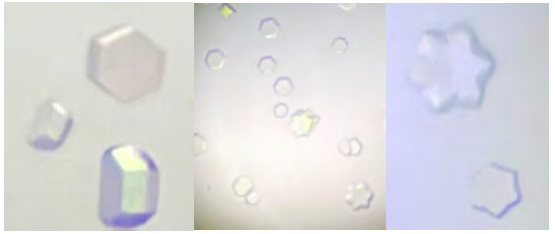
Modelling

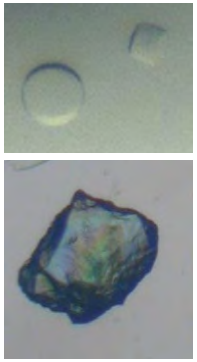




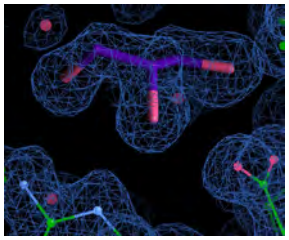
Bermeo, ACS Chem Biol, 2022; Mazzota, Molecules 2023; Antonini, Frontiers, 2023, in press

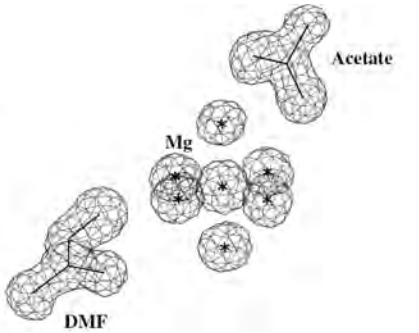
Miscellaneous





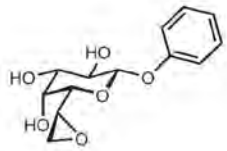
- Crystal shape can be misleading
  - Bc2lCnt 0.9-1.2 Trisodium citrate pH 7
    - Hexagons OK - flower not OK
  - Coda: 1 crystal after several month and broken coverslip
    - Not multiple, 1.3 Å

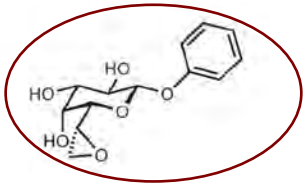


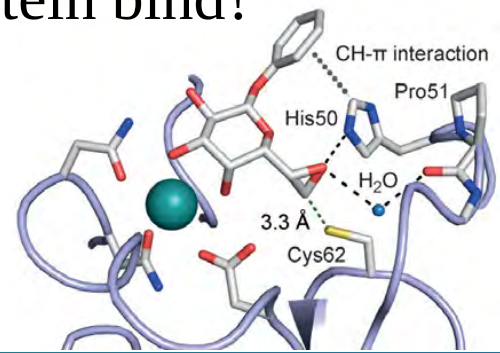


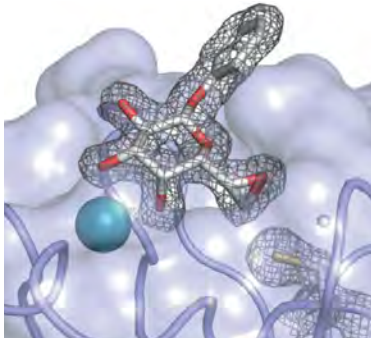
- Components from purification or crystallisation
  - Electron density of chemical can help
    - Glycerol from cryo in TrfbL1 native
    - Cel6A D405N-SDP5: 1OC7, 1.1Å

- Which diastereoisomer did the protein bind?
  - LecA with potential covalent epoxide
    - Crystal at unreactive pH : 4.5









Varrot, Structure, 2003; Gourdine, JBC, 2008; Wagner, Angewand Chem, 2017

Conclusions

- Still need for X-ray crystallography to gain access to protein-sugar interactions at the atomic level
  - Highest the resolution the better to draw accurate conclusions
    - Distorsion / High energy conformation
      - Real or artefact from user/program errors
  - Binding site location and architecture
    - → Structure based drug design
    - → Site directed mutagenesis: new specificity
  - Quaternary structure and multivalency
    - → Gain fondamental knowledge on protein folding and function
    - → Design new molecular tools

- Could be tricky for non glycobiologists
  - No « Structural glycobiology for dummies »
  - Do not hesitate to contact experts

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