Synthesis of Carbohydrate Based Therapeutics
~ Toward Automated Synthesis of Oligosaccharides ~

040922 Reiko Wada (D2)

Introduction

Carbohydrates play a major role in cellular recognition and signal transduction causing inflammation, immune response, metastasis, bacterial and viral infection and so on. In spite of its important biological activities, major impediment to carbohydrate research has been the difficultues in both synthesis and characterization. Today's seminar will cover recent developments toward automated synthesis of oligosaccharides, in particular therapeutics.

Difficulties Met with Carbohydrate Research

1. Synthesis
   - stereoselectivity (α/β)
   - regioselectivity (differentiation of OH groups)
   - protecting group manipulations

2. Analysis
   - branched structures : complicated structural characterization
   - microheterogeneity : diffucult to define biologically relevant motif
   - no genetic information : Enzymes compete to produce diverse products.

↕

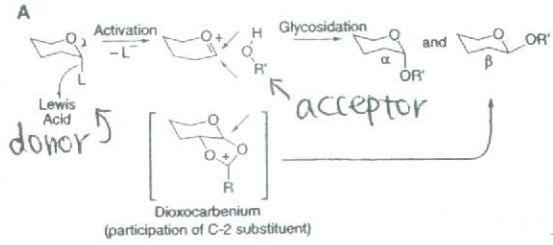
other biopolymers : DNA and proteins

Automated synthesis of these biopolymers has enabled a great progress in research in these fields.

Contents

I Synthesis of Oligosaccharides

1. General Mechanism of O-Glycosidation



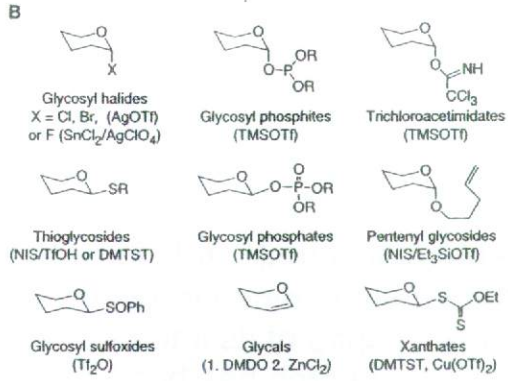
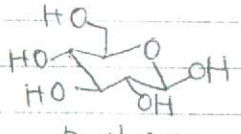


Fig. 1. (A) Common mechanisms for glycosidation. (B) Commonly used glycosidation reagents and their activators (in parentheses). Some of these glycosidation reagents can be used orthogonally. For example, the activator for glycosyl fluorides or phosphites will not activate thioglycosides or pentenyl glycosides. Abbreviations are as follows: DMDO, 3,3-dimethyldioxirane; DMTST, dimethylthiosulfonium triflate; Et, ethyl; L, leaving group; NIS, *N*-iodosuccinimide; R, variable group; OTf, triflate; TfOH, triflic acid; and TMSOTf, trimethylsilyl triflate.

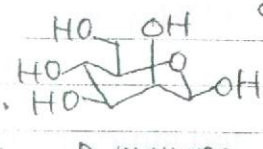
Most frequently used donors

1. glycosyl halides (koenigs-knorr method)
   reactive, but unstable. Difficult to control stereoselectivity.
2. Trichloroimidates
   Stable. α and β isomers can be separately prepared. Cheap activator.
3. Thioglycosides

General rules of stereoselective O-glycosidation (X=halide for simplicity)



D-glucose

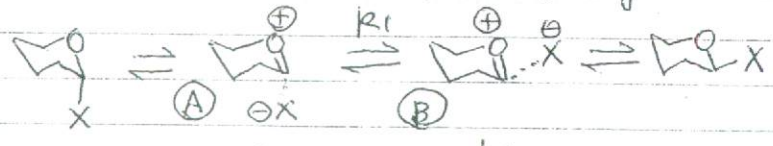


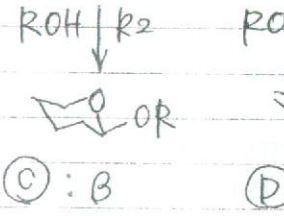
D-mannose

表 3・1 O-グリコシドの分類と合成法

1,2-*cis*	α-グルコ型	(1) *in situ* anomerization 法
	β-マンノ型	
		(2) S_N2 型反転
1,2-*trans*	α-マンノ型	(3) 隣接基関与
	β-グルコ型	

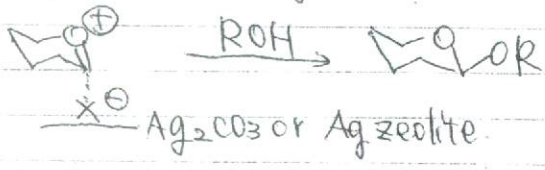
(1) in situ anomerization, : α linkage





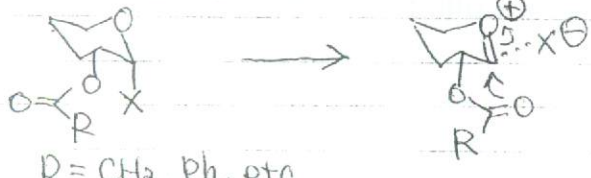
- reactivity Ⓐ < Ⓑ (anomer effect)
- $k_1 \gg k_2$ ⇒ Ⓐ → Ⓑ → Ⓓ α selective.
- high temp., strong activator
  ⇒ Ⓐ → Ⓒ β dominates.

(2) SN2 type : β linkage

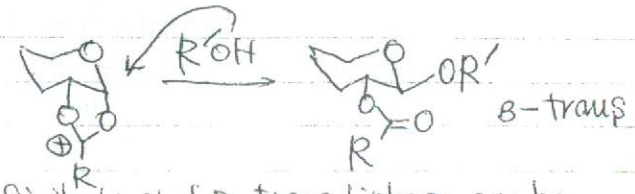


Equilibrium between Ⓐ and Ⓑ ((1)) is inhibited by stabilization of $X^{\ominus}$ to insoluble metal surface.

(3) neighboring group participation. (1,2-trans)



R = CH_3, Ph, etc



* Similarly, α-1,2-trans linkage can be constructed.

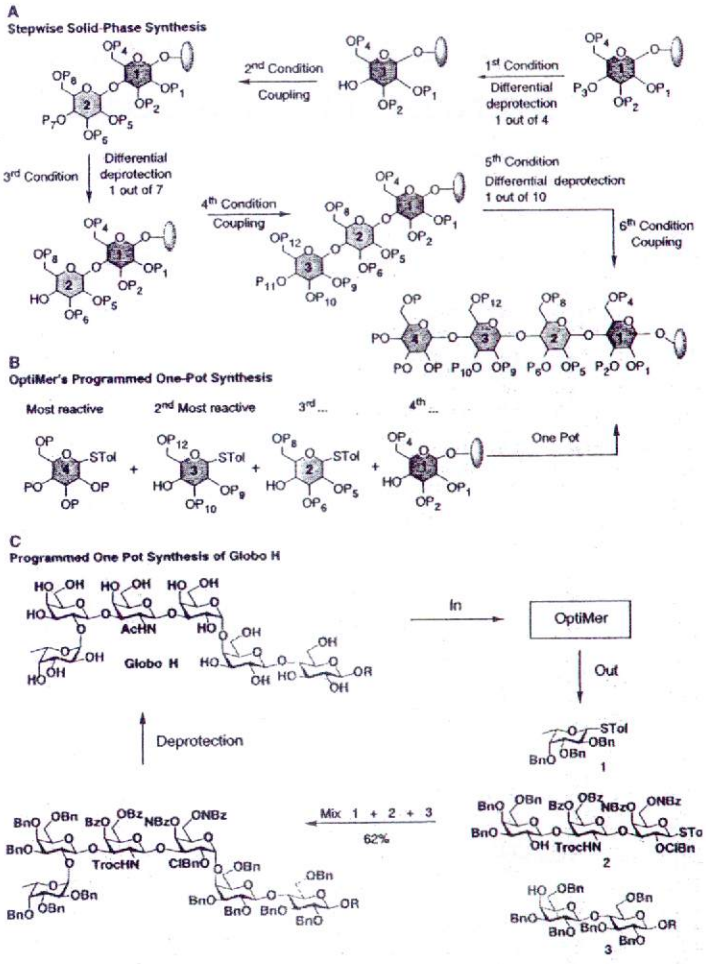
2. Strategies for Automated Synthesis

Solid phase

P. H. Seeberger et al Science, 2001, 291, 1523,
precedents on solid phase oligosaccharide synthesis
- Frechet JACS, 1971, 93, 492
- Danishefsky Science, 1993, 260, 1307
- Kahne Science, 1996, 274, 1520
- K.C. Nicolaou Angew. Chem. Int. Ed. 1995, 34, 2289

Advantages : ◦ rapid removal of reactants
◦ relatively easy purification

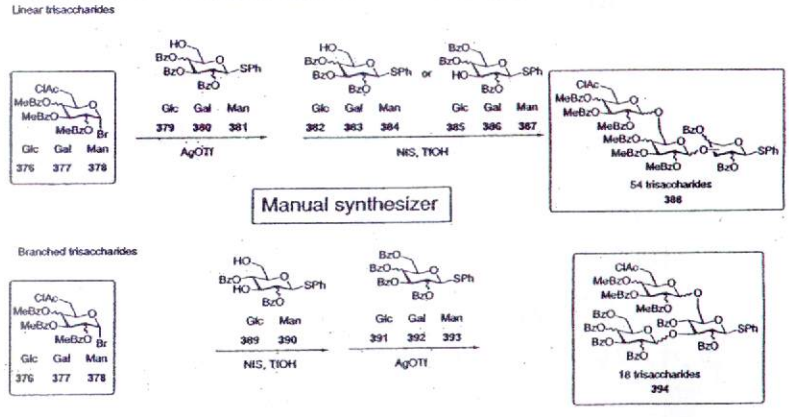
Disadvantages : ◦ low yield (steric hindrance)
◦ Monitoring the reaction progress is not trivial.
◦ protecting group manipulation is extremely difficult.
ex. Pd/C is not effective. debenzylation.
(Pd nanoparticles were effective in some cases)



Solution phase

<Programmed 1 pot Synthesis>
Wong et al. JACS, 1999, 121, 734-753,
◦ Reactivity profile of differently protected sugars are used to predict the outcome.
◦ OptiMer (computer program)
>100 data of STol

<1 pot Sequential Synthesis of trisaccharide libraries>
Takahashi et al. TL, 2000, 41, 2599.
◦ 72 trisaccharides were constructed using Quest 210™ manual synthesizer.
(↔ oligosaccharide - enediyne analogues)



Enzymatic Synthesis

Advantages. ◦ highly regio and stereoselective.
◦ protecting group manipulation is not complex.

Disadvantages ◦ expensive
◦ only natural sugars can be used.
◦ Many kinds of enzymes are required.

II Synthesis of Carbohydrate Based Therapeutics

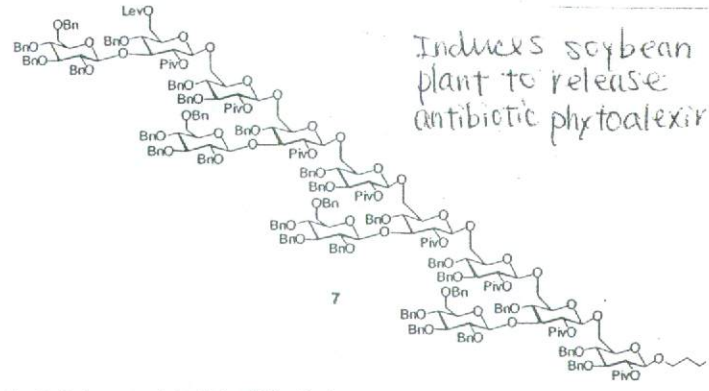
1. Malaria Vaccine Candidate.

1.1. Automated Synthesis on Solid Phase

Automated Solid-Phase Synthesis of Oligosaccharides

Obadiah J. Plante, Emma R. Palmacci, Peter H. Seeberger*

Science, 2001, 291, 1523-1527.



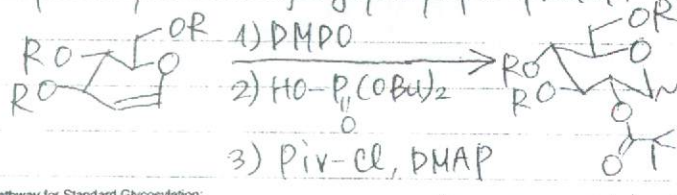
Induces soybean plant to release antibiotic phytoalexin

Fig. 3. Dodecamer phytoalexin elicitor β-glucan.

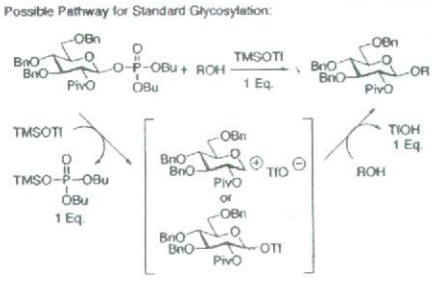
* peptide synthesizer was modified to apply to oligosaccharide synthesis.
  - reactant bottles
  - temperature control devise (peptide synthesis : rt)
* key features.

1. choice of leaving group (phosphates + trichloroacetimidates) ← not shown this time.

JACS, 2001, 123, 9545-9554. Seeberger et al. (cf. Use of phosphates as glycosyl
1 pot synthesis of glycosyl phosphates. reagent: S. Ikegami Chem. Comm. 1989, 685)



for procedure 2)
CH_2Cl_2 : β only
THF : α enriched (isomerization after addition)



← By C2 participation, glycosidation occurs β selectively.

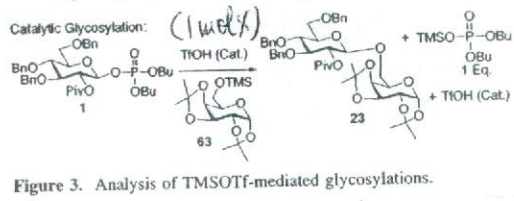
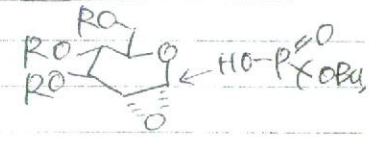
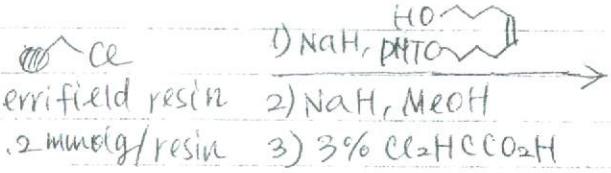


Figure 3. Analysis of TMSOTf-mediated glycosylations.



← Attempts for catalytic activation

2. choice of octenediol linker on resin (OL, 1999, 1, 1811 Seeberger et al.)



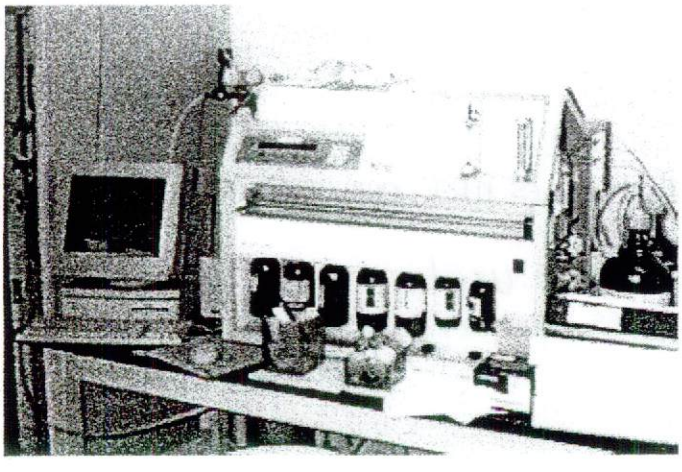
Merrifield resin
1.2 mmol/g resin

0.5 mmol/resin

- high loading
- stable under various glycosidation conditions.
- easily removable

peptide synthesizer (Applied Biochemicals. Inc, Model 433A)

* DMT = dimethoxytrityl



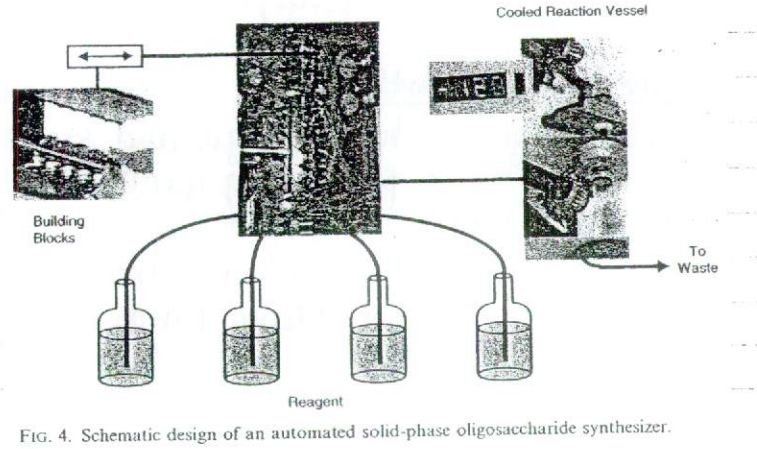
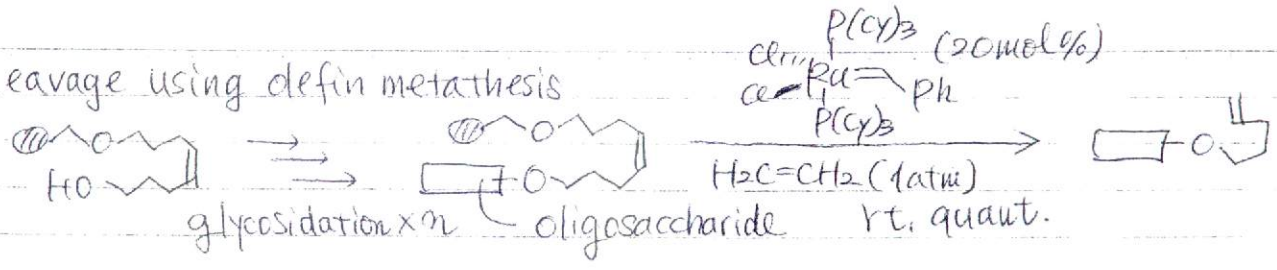
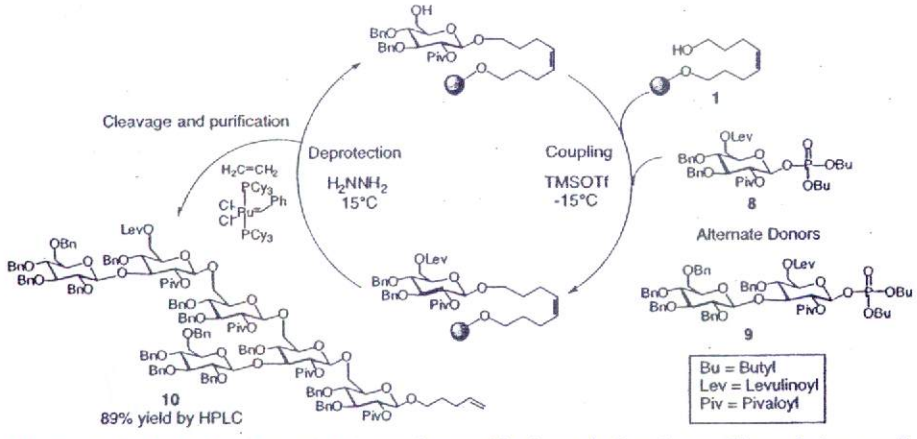


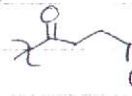
FIG. 4. Schematic design of an automated solid-phase oligosaccharide synthesizer.

cleavage using defin metathesis





Scheme 2. Automated oligosaccharide synthesis with glycosyl phosphates. Glycosylation conditions: 25 μmol scale: 25 μmol resin (83 mg, 0.30 mmol/g loading); 5 equiv. donor **8** or **9** (90 and 170 mg, respectively); 5 equiv. TMSOTf (1 ml, 0.125 M TMSOTf in CH_2Cl_2) repeated two times for 15 min each at –15°C. Deprotection conditions: 25 μmol scale: 4 ml, 0.25 M N_2H_4 in pyridine:acetic acid (3:2) repeated two times for 15 min each at 15°C.

- 8 and 9 are manually synthesized
- Lev = 
  easily cleaved by H_2N-NH_2
- cleavage from resin is performed in a separate flask.
- 10h, >80% y.
  (for dodecamer 7, 17h, >50% y)

↕

4 or 5 times higher yield compared to manual solid phase synthesis

Table 1. Cycles used with trichloroacetimidate and phosphate donors.

Step	Function	Reagent	Time (min)
		Trichloroacetimidate cycle	
1	Couple	10 equiv. donor and 0.5 equiv. TMSOTf	30
2	Wash	Dichloromethane	6
3	Couple	10 equiv. donor and 0.5 equiv. TMSOTf	30
4	Wash	Dichloromethane	6
5	Wash	1:9 methanol:dichloromethane	6
6	Deprotection	2 × 10 equiv. NaOMe (1:9 methanol:dichloromethane)	60
7	Wash	1:9 methanol:dichloromethane	4
8	Wash	0.2 M acetic acid in tetrahydrofuran	4
9	Wash	Tetrahydrofuran	4
10	Wash	Dichloromethane	6
		Phosphate cycle	
1	Couple	5 equiv. donor and 5 equiv. TMSOTf	30
2	Wash	Dichloromethane	6
3	Couple	5 equiv. donor and 5 equiv. TMSOTf	30
4	Wash	1:9 methanol:dichloromethane	4
5	Wash	Tetrahydrofuran	4
6	Wash	3:2 pyridine:acetic acid	3
7	Deprotection	2 × 20 equiv. hydrazine (3:2 pyridine:acetic acid)	30
8	Wash	3:2 pyridine:acetic acid	3
9	Wash	1:9 methanol:dichloromethane	4
10	Wash	0.2 M acetic acid in tetrahydrofuran	4
11	Wash	Tetrahydrofuran	4
12	Wash	Dichloromethane	6

1.2. Synthesis of Malaria Vaccine Candidate.

Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria

Louis Schofield*, Michael C. Hewitt†, Krystal Evans*, Mary-Anne Siomos* & Peter H. Seeberger†

** Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia*
† Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

Nature, 2002, 418, 785 (manual synthesis + evaluation)
JACS, 2002, 124, 13434 (automated synthesis)

- Malaria parasite plasmodium falciparum infects 5-10% of the world's population. (kills 2 million people per year)
- GPI (glycosylphosphatidyl inositol) is proposed to be a toxin
- precise mechanism is not known.

↓

Synthetic GPI = vaccines & biological tools?

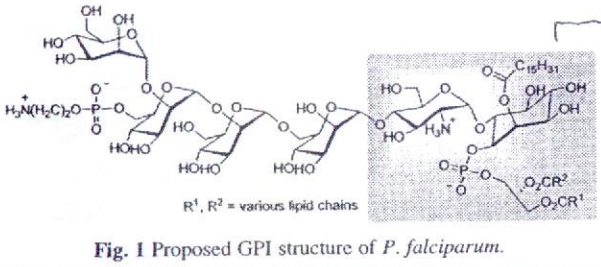


Fig. 1 Proposed GPI structure of *P. falciparum*.

→ Correct structure is not known.

- Synthetic GPI served as an effective anti-toxin malaria vaccine in rodent model.
  → GPI is a dominant proinflammatory toxin!

GPI: a class of glycolipids that anchor proteins to cell membranes.

Cf. Other synthetic studies.
Fraser-Reid JACS, 2004, 126, 7540-7547
Zhongwu Guo JACS, 2003, 125, 16334-16339.

6/11

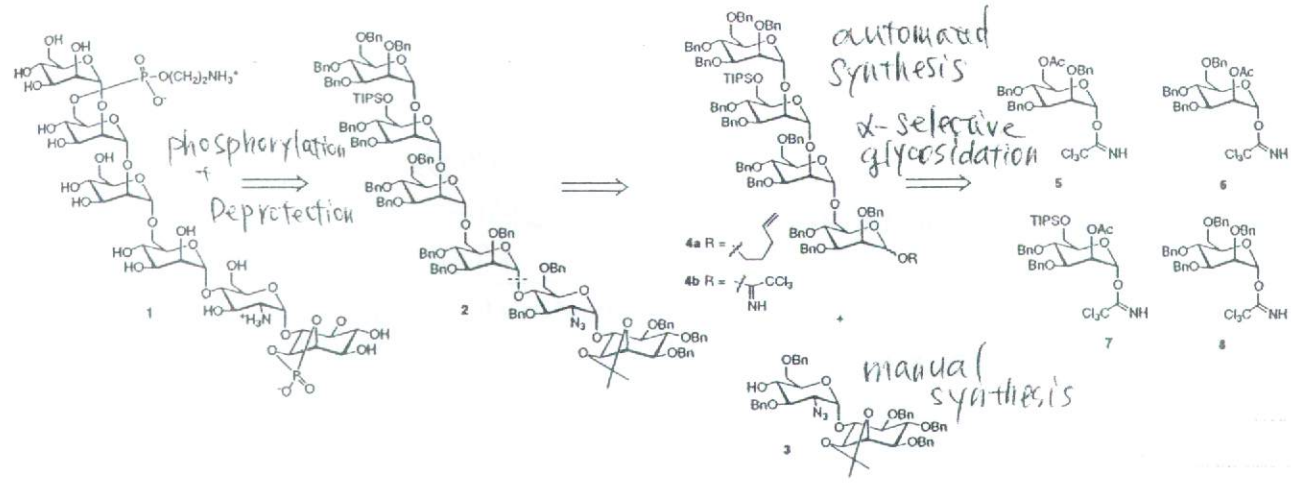
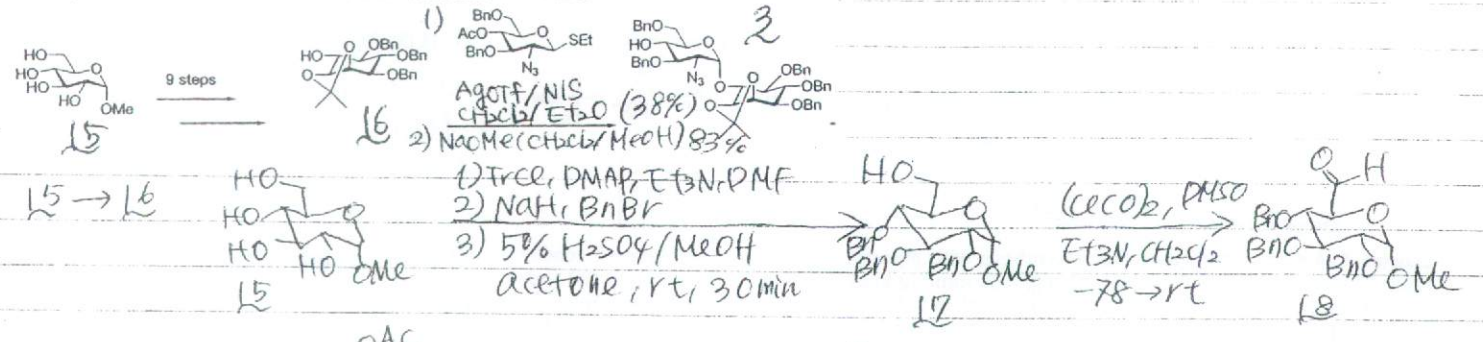
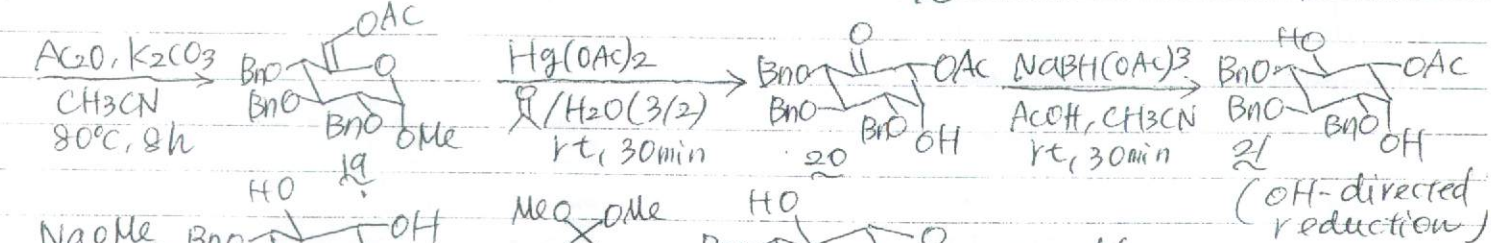
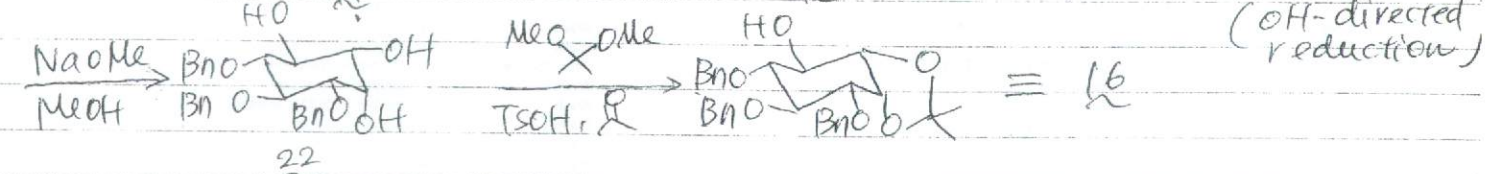


Figure 1. Retrosynthesis of GPI malarial toxin **1**.

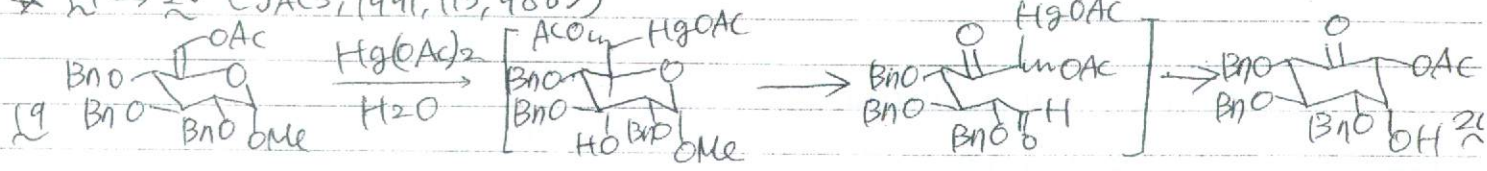
◦ Synthesis of 3







☆ 19 → 20 (JACS, 1991, 113, 9883)



◦ Automated Synthesis of 4a ◦ 3 + 4b → 2

◦ Completion.

Scheme 1

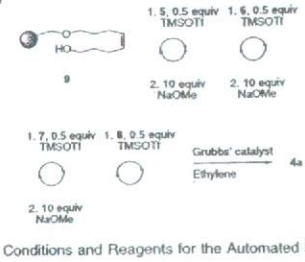
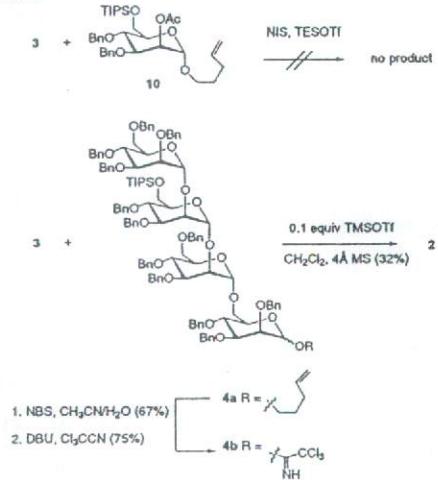


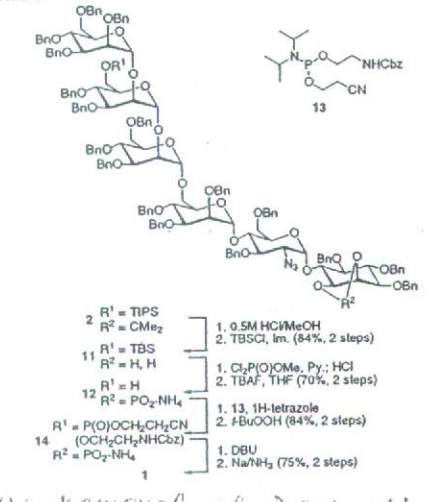
Table 1. Conditions and Reagents for the Automated Synthesis of 4a

function	reagent	time (min)
glycosylation	5 equiv **5**, **6**, **7**, or **8** and 0.5 equiv TMSOTf	20
wash	CH_2Cl_2	9
glycosylation	5 equiv **5**, **6**, **7**, or **8** and 0.5 equiv TMSOTf	20
wash	CH_2Cl_2	9
deprotection	2 × 10 equiv NaOMe	60
wash	0.2M AcOH/0.2M MeOH/THF	9
wash	THF	9
wash	CH_2Cl_2	9

Scheme 2



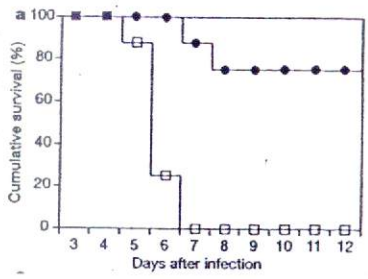
Scheme 3



DBU: removal of 2-CN

- part of biological evaluations
  kaplan-Meier survival plots
  - ● : immunized mice
  - □ : non-immunized mice

  (for more details, please see the text.)



← nearly 75% survived.

2. Anti HIV-1 Antibodies.

2.1. Programmable One-Pot Oligosaccharide Synthesis.

J. Am. Chem. Soc. **1999**, *121*, 734−753

Programmable One-Pot Oligosaccharide Synthesis

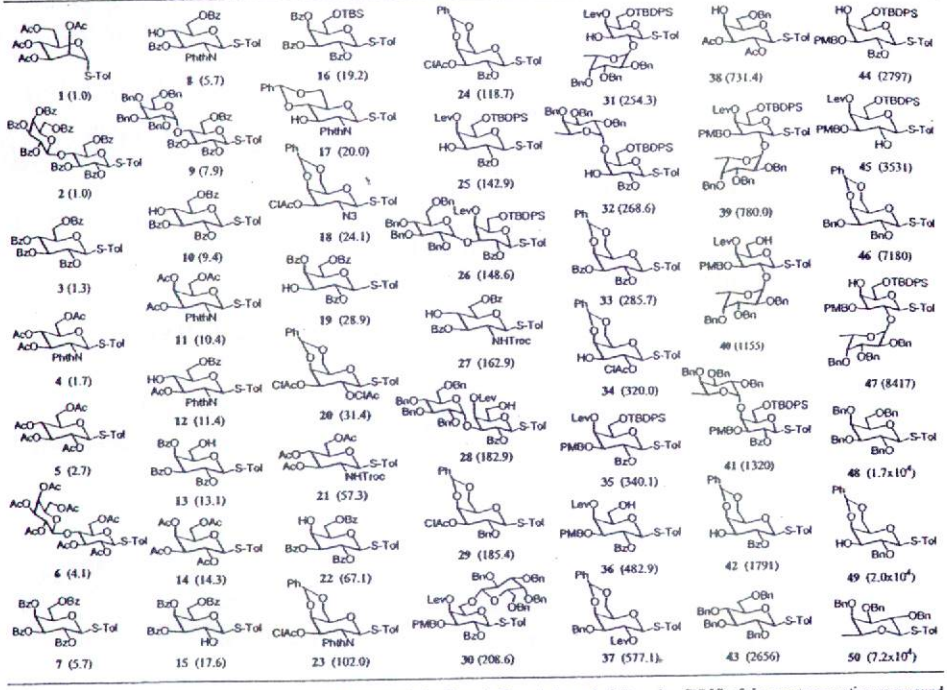
Zhiyuan Zhang, Ian R. Ollmann, Xin-Shan Ye, Ralf Wischnat, Timor Baasov,† and Chi-Huey Wong*

Contribution from the Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

RRV = relative reactivity value.
RRV was determined by competition experiment with reference donor.

Dx —MeOH→ —NIS→ —TfOH→
Pref H.Sieves CH_3CN 2h.
CH_2Cl_2.
↓ ↓
HPLC. Crude in CH_2Cl_2
HPLC.

Table 1. Relative Reactivity Values of Thioglycosides[a]



[a] This table was constructed on the basis of the manner shown in Chart 1. The relative reactivity value (RRV) of the most unreactive compound, acetylated mannoside, was defined as 1.0. Compounds are numbered as the decreased reactivity trend, and the RRVs are shown in parentheses.

- STol (−S-⟨⟩-CH_3) was chosen as a leaving group.
  - stable
  - high UV-absorbancy
  - easily prepared.
  - activated by various promotors.
- So far, up to tetra oligosaccharides have been possible to synthesize.

(Globo H, Lewis Y, N-acetyllactosamine oligomer, fucosy-GM were prepared by this method.)

They recently developed a new method for the preparation of antigens which bind to HIV-1 Antibody 2G12.

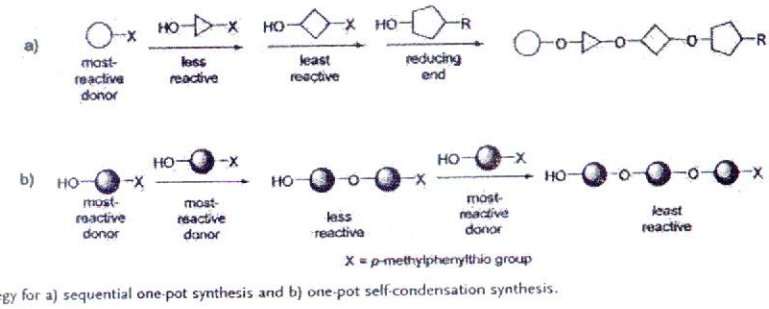


Figure 2. Strategy for a) sequential one-pot synthesis and b) one-pot self-condensation synthesis.

8/11

2.2. Synthesis of Antigens against HIV-1 Antibody.

Drug Development

Reactivity-Based One-Pot Synthesis of Oligomannoses: Defining Antigens Recognized by 2G12, a Broadly Neutralizing Anti-HIV-1 Antibody**

*Hing-Ken Lee, Christopher N. Scanlan, Cheng-Yuan Huang, Aileen Y. Chang, Daniel A. Calarese, Raymond A. Dwek, Pauline M. Rudd, Dennis R. Burton, Ian. A. Wilson, and Chi-Huey Wong**

Angew. Chem. Int. Ed. 2004, 43, 1000.

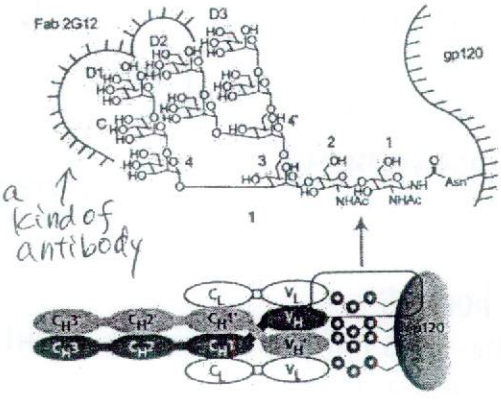


Figure 1. The structure of $Man_9GlcNAc_2$ (1; Man = mannose; Glc = glucose; Ac, acetyl) on gp120 interacting with the Fab fragment of the broadly neutralizing 2G12 antibody.

gp120 is known to be on the surface of HIV virus.

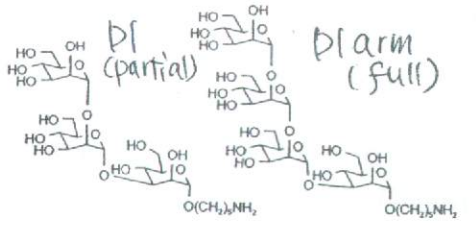
oligomannose structure is responsible for the viral entry into host cells

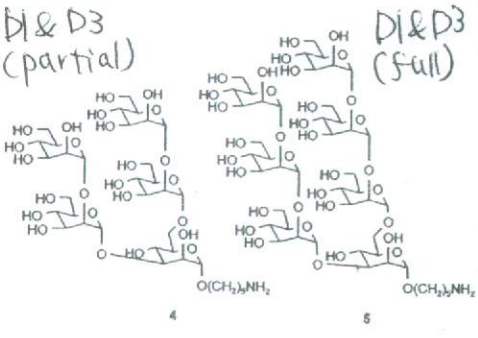
- X-ray crystal structure was taken. (2003)

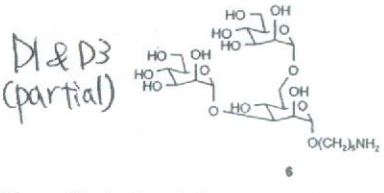
↓

Based on this information, several antigens were designe

Designed antigens.

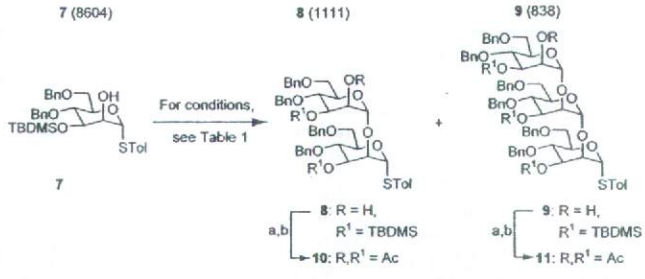






Scheme 1. The structures of oligomannoses 2–6.

- One-pot condensation strategy.



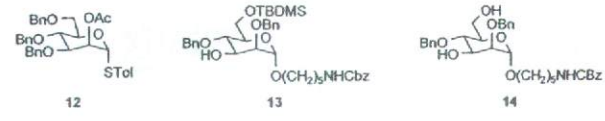
Scheme 3. One-pot self-condensation synthesis of building blocks 10 and 11. a) Tetrabutylammonium fluoride, tetrahydrofuran, RT, 24 h; b) Ac_2O, Et_3N, 4-dimethylaminopyridine, CH_2Cl_2, RT, 2 h.

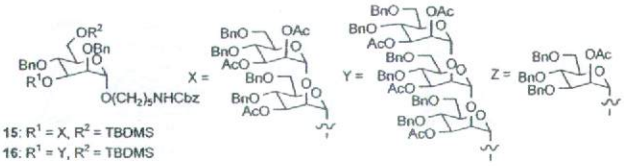
Conditions:
0.6 eq. NIS, −40°C
yield: 8 (38%) (1h)
9 (30%)

More NIS : decomp.
higher temp : polymer-zation
lower temp. : No reacti

* Very difficult to control the reaction, while getting sufficient yields.

- Assembly of the Saccharides.





Inhibition assay

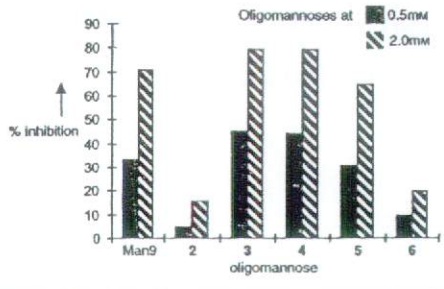


Figure 3. Inhibition (%) of 2G12 binding to gp120. Man9 = $Man_9GlcNAc_2$ (1).

← these results are consistent with Crystal structure.

Table 2: The reaction conditions for the synthesis of oligosaccharides 15–18 and 2–6.

Donor	Acceptor	NIS [equiv]	TfOH[b] [equiv]	T [°C]	t [h]	Protected oligosaccharide (yield [%])	Deprotected oligosaccharide (yield [%])
10	13	1.3	0.13	−20	2	15 (85)	2 (75)[b]
11	13	1.3	0.13	−10	4	16 (83)	3 (72)[b]
10	14	2.6	0.26	−20	2	17 (65)	4 (68)[b]
11	14	2.6	0.26	−10	4	18 (63)	5 (65)[b]
12	14	2.6	0.26	0	24	19 (50)	6 (60)[b]

[a] TfOH, trifluoroacetic acid. [b] a) 80% acetic acid, RT, 4 h; b) NaOMe, RT, 2 h; c) Pd black, 5% formic acid/MeOH, H_2, RT, 24 h. [c] a) NaOMe, RT, 2 h; b) Pd black, 5% formic acid/MeOH, H_2, RT, 24 h.

2 & 4 showed higher inhibition of 2G12 binding to gp120.
→ these may be candidates toward HIV vaccine development.

2.3 Linear Synthesis of High-Mannose Oligosaccharides.

A Linear Synthesis of Branched High-Mannose Oligosaccharides from the HIV-1 Viral Surface Envelope Glycoprotein gp120

Daniel M. Ratner,[a] Obadiah J. Plante,[a] and Peter H. Seeberger*[a]

Eur. J. Org. Chem. 2002, 826-833.

Application to automated synthesis in mind they developed efficient, linear synthesis of nonasaccharides from only 3 building blocks

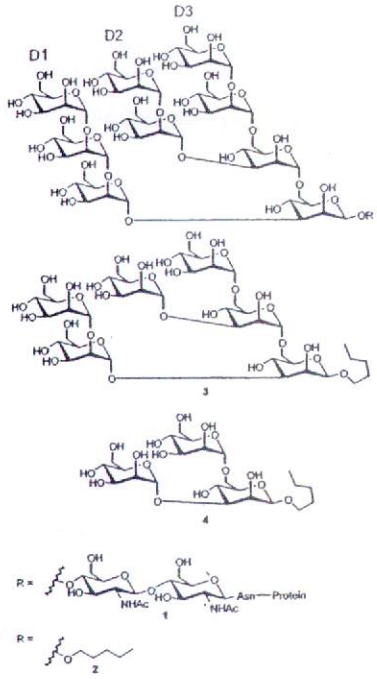
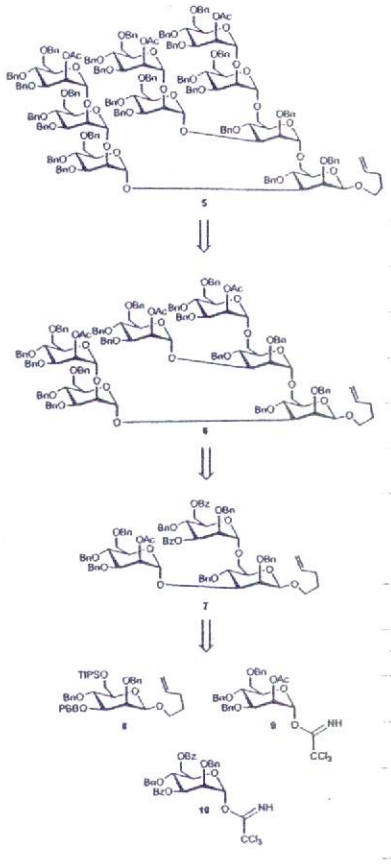
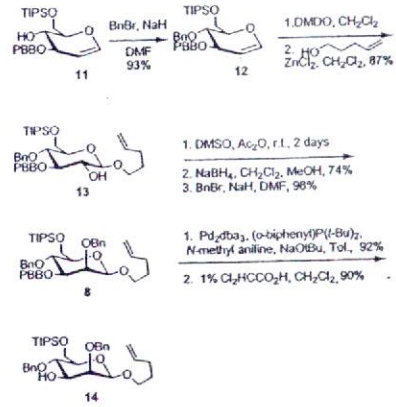


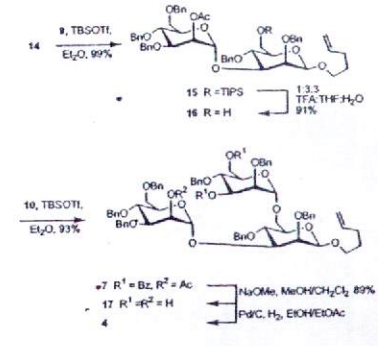
Figure 1. High-mannose oligosaccharide targets



Scheme 1. Retrosynthesis of nonamannoside 5

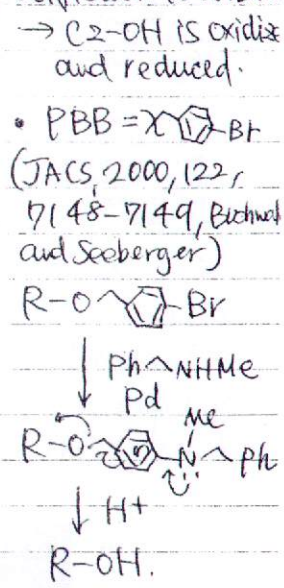


Scheme 2. Synthesis of the core β-mannoside 8

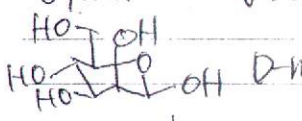


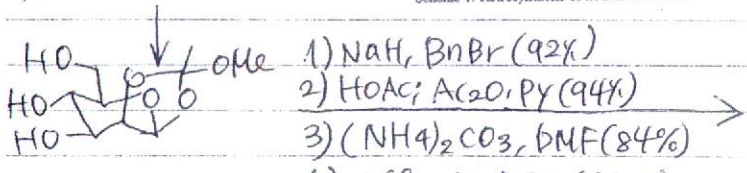
Scheme 3. Assembly of core trimannoside 7

- B-mannoside is difficult to constru → C2-OH is oxidiz and reduced.
- PBB =  (JACS 2000, 122, 7148-7149, Eichwal and Seeberger)



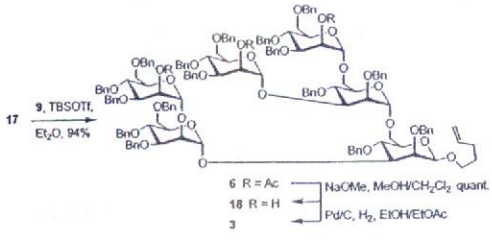
- Synthesis of 2

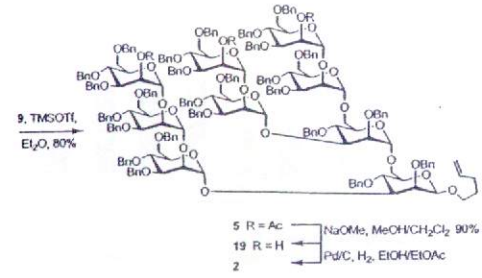




- Selective deacylation at C1 using $(NH_4)_2CO_3$
- α-imidate exlusively. (thermodynamically stable)

Assembly.





2.4 Oligosaccharide/protein interactions

Oligosaccharide and Glycoprotein Microarrays as Tools in HIV Glycobiology: Glycan-Dependent gp120/Protein Interactions

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Switzerland

Chemistry & Biology
2004, 11, 875-881

gp120 ↔ 2G12
HIV ↔ Scytovirin
↔ cyanovirin
↔ DC-SIGN

Microarray technique for oligosaccharides has recently developed.
(Nature Biotech, 2002, 275)
This has gradually become convenient tool to search sugar-protein interactions.

- Only a small amount of oligosaccharide is needed.
- Fluorescence detection

★ 4 gp120-binding proteins were used.
1. 2G12 (antibody, very strong interaction)
2. Scytovirin (found in 2003)
3. Cyanovirin N (found by HTS)
4. DC-SIGN (found in 2000)

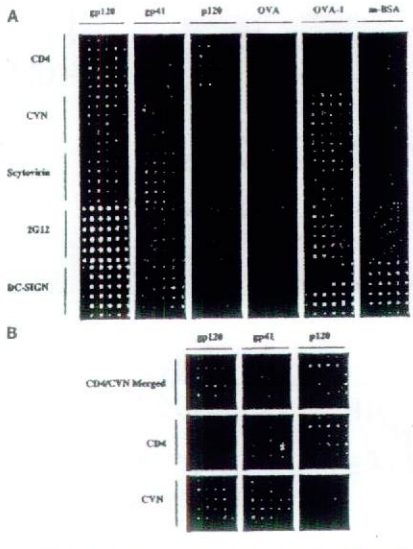

Figure 1. Analyzing Glycoprotein Binding Individually and Competitively
(A) Glycoprotein microarrays (including deglycosylated gp120, p120) were incubated with fluorophore-labeled proteins, and binding events were detected with a DNA-microarray scanner.
(B) A glycoprotein array (plus p120) incubated with CD4 (25 μg/ml) and then coumarin-CV-N (25 μg/ml) analyzed for displacement of CD4 binding by CV-N.

- CVN and 2G12
  → requirement for Manα1-2Man.
- Scytovirin.
  Terminal α1-2 mannose linkage is necessary (D3 arm)

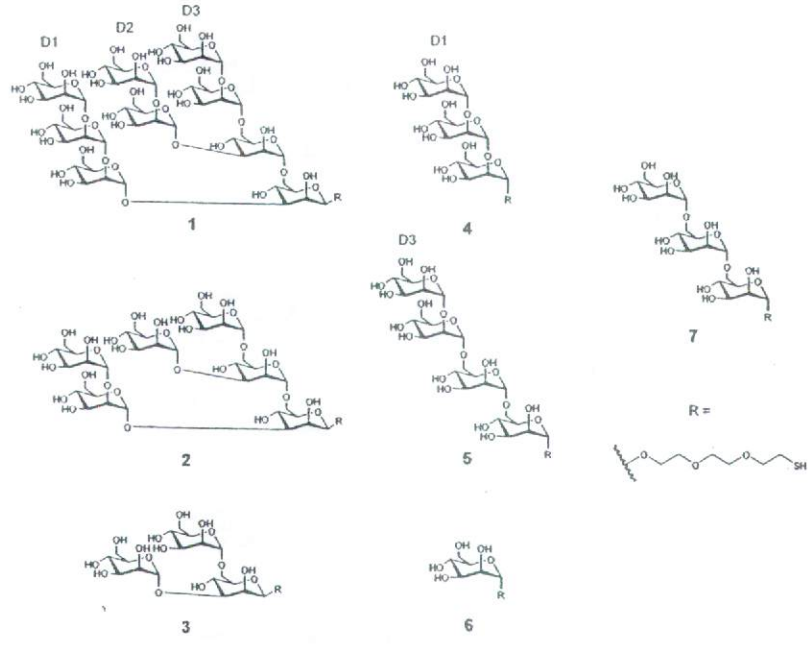

Figure 2. High-Mannose Oligosaccharide 1 and Synthetic Substructures Utilized in This Study
Stereochemistry as indicated at reducing end. The branched outer-trimannoside unique to high-mannose oligosaccharides is highlighted i blue. Reducing-end stereochemistry accurately represented by –R.

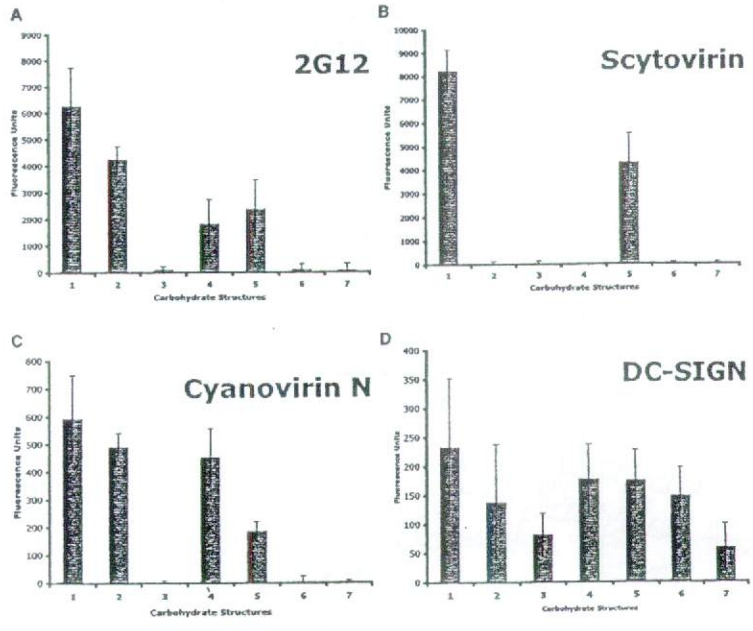

Figure 3. Analysis of High-Mannose Binding Proteins with Carbohydrate Microarrays Reveals Structural Requirements for Binding
(A) 2G12, (B) Scytovirin, (C) CVN, (D) DC-SIGN. Each protein was incubated with microarrays bearing carbohydrates 1–7 and analyzed as described (Experimental Procedures).

Total synthesis of Oligomannosides + Microarray technology

↳ Even unnatural carbohydrates can be screened.

11/11

3. Haemophilus Influenza Type b vaccine.

A Synthetic Conjugate Polysaccharide Vaccine Against *Haemophilus influenzae* Type b

V. Verez-Bencomo,[1*] V. Fernández-Santana,[1*] Eugenio Hardy,[2*] Maria E. Toledo,[3*] Maria C. Rodríguez,[1] Lazaro Heynngnezz,[2] Arlene Rodriguez,[2] Alberto Baly,[3] Luis Herrera,[2] Mabel Izquierdo,[2] Annette Villar,[1] Yury Valdés,[1] Karelia Cosme,[2] Mercedes L. Deler,[1] Manuel Montane,[2] Ernesto Garcia,[1] Alexis Ramos,[1] Aristides Aguilar,[2] Ernesto Medina,[2] Gilda Toraño,[3] Iván Sosa,[2] Ibis Hernandez,[3] Raydel Martínez,[3] Alexis Muzachio,[2] Ania Carmenates,[4] Lourdes Costa,[2] Félix Cardoso,[1] Concepción Campa,[5] Manuel Diaz,[3] René Roy[6*]

- Large scale synthesis, pharmaceutical development, and clinical evaluation (phase II)
- First fully chemically synthesized glycoconjugate vaccine.

Haemophilus influenza Type b

- vaccines became available in 1990's (enzymatic synthesis)
- 600,000 infants deaths annually as a result of Hib-induced pneumonia or meningitis (70%).

Nature, 2004, 305, 522.

Several attempts for chemical synthesis.

- Stepwise reaction
- Succeeded to immunize animals.

↳ But many steps were required and scale up was impossible.

<This time>

- 100g scale per batch
- Only 2 chromatographies
- Single step, high yielding polycondensation
- Deprotection - good y.
- Conjugation to carrier protein - good y.

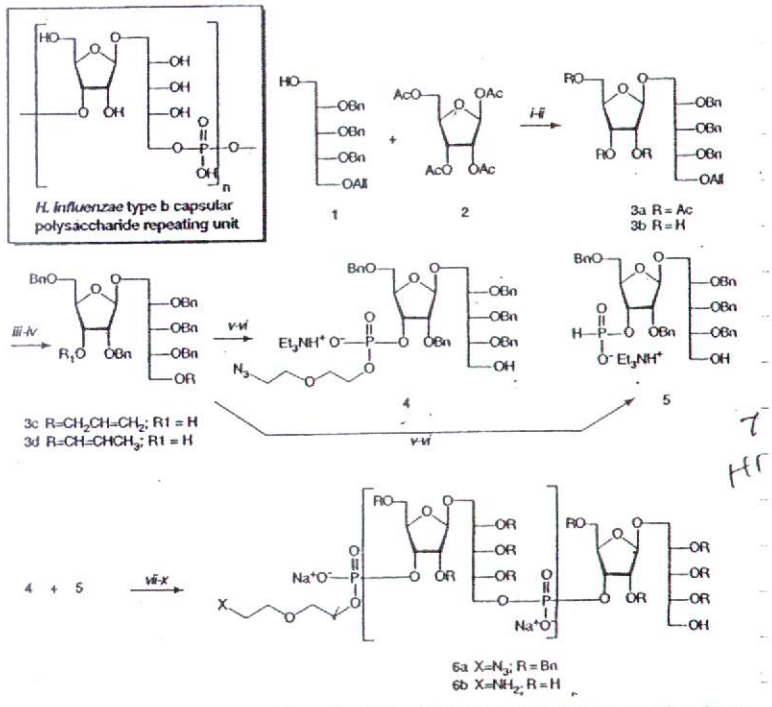

Fig. 1. Synthetic pathway leading to oligomeric polyribosylribitol phosphate 6. Reagents and conditions (Et, ethyl; Bn, benzyl; Bu, butyl; Ac, acetyl; and Piv, pivaloyl): (i) BF_3Et_2O and CH_2Cl_2; (ii) CH_3ONa and CH_3OH; (iii) BnCl, Bu_2SnO, NaH, and Bu_4NI; (iv) tBuOK and dimethyl sulfoxide (DMSO) at 100°C; (v) PCl_3, imidazole, CH_3CN for **5** and $N_3(CH_2)_2O(CH_2)_2OH$ for **4**, followed by I_2 oxidation; (vi) AcOH-H_2O at 80°C; (vii) PivCl and pyridine; (viii) I_2, pyridine, H_2O then gel filtration on LH-20; (ix) H_2, Pd-C, and EtOH-H_2O-EtOAc-AcOH at 1.5 atm; (x) cation exchange chromatography on Sephadex SP-C25.

one-step polycondensation using pivaloyl-chloride.

↳ Surprisingly, 6a with an average of 8 repeating units were reproducibly obtained in 80% y. after size exclusion chromatography.

* O-acylation did not take place.

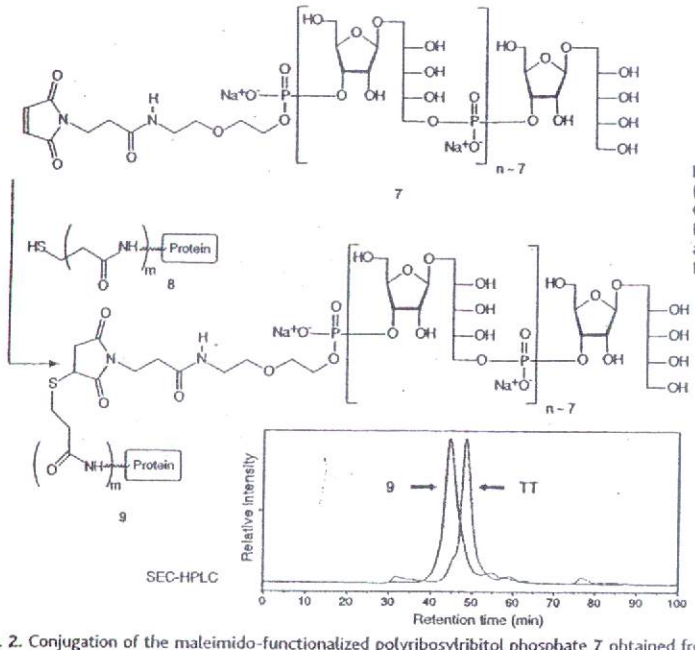

Fig. 2. Conjugation of the maleimido-functionalized polyribosylribitol phosphate **7** obtained from **6b** after coupling with 3-maleimidopropionic acid N-hydroxysuccinimide (DMSO, >95% conversion). 1,4-Conjugate addition of thiolated protein **8** onto **7** provided conjugate **9**. The shift in the molecular weight for TT could be observed in size exclusion chromatography–high performance liquid chromatography (TSK-5000-1 column) for conjugate **9** (PRP/TT ratio of 1/2.6).

Sin azúcar no hay país—no sugar, no country. That Cuban saying reflects the country's historic dependence on producing sugar, an industry hit hard in recent years by falling sugar prices. But some Cuban researchers now see economic—and medical—promise in another type of sugar, the kind found on the surfaces of microbes.