

# Journal of Visualized Experiments

## Guideline and demonstration for hierarchical and programmable one-pot synthesis of oligosaccharides --Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE59987R2
<b>Full Title:</b>	Guideline and demonstration for hierarchical and programmable one-pot synthesis of oligosaccharides
<b>Keywords:</b>	One-pot oligosaccharide synthesis Carbohydrate chemistry Auto-CHO SSEA-4 RRV prediction Machine learning Software
<b>Corresponding Author:</b>	Wen-Lian Hsu Academia Sinica Taipei, TAIWAN, PROVINCE OF CHINA
<b>Corresponding Author's Institution:</b>	Academia Sinica
<b>Corresponding Author E-Mail:</b>	hsu@iis.sinica.edu.tw
<b>Order of Authors:</b>	Cheng-Wei Cheng Yixuan Zhou Wen-Harn Pan Supriya Dey Chung-Yi Wu Wen-Lian Hsu Chi-Huey Wong
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Taipei, Taiwan

**TITLE:**

Hierarchical and Programmable One-Pot Synthesis of Oligosaccharides

**AUTHORS AND AFFILIATIONS:**

Cheng-Wei Cheng<sup>1</sup>, Yixuan Zhou<sup>1</sup>, Wen-Harn Pan<sup>2</sup>, Supriya Dey<sup>3</sup>, Chung-Yi Wu<sup>1</sup>, Wen-Lian Hsu<sup>4</sup>, Chi-Huey Wong<sup>1,3</sup>

<sup>1</sup>Genomics Research Center, Academia Sinica, Taipei, Taiwan

<sup>2</sup>Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

<sup>3</sup>The Scripps Research Institute, La Jolla, CA, USA

<sup>4</sup>Institute of Information Science, Academia Sinica, Taipei, Taiwan

**Corresponding Authors:**

Wen-Lian Hsu (hsu@iis.sinica.edu.tw)

Chi-Huey Wong (chwong@gate.sinica.edu.tw)

**Email Addresses of Co-authors:**

Cheng-Wei Cheng (chengwei@iis.sinica.edu.tw)

Yixuan Zhou (zhouyixuan@gate.sinica.edu.tw)

Wen-Harn Pan (pan@ibms.sinica.edu.tw)

Supriya Dey (supriyadey2000@gmail.com)

Chung-Yi Wu (cyiwu@gate.sinica.edu.tw)

**KEYWORDS:**

one-pot oligosaccharide synthesis, carbohydrate chemistry, Auto-CHO, SSEA-4, RRV, prediction, machine learning, software

**SUMMARY:**

This protocol demonstrates how to use the Auto-CHO software for hierarchical and programmable one-pot synthesis of oligosaccharides. It also describes the general procedure for competition experiments and one-pot glycosylation of SSEA-4.

**ABSTRACT:**

This article presents a general experimental protocol for programmable one-pot oligosaccharide synthesis and demonstrates how to use Auto-CHO software for generating potential synthetic solutions. The programmable one-pot oligosaccharide synthesis approach is designed to empower fast oligosaccharide synthesis of large amounts using thioglycoside building blocks (BBLs) with the appropriate sequential order of relative reactivity values (RRVs). Auto-CHO is cross-platform software with a graphical user interface that provides possible synthetic solutions for programmable one-pot oligosaccharide synthesis by searching a BBL library (containing about 150 validated and >50,000 virtual BBLs) with accuracy predicted RRVs by support vector regression. The algorithm for hierarchical one-pot synthesis has been implemented in Auto-CHO and uses fragments generated by one-pot reactions as new BBLs. In addition, Auto-CHO allows users to give feedback for virtual BBLs to keep valuable ones for further use. One-pot synthesis of stage-specific embryonic antigen 4 (SSEA-4), which is a pluripotent human embryonic stem cell marker, is demonstrated in this work.

## INTRODUCTION:

Carbohydrates are ubiquitous in nature<sup>1,2</sup>, but their presence and mode of action remain uncharted territory, mainly due to difficult access to this class of molecules<sup>3</sup>. Unlike automated synthesis of oligopeptides and oligonucleotides, the development of automated synthesis of oligosaccharides remains a formidable task, and progress has been relatively slow.

To tackle this problem, Wong et al. developed the first automated method for the synthesis of oligosaccharides using a programmable software program called Optimizer<sup>4</sup>, which guides the selection of BBLs from a library of ~50 BBLs for sequential one-pot reactions. Each BBL was designed and synthesized with well-defined reactivity tuned by various protecting groups. Using this approach, the complexities of protecting manipulation and intermediate purification can be minimized during synthesis, which have been considered the most difficult issues to overcome in the development of automated synthesis. Despite this advance, the method is still quite restricted, as the number of BBLs is too small and the Optimizer program can only handle certain small oligosaccharides. For more complex oligosaccharides that require more BBLs and multiple passes of one-pot reactions and fragment condensation, an upgraded version of the software program, Auto-CHO<sup>5</sup>, has been developed.

In Auto-CHO, more than 50,000 BBLs with defined reactivity to the BBL library have been added, including 154 synthetic and 50,000 virtual ones. These BBLs were designed by machine learning based on basic properties, calculated NMR chemical shifts<sup>6, 7</sup>, and molecular descriptors<sup>8</sup>, which affect the structure and reactivity of the BBLs. With this upgraded program and new set of BBLs available, synthesis capacity is expanded, and as demonstrated, several oligosaccharides of interest can rapidly be prepared. It is believed that this new development will facilitate the synthesis of oligosaccharides for studying of their roles in various biological processes and their impacts on the structures and functions of glycoproteins and glycolipids. It is also thought that this work will benefit the glycoscience community significantly, given that this method is available to the research community free of charge. Synthesis of the essential human embryonic stem cell marker, SSEA-4<sup>5</sup>, is demonstrated in this work.

## PROTOCOL:

### 1 Auto-CHO software manipulation

1.1 Java Runtime Environment installation: make sure the Java Runtime Environment (JRE) has been installed in the device. If JRE has been installed, go to the next step, "software initialization"; otherwise, download and install JRE according to the user's operating system found at: <<https://www.oracle.com/technetwork/java/javase/downloads/index.html>>.

1.2 Software initialization: go to the Auto-CHO website at <<https://sites.google.com/view/auto-cho/home>> and download the software according to the operating system. Currently, Auto-CHO supports Windows, macOS, and Ubuntu. The latest PDF user guide is provided on the Auto-CHO website.

1.2.1 For Windows users, unzip the **Auto-CHO\_Windows.zip** and double-click on **Auto-CHO.jar** in the **Auto-CHO\_Windows** folder to start the program.

NOTE: The user needs to install unzip software, such as 7-Zip, found at <<https://www.7-zip.org>>, for unpacking the zip file. The user may also use the **java -jar Auto-CHO.jar** command to start the program by the Windows Command Prompt.

1.2.2 For macOS users, right-click on **Auto-CHO.jar** and choose **Open** to start the program.

1.2.3 For Ubuntu users:

1.2.3.1 Install libcanberra-gtk using the following command:

```
$ sudo apt-get install libcanberra-gtk*
```

1.2.3.2 Change the access permission of **Auto-CHO\_Ubuntu.sh**:

```
$ chmod 755 Auto-CHO_Ubuntu.sh
```

1.2.3.3 Run the Auto-CHO program:

```
$ ./Auto-CHO_Ubuntu.sh
```

1.3 Input the desired glycan structure. Choose to draw a glycan structure or read an existing structure file.

1.3.1 Input by drawing:

1.3.1.1 Click on **Edit Glycan by GlycanBuilder**<sup>9,10</sup> (Figure 1, btn1; Figure 2A) or the area of **Click Here to Edit the Synthetic Target** to draw and edit the query structure by GlycanBuilder. Linkage and chirality information should not be ignored. Click on the **Globo-H**, **SSEA-4**, or **OligoLacNAc** buttons (Figure 1, examples) to display the examples.

1.3.1.2 Select **File | Export to sequence formats | Export to GlycoCT condensed** to save the edited structure (optional).

1.3.1.3 Close the GlycanBuilder dialog to complete editing.

1.3.2 Input by reading a file:

1.3.2.1 Click on **Edit Glycan by GlycanBuilder** (Figure 1, btn1; Figure 2A) or the area of **Click Here to Edit the Synthetic Target** to edit the query structure.

1.3.2.2 Select **File | Import from sequence formats** to choose the query structure file with the corresponding format.

1.4 Search parameter settings (optional).

1.4.1 Define the search parameters in the “**Parameter Settings**” tab (Figure 1, tab2) to get reasonable search results.

NOTE:

**Threshold of High-Class RRV** must be a real number and  $\geq 0$ .

**Threshold of Medium Class RRV** must be a real number and  $\geq 0$ .

**Threshold of High-Class RRV** should be  $>$  **Threshold of Medium Class RRV**.

**Max Fragment Number** should be an integer and  $\geq 1$ .

**Min BBL Number in a Fragment** must be an integer and between 1 and 3.

**Max BBL Number in a Fragment** must be an integer and between 1 and 3.

**Max BBL Number in a Fragment** must be  $\geq$  **Min BBL Number in a Fragment**.

**Min Donor/Acceptor RRV Difference** must be a positive real number.

**Min Donor/Acceptor RRV Ratio** must be a positive real number.

**Max Donor/Acceptor RRV Ratio** must be a positive real number.

**Max Donor/Acceptor RRV Ratio** must be  $>$  **Min Donor/Acceptor RRV Ratio**.

1.4.2 Click on the **OK** button to enable the new settings.

1.5 Select the building block library (**Figure 1**, tab5). The default setting is to search the experimental library only. If it is desired to search both the experimental and virtual libraries, check the following steps.

1.5.1 Select the **Virtual Building Block Library** tab (**Figure 2C**, tab5). Experimental and virtual building blocks can work together to enhance the searching ability of Auto-CHO. Currently, Auto-CHO provides more than 50,000 virtual building blocks with predicted RRVs in the library.

1.5.2 Select **Use Experimental and Virtual Libraries** and apply **Filtering** to display virtual building blocks with certain criteria. Click on the **Show Selected Virtual BBL(s)** button (**Figure 2C**, btn5) to show only the selected virtual building block(s).

1.5.3 Click on the **Show Filtered Virtual BBL(s)** button (**Figure 2C**, btn6) to show only virtual building blocks with certain criteria defined by the user.

1.5.4 Click on the **Show All Virtual BBL(s)** button (**Figure 2C**, btn7) to show all available virtual building blocks and reset the filter.

1.5.5 Check one or multiple desired virtual building blocks that the user would like to use for searching.

1.6 Select the **Query Structure** tab (**Figure 1**, tab1) and click on the **Search Building Block Library** button (**Figure 1**, btn2) to find the one-pot synthetic solutions for the query structure. Then, confirm the parameter settings.

1.7 Search the result viewer.

NOTE: The search result is shown in the **Result Visualization** tab (**Figure 1**, tab6). The reducing end acceptors of different residue numbers are displayed in the **Reducing End Acceptor** column (**Figure 1**, viewer1).

1.7.1 Select a reducing-end acceptor, and solutions are displayed on the **Synthetic Solution List** (**Figure 1**, viewer2). Fragments are shown in the **Fragment List** (**Figure 1**, viewer3) to suggest how many fragments should be used in the synthesis.

NOTE: The system provides detailed information of each fragment, including the RRV of the fragment, computational yield as well as which protecting group should be deprotected for the subsequent use of the fragment in the one-pot reaction. The building blocks used to assemble the selected fragment are shown in the viewer4 of **Figure 1**. The viewer5 of **Figure 1** also displays the fragment connection information.

1.7.2 View and check chemical structures and detailed information of the selected building blocks in the regions of **Chemical Structure of Building Block** and **Building Block Browser**, respectively, for experimental building blocks (**Figure 1**, tab4).

1.8 Output the search result to text (optional).

1.8.1 Select the **Result Text** tab (**Figure 1**, tab7).

1.8.2 Click on **Save Result Text** (**Figure 2B**, btn4) and choose the text file destination.

1.9 Feedback for virtual building blocks (optional).

NOTE: Feedback can be given on virtual building blocks through the online questionnaire. Feedback can help the community to keep useful virtual building blocks and remove ineffectual ones.

1.9.1 Select the **Virtual Building Block** tab (**Figure 1**, tab5).

1.9.2 Click on the **To rate** link of the virtual building block of which it is desired to rate or comment in the **Feedback** column.

1.9.3 Fill in the feedback form after the system opens up a webpage and submit it.

NOTE: Do not change the virtual BBL ID.

## 2 Competition experiments

2.1 In a 1 mL round-bottom flask, combine the two thioglycoside donors (0.02 mmol of each:  $D_{r4}$  is the reference donor with known RRV;  $D_{x1}$  is the donor molecule of unknown RRV), absolute methanol (0.10 mmol), and Drierite in dichloromethane (DCM, 1.0 mL), then stir at room temperature (RT) for 1 h.

2.2 Take an aliquot of this mixture (30  $\mu$ L) and inject the mixture into high performance liquid chromatography (HPLC) in three separate injections (10  $\mu$ L for each injection). Measure the coefficient ( $\alpha$ ) between the absorption ( $A$ ) and concentration of the donor molecule [**D**] under the baseline separation conditions (ether acetate/*n*-Hexane = 20/80).

2.3 Add a solution of 0.5 M *N*-Iodosuccinimide (NIS) in acetonitrile (40  $\mu$ L, 0.02 mmol) into the reaction mixture, followed by addition of a 0.1 M trifluoromethanesulfonic acid (TfOH) solution (20  $\mu$ L, 0.002 mmol), and stir the mixture at RT for 2 h.

2.4 Dilute the reaction mixture with DCM (4.0 mL), filter, and wash with saturated aqueous sodium thiosulfate containing 10% sodium hydrogen carbonate (2x with 5 mL volume each). Extract the aqueous layer with DCM (3x with 5 mL). Combine all organic layer, wash it with 5 mL of brine, and dry it with approximately 200 mg of anhydrous magnesium sulfate.

2.5 Shake the mixture mildly for 30 s, filter it through a funnel with a fluted filter paper in order to remove the magnesium sulfate, then collect the filtrate in a 25 mL round-bottom flask. Remove the solvent using a rotary evaporator.

2.6 Dissolve the residue in DCM (1.0 mL). Take an aliquot of this mixture (30  $\mu$ L) and inject it into HPLC in three separate injections (10  $\mu$ L for each injection). Measure the concentrations of the remaining donors ( $[D_x]$  and  $[D_{ref}]$ ) by HPLC under the same separation conditions (ether acetate/*n*-Hexane = 20/80) ( $A_{ref}$ )<sub>t</sub> = 24417.0, ( $A_x$ )<sub>t</sub> = 23546.3.

2.7 Measure the relative reactivity between  $D_{x1}$  vs.  $D_{r4}$ ,  $k_{x1}/k_{r4} = 0.0932$ . Based on the relative reactivity value of  $D_{r4}$ , the relative reactivity value of  $D_{x1}$  is 3.

NOTE:  $a = A/[D]$ , ( $A_{ref}$ )<sub>0</sub> = 74530.1, ( $A_x$ )<sub>0</sub> = 26143.0.  $k_x/k_{ref} = (\ln[D_x]_t - \ln[D_x]_0)/(\ln[D_{ref}]_t - \ln[D_{ref}]_0) = (\ln[A_x]_t - \ln[A_x]_0)/(\ln[A_{ref}]_t - \ln[A_{ref}]_0) = 0.0932$ .

### 3 One-pot glycosylation of SSEA-4

3.1 Place a 1 mL round-bottom flask under vacuum, flame-dry it, and allow the flask to cool to RT while still under vacuum. Remove the rubber septum to add a mixture of disaccharide **1** donor (38 mg, 1.1 eq., 0.057 mmol), the first acceptor **2** (40 mg, 1.0 eq., 0.053 mmol) and a Teflon-coated magnetic stir bar into the flask.

3.2 Transfer 100 mg of powdered molecular sieves 4 Å into a 5 mL round-bottom flask. Keep this flask under vacuum, flame-dry it, and allow the flask to cool to RT while still under vacuum. Transfer the freshly dried 4 Å molecular sieves into the first flask that contains the starting material.

3.3 Transfer 1 mL of freshly dried DCM into the flask. Stir the reaction mixture for 1 h at RT and then place it under a temperature of -40 °C. Transfer the NIS (13 mg, 1.1 eq., 0.057 mmol) into the flask.

3.4 Inject TfOH (34  $\mu$ L, 0.3 eq., 0.017 mmol, 0.5 M in ether) into the flask through the septum using a micro-volume syringe at -40 °C. Keep stirring at -40 °C for 3 h.

3.5 After the first acceptor **2** is almost consumed, inject the solution of acceptor **3** in DCM into the flask through the septum.

3.6 Warm the reaction mixture up to -20 °C and transfer NIS (19 mg, 1.6 eq., 0.083 mmol) into the flask. Inject TfOH (34 µL, 0.3 eq., 0.017 mmol, 0.5 M in ether) into the flask through the septum at -20 °C. Keep stirring at -20 °C for 3 h.

3.7 After the product of the first step reaction is consumed, quench the reaction by injecting two equivalents of triethyl amine. Remove the molecular sieves through a filter funnel packed with Celite, collect the filtrate into a 25 mL round-bottom flask and further wash the filter with 10 mL of DCM.

3.8 Transfer the filtrate into a separatory funnel and wash it with saturated aqueous sodium thiosulfate containing 10% NaHCO<sub>3</sub> (2x with 10 mL each). Extract the aqueous layer with DCM (3x with 10 mL). Combine the organic layers and wash the mixture with brine (10 mL) and dry it by adding anhydrous MgSO<sub>4</sub>. Filter it and collect the filtrate in a 100 mL round-bottom flask.

3.9 Remove the solvent using a rotary evaporator. Dissolve the crude mixture with approximately 1 mL of DCM and load it on top of the silica bed. Elute the product with a mixture of ethyl acetate and toluene (EtOAc/toluene, 1/4 to 1/2) and collect the fractions.

3.10 Remove the solvent using a rotary evaporator. Dry the residue under reduced pressure to give fully protected SSEA-4 derivative **4** (74 mg, 50% based on acceptor **2**) as white foam.

#### REPRESENTATIVE RESULTS:

The Auto-CHO search result based on default parameter settings indicates SSEA-4 can be synthesized by a [2 + 1 + 3] one-pot reaction. **Figure 3** shows the software screenshot of the SSEA-4 search result. When a trisaccharide reducing end acceptor is selected (**Figure 3**, label 1), the program shows four potential solutions for the query. The first solution has one fragment (**Figure 3**, label 2), and its calculated yield is about 94%. The fragment can be synthesized by two BBLs (**Figure 3**, label 3). The RRV of the first disaccharide BBL is 1462 and the RRV of the second monosaccharide is 32.0. Label 4 of **Figure 3** shows the chemical structure of the first suggested BBL used in the one-pot reaction. The one-pot experiment shows that SSEA-4 can be synthesized in 43% yield by this suggestion successfully (**Figure 4**) and it has also been demonstrated in previous work<sup>5</sup>. The detail experimental procedures and the characterization of mentioned compounds, particularly SSEA-4 can be found in the cited reference<sup>5</sup>.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Auto-CHO screenshot.** Users can edit query glycan structure, browse experimental and virtual building block information, and view one-pot synthetic solutions provided by the software.

**Figure 2: Partial screenshots of Auto-CHO software. (A)** Click “Edit Glycan by GlycanBuilder” button (btn1) in the “Query Structure” tab (tab1) and the system pops up the GlycanBuilder dialog. **(B)** Select “Result Text” (tab7) and click “Save Result Text” (btn4) to save the text



search results. (C) Select “Virtual Building Block Library” (tab5) and check desirable virtual building blocks for searching by filtering options.

**Figure 3: One-pot synthetic blueprint given by the Auto-CHO program.** Label 1: Reducing end acceptor. Label 2: Fragment. Label 3: Building block of the fragment. Label 4: Chemical structure of the selected building block.

**Figure 4: The [2 + 1 + 3] one-pot synthetic strategy for SSEA-4.** SSEA-4 can be synthesized by three units suggested by Auto-CHO: sialyl disaccharide building block **1** (RRV=1,462), monosaccharide building block **2** (RRV=32.0), and reducing end acceptor **3** (RRV=0). This figure was modified from a previous publication with permission<sup>5</sup>.

## DISCUSSION:

The Auto-CHO software was developed for assisting chemists to proceed hierarchical and programmable one-pot synthesis of oligosaccharides<sup>5</sup>. Auto-CHO was built by Java programming language. It is a GUI software and cross-platform, which currently supports Windows, macOS, and Ubuntu. The software can be downloaded free of charge for the Auto-CHO website at <<https://sites.google.com/view/auto-cho/home>>, and its source code with MIT license can be accessed from the GitHub at <<https://github.com/CW-Wayne/Auto-CHO>>.

The BBL library of Auto-CHO contains 154 experimental BBLs and more than 50,000 virtual BBLs with accurately predicted RRVs. Currently, sugar types of virtual BBLs include Gal, Glc, Man, GalNAc, GlcNAc, GlcN, and GlcA. All library searches are processed in the local machine and we do not collect any query structure from users. Since Auto-CHO cannot guarantee the success of high-yield synthesis among virtual BBLs given by the program (due to many structural constraints or unknown factors in the chemical reactions), Auto-CHO provides online feedback questionnaire for virtual BBLs. It is believed that user feedback from the research community can help keep valuable virtual BBLs and eliminate unsuitable ones. An email address is provided for technical assistance in the software user guide. Users can contact this address if they encounter technical questions or problems.

Two search strategies are provided here. For parameter settings (section 1.4), it is suggested to set parameters with stricter criteria at the beginning. If Auto-CHO does not return satisfied synthetic solutions, it is advised to use more flexible parameters in the next search run. For the selection of BBL library (section 1.5), it is suggested to search the experimental library only at first. If the software does not return any suitable solution, it is advised to search experimental and virtual libraries in the following iterations.

In summary, this protocol demonstrates the operation of Auto-CHO software and use of Auto-CHO for one-pot synthesis of the SSEA-4 molecule. In addition, the programmable one-pot protocol is described. Auto-CHO is the GUI and open-source software with library includes validated and virtual BBLs, and it supports hierarchical one-pot synthesis of oligosaccharides. It is believed that this software can benefit the research community and more essential oligosaccharides can be synthesized by one-pot reactions through Auto-CHO for further research.

**ACKNOWLEDGMENTS:**

This work was supported by Academia Sinica including the Summit Program, Ministry of Science and Technology [MOST 104-0210-01-09-02, MOST 105-0210-01-13-01, MOST 106-0210-01-15-02].

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1. Apweiler, R., Hermjakob, H., Sharon, N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochimica Et Biophysica Acta*. **1473** (1), 4–8 (1999).
2. Sears, P., Wong, C. -H. Toward Automated Synthesis of Oligosaccharides and Glycoproteins. *Science*. **291** (5512), 2344–2350, doi: 10.1126/science.1058899 (2001).
3. Kulkarni, S. S. et al. “One-Pot” Protection, Glycosylation, and Protection–Glycosylation Strategies of Carbohydrates. *Chemical Reviews*. **118** (17), 8025–8104, doi: 10.1021/acs.chemrev.8b00036 (2018).
4. Zhang, Z. et al. Programmable One-Pot Oligosaccharide Synthesis. *Journal of the American Chemical Society*. **121** (4), 734–753, doi: 10.1021/ja982232s (1999).
5. Cheng, C. -W. et al. Hierarchical and programmable one-pot synthesis of oligosaccharides. *Nature Communications*. **9** (1), 5202, doi: 10.1038/s41467-018-07618-8 (2018).
6. ChemDraw (PerkinElmer Informatics).
7. Cheeseman, J. R., Frisch, Æ. Predicting magnetic properties with chemdraw and gaussian. (2000).
8. Yap, C. W. PaDEL-descriptor: An open source software to calculate molecular descriptors and fingerprints. *Journal of Computational Chemistry*. **32** (7), 1466–1474, doi: 10.1002/jcc.21707 (2011).
9. Ceroni, A., Dell, A., Haslam, S. M. The GlycanBuilder: a fast, intuitive and flexible software tool for building and displaying glycan structures. *Source Code for Biology and Medicine*. **2**, 3, doi: 10.1186/1751-0473-2-3 (2007).
10. Damerell, D. et al. The GlycanBuilder and GlycoWorkbench glycoinformatics tools: updates and new developments. *Biological Chemistry*. **393** (11), 1357–1362, doi: 10.1515/hsz-2012-0135 (2012).

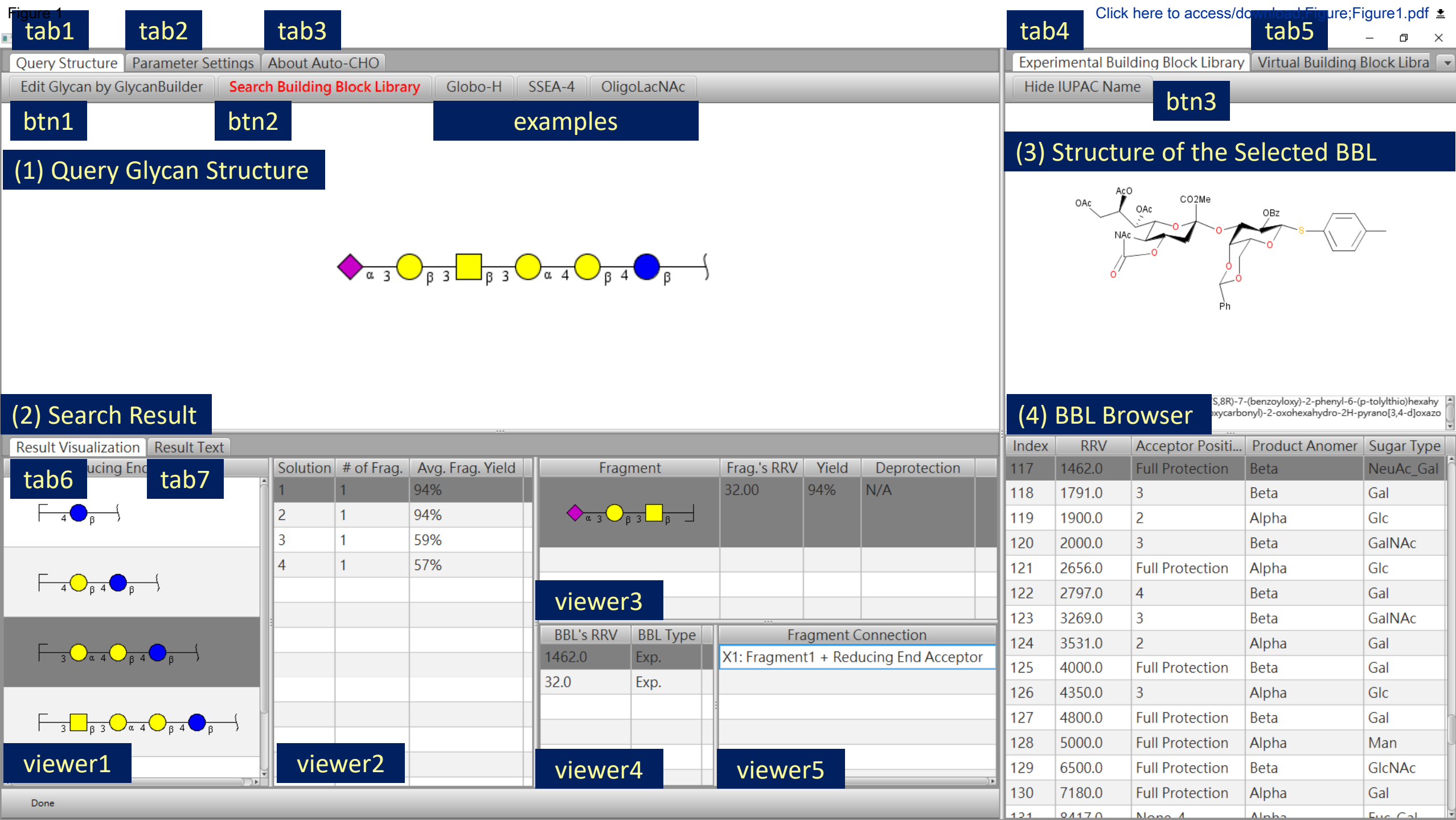


Figure 2

**a) tab1**

**btn1**

**GlycanBuilder**

File Edit Structure View Help

Linkage ? ? ? ? ? Chirality ? Ring ? 2nd bond ? ? ? ? ?

m/z: 1491.7302 [MONO,perMe,Na,0,freeEnd]

**b) tab7**

**btn4**

Result Visualization Result Text

Save Result Text

Building Blocks for Searching: ALL

Block(s) for Searching:

GalNAc[NPhth,OH,OCIAc,OTIPS] PredictedRRV=11.971

Index9941 GalNAc[NPhth,OH,NO2Bz,OTBDPS] PredictedRRV=25.804

Index10735 GalNAc[NPhth,OH,NO2Bz,OTBS] PredictedRRV=29.159

Index15044 GalNAc[NPhth,OH,OTBDPS,OCIAc] PredictedRRV=55.4

RESULT:

ResidueID:[110041] Sugar:[D-Glc-b] ParentID:[Root] ChildID:[Pos4=ID110042]

--

ResidueID:[110042] Sugar:[D-Gal-b] ParentID:[110041] ChildID:[Pos4=ID110043]

--

ResidueID:[110043] Sugar:[D-Gal-a] ParentID:[110042] ChildID:[Pos3=ID110044]

--

ResidueID:[110044] Sugar:[D-GalNAc-b] ParentID:[110043] ChildID:[Pos3=ID110045]

Solution 1:

Fragment 1[Fragment Yield=94%][Deprotect=N/A][HasSatbleProductAnomer=Yes][LeavingGroup=STol]

BBL RRV=1462.00 Type=Exp. Index=117 ProtectingGroup=[2:OBz,3:2,4:OPh,5:-,6:OPh]

BBL RRV=32.00 Type=Exp. Index=45 ProtectingGroup=[2:NHTroc,3:OH,4:OBz,5:-,6:OBz]

Solution 2:

Fragment 1[Fragment Yield=94%][Deprotect=N/A][HasSatbleProductAnomer=Yes][LeavingGroup=STol]

BBL RRV=1306.00 Type=Exp. Index=114 ProtectingGroup=[2:OLev,3:2,4:OPh,5:-,6:OPh]

BBL RRV=32.00 Type=Exp. Index=45 ProtectingGroup=[2:NHTroc,3:OH,4:OBz,5:-,6:OBz]

Done

**c) tab5**

**btn5**

**btn6**

**btn7**

**Filtering Options**

Library for Searching

Use Experimental Library Only

Use Experimental and Virtual Libraries

Filtering

Sugar Type

Product Anomer

R2

R3

R4

R6

Show Selected Virtual BBL(s)

Show Filtered Virtual BBL(s)

Show All Virtual BBLs

Selection	Sugar Type	Product Anomer	R2	R3	R4	R6	Predicted RRV	Feedback [BBL ID]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OCIAc	OTBS	6.774	To rate [3666]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OCIAc	OCIAc	8.366	To rate [4393]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	NO2Bz	NO2Bz	9.42	To rate [4849]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OCIAc	OTBDPS	9.516	To rate [4891]
<input checked="" type="checkbox"/>	GalNAc	Beta	NPhth	OH	OCIAc	OTIPS	11.971	To rate [5885]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OBz	OBz	14.324	To rate [6715]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	NO2Bz	OCIAc	15.354	To rate [7068]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OBz	OCIAc	15.775	To rate [7216]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OCIAc	OBz	16.861	To rate [7588]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	NO2Bz	OBz	16.942	To rate [7611]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OCIAc	NO2Bz	17.748	To rate [7851]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OBz	NO2Bz	20.016	To rate [8492]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OBz	OTBS	21.088	To rate [8769]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OBz	OTBDPS	22.91	To rate [9253]
<input checked="" type="checkbox"/>	GalNAc	Beta	NPhth	OH	NO2Bz	OTBDPS	25.804	To rate [9942]
<input checked="" type="checkbox"/>	GalNAc	Beta	NPhth	OH	NO2Bz	OTBS	29.159	To rate [10736]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OTBS	OCIAc	44.817	To rate [13596]
<input type="checkbox"/>	GalNAc	Beta	NPhth	OH	OBz	OTIPS	48.94	To rate [14190]

Figure 3

[Click here to access/download;Figure;Figure3.pdf](#)

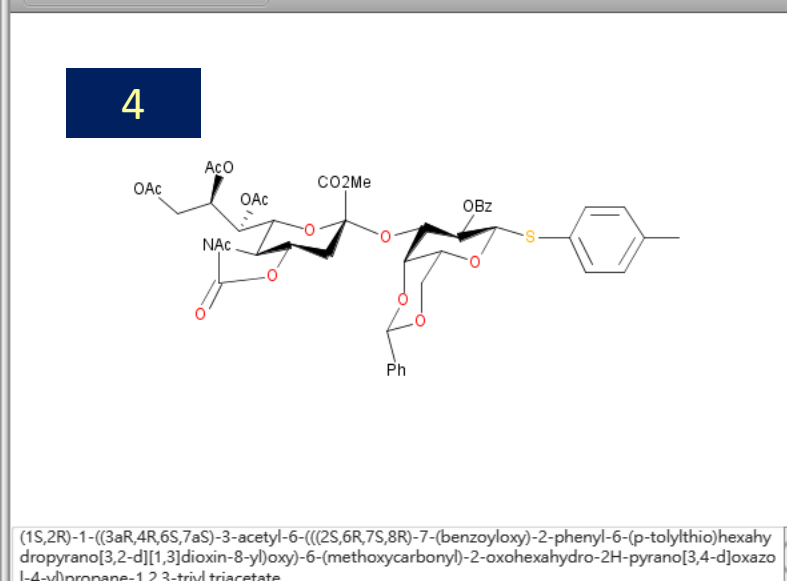
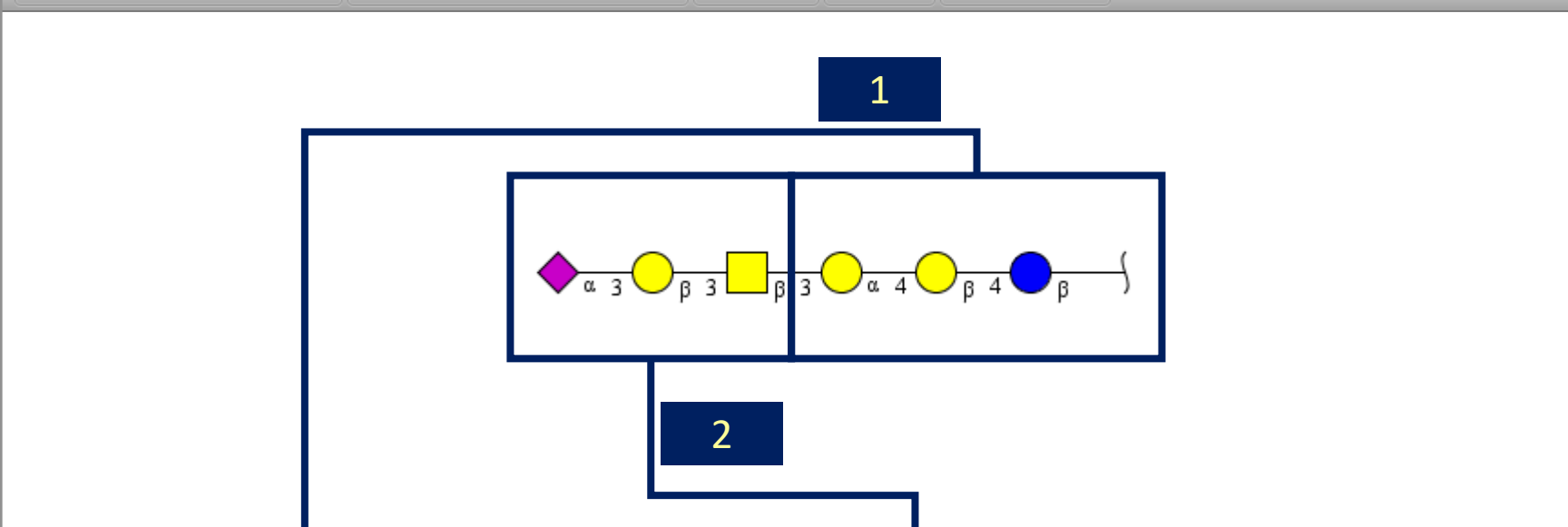
Auto-CHO

Query Structure   Parameter Settings   About Auto-CHO

Edit Glycan by GlycanBuilder   **Search Building Block Library**   Globo-H   SSEA-4   OligoLacNAc

Experimental Building Block Library   Virtual Building Block Library

Hide IUPAC Name



Result Visualization   Result Text

Reducing End Acceptor

Fragment

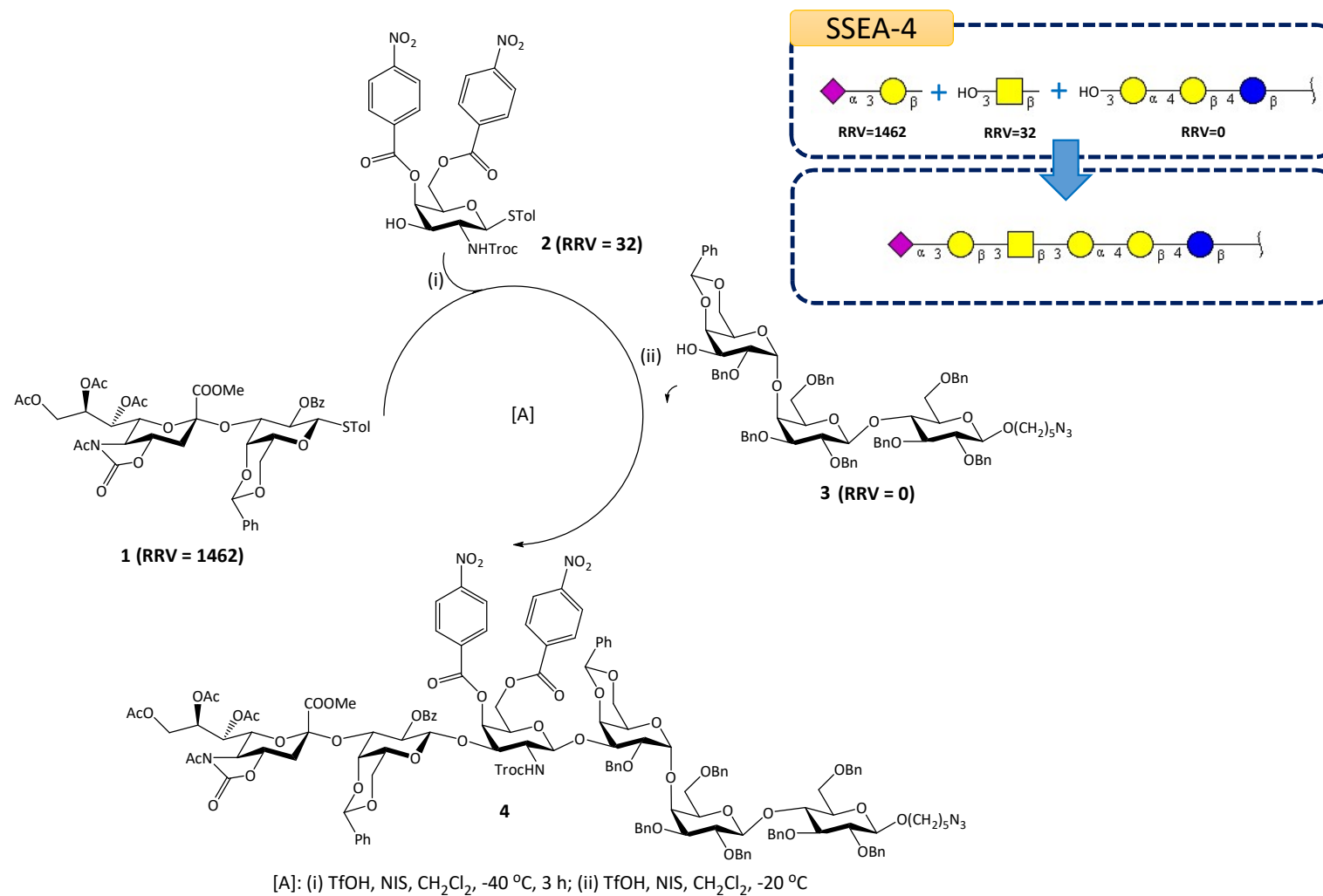
3

Solution	# of Frag.	Avg. Frag. Yield	Fragment	Frag.'s RRV	Yield	Deprotection
1	1	94%		32.00	94%	N/A
2	1	94%				
3	1	59%				
4	1	57%				

BBL's RRV	BBL Type	Fragment Connection
1462.0	Exp.	X1: Fragment1 + Reducing End Acceptor
32.0	Exp.	

Index	RRV	Acceptor Positi...	Product Anomer	Sugar Type
117	1462.0	Full Protection	Beta	NeuAc_Gal
118	1791.0	3	Beta	Gal
119	1900.0	2	Alpha	Glc
120	2000.0	3	Beta	GalNAc
121	2656.0	Full Protection	Alpha	Glc
122	2797.0	4	Beta	Gal
123	3269.0	3	Beta	GalNAc
124	3531.0	2	Alpha	Gal
125	4000.0	Full Protection	Beta	Gal
126	4350.0	3	Alpha	Glc
127	4800.0	Full Protection	Beta	Gal
128	5000.0	Full Protection	Alpha	Man
129	6500.0	Full Protection	Beta	GlcNAc
130	7180.0	Full Protection	Alpha	Gal
131	8417.0	None	Alpha	Fuc_Gal

Done



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetonitrile	Sigma-Aldrich	75-05-8	
Anhydrous magnesium sulfate	Sigma-Aldrich	7487-88-9	
Cerium ammonium molybdate	TCI	C1794	
Dichloromethane	Sigma-Aldrich	75-09-2	
Drierite	Sigma-Aldrich	7778-18-9	
Ethyl acetate	Sigma-Aldrich	141-78-6	
Methanol	Sigma-Aldrich	67-56-1	
Molecular sieves 4 Å	Sigma-Aldrich		
<i>n</i> -Hexane	Sigma-Aldrich	110-54-3	
<i>N</i> -Iodosuccinimide	Sigma-Aldrich	516-12-1	
Sodium bicarbonate	Sigma-Aldrich	144-55-8	
Sodium thiosulfate	Sigma-Aldrich	10102-17-7	
Toluene	Sigma-Aldrich	108-88-3	
Trifluoromethanesulfonic acid	Sigma-Aldrich	1493-13-6	



1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
[www.jove.com](http://www.jove.com)

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Guideline and demonstration for hierarchical and programmable one-pot synthesis of oligosaccharides
Author(s):	Cheng-Wei Cheng, Yixuan Zhou, Wen-Harn Pan, Supriya Dey, Chung-Yi Wu, Wen-Lian Hsu, Chi-Huey Wong

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:



Standard Access



Open Access

Item 2: Please select one of the following items:



The Author is **NOT** a United States government employee.



The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.



The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.



## ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

## ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

### CORRESPONDING AUTHOR

Name:

Chi-Huey Wong

Wen-Lian Hsu

Department:

Genomics Research Center

Institute of Information Science

Institution:

Academia Sinica

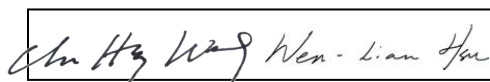
Academia Sinica

Title:

Distinguished Research Fellow

Distinguished Research Fellow

Signature:



Date:

March 12, 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**Response:** We have proofread the manuscript and all changes has been highlighted by the tracking system.

2. Please avoid long steps (more than 4 lines).

**Response:** We have modified the manuscript to meet the requirement.

3. Step 1.4.1: Please write this step in the imperative tense.

**Response:** We have modified the manuscript to meet the requirement.

4. 1.5.1: Please write this step in the imperative tense.

**Response:** We have modified the manuscript to meet the requirement.

5. 1.7.2: Please write this step in the imperative tense.

**Response:** We have modified the manuscript to meet the requirement.

6. 1.9.3: Please write this step in the imperative tense.

**Response:** We have modified the manuscript to meet the requirement.

7. 3.1: Please write this step in the imperative tense.

**Response:** We have modified the manuscript to meet the requirement.

8. 3.2: Please write this step in the imperative tense.

**Response:** We have modified the manuscript to meet the requirement.

9. 3.3: Please write this step in the imperative tense.

**Response:** We have modified the manuscript to meet the requirement.