Method Article – Title Page

Title	*Two-step conversion of unprotected oligosaccharides to generate bioorthogonal oligosaccharide tool compounds*
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Keywords	• *Complex carbohydrates* • *Bioorthogonal probes* • *Facile synthesis*
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ABSTRACT
Complex oligosaccharides have invaluable effects within biological systems, but their complex nature makes them difficult to convert into chemical tools. We have developed a two-step conversion of unprotected oligosaccharides that is highly amenable to generating a variety of complex oligosaccharide tool compounds. This sequence features an optimized Kochetkov amination procedure and subsequent amide coupling. With these simple synthetic conversions, the creation of novel bioorthogonal carbohydrate probes becomes easily accessible and new avenues for chemical biology may be opened.
- Optimized Kochetkov amination procedure for complex oligosaccharides
- Highly versatile amide coupling for facile probe synthesis

SPECIFICATIONS TABLE

Subject Area	Chemistry
More specific subject area	*Carbohydrate Chemistry and Bioorthogonal Application*
Method name	*Two-step conversion of deprotected oligosaccharides to generate bioorthogonal tools for chemical biology applications*
Name and reference of original method	*Kochetkov amination*[1]
Resource availability	*All reagent grade chemicals can be purchased through Millipore-Sigma. https://www.sigmaaldrich.com/united-states.html*

Method Details
Detailed below are the optimized reaction methods for the two-step sequence outlined in Figure 1. The first reaction is a Kochetkov amination optimized for complex oligosaccharide derivatives, while the second reaction is a carbodiimide mediated amide coupling amenable to a variety of functional tags.

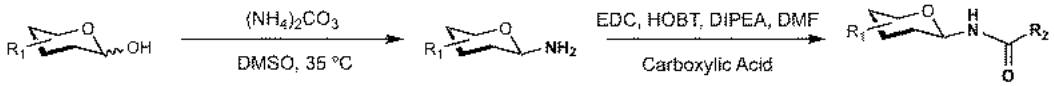


Figure 1: Two-step conversion of oligosaccharides into functional bioorthogonal probes. R_1 denotes any complex carbohydrate scaffold, while R_2 is representative of a desired functional handle (i.e. fluorophore, alkyne, azide)

General Procedure of Modified Kochetkov Amination
1. To an oven dried round-bottom equipped with a stir bar, oligosaccharide (100 mg) is dissolved in DMSO (1.0 M; dried repeatedly over activated 3 Å molecular sieves from Millipore-Sigma).

2. Ammonium carbonate (500 mg, 5 x mass of oligosaccharide) is then added and the reaction stirred at 35 °C for 3 days under an atmosphere of argon.

3. The solution is then diluted in a minimal amount of water and frozen immediately in liqid nitrogen to avoid unwanted hydrolysis.

4. The frozen solid is then lyophilized to dryness and the process repeated (at minimum x3) until a constant mass of white solid is obtained.

4. NMR analysis is conducted in D_2O solvent. Experiments are run immediately upon sample preparation to avoid unwanted hydrolysis. Ratios of conversion are determined by integration of C-1 anomeric protons of the starting material to that of the desired product. Mass of residual DMSO is accounted for by calculation from corresponding integration values and is represented in final yields.

General Procedure of Amide Coupling

1. Glycosyl amine (10 mg, 1 equiv.) in anhydrous DMF (0.1 M; dried repeatedly over activated 3 Å molecular sieves from Millipore-Sigma) is stirred in an oven dried round bottom flask at room temperature under an atmosphere of argon.

2. EDC (1.25 equiv.), DIPEA (1.25 equiv.), HOBT (1.25 equiv.), and carboxylic acid (1.25 equiv.) are added to the reaction sequentially. The resulting slurry is then stirred at room temperature and kept in the dark (if light sensitive).

3. The reaction mixture is monitored by TLC for remaining glycosyl amine (60:30:5:1 DCM:MeOH:H_2O:AcOH; Ninhydrin Stain).

The glycosyl amine appears bright orange to red in color upon staining and is an easy indicator of consumption in the reaction conditions. Typical reaction times are between 4-12 hours.

4. Upon consumption, the reaction is concentrated *in vacuo* via co-evaporation with toluene (x3).

5. The resulting solid is dissolved in a minimal amount of 10% MeCN/H_2O and purified by reverse phase preparative HPLC (5%-40% MeCN/H_2O over 30 min; flow rate 5.00 mL min^{-1}; Hypersil GOLD 150mm x 10mm).

HOBT byproducts are the most difficult to remove. Alternative gradients (5-20% MeCN/H_20 over 30 min; flow rate 5.00 mL min^{-1}; Hypersil GOLD 150mm x 10mm) may be used in some cases. Additionally, ease of separation depends on the nature of the coupling partner, so subsequent considerations should be made accordingly.

6. The purified fractions are detected by mass spectrometry. Hydrolyzed and unreacted oligosaccharide can also be detected and

recovered.

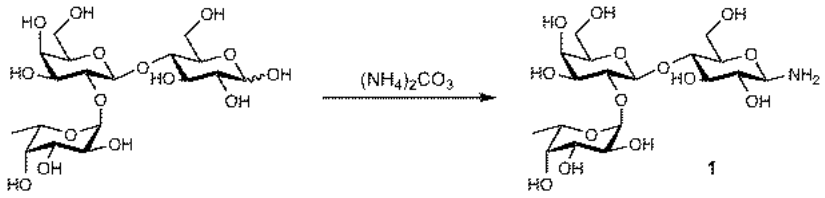
7. Purified fractions are then lyophilized to yield the desired product as a white solid, which is stored at -20 °C in the dark (if necessary) until use.

Method Validation

2'-fucosyllactose was chosen as a model oligosaccharide due to its high level of availability relative to other potential carbohydrates. The optimized conditions were then performed and compared to alternative methods.

Synthesis of 2'-fucosyllactose amine (1)

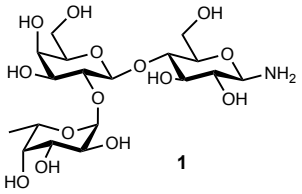
The procedure listed above was utilized in the conversion of 2'-fucosyllactose to the corresponding glycosyl amine **1** (**Table 1**). The conditions developed (**Entry 1**) produced a 78% yield with a ratio of 1 to 2.4 starting material to product (SM:P). Contrarily, when this reaction is performed in H_2O as a solvent or under microwave irradiation conditions, the conversion ratio to desired product significantly decreased (**Entries 2-3**). Additionally, when the reaction was performed at a higher temperature, the formation of unwanted diglycosylamine products predominated. As such the conditions developed herein represent the most efficient method for conversion of complex oligosaccharides to their corresponding amines.



Entry	Solvent	Temperature	Time	Yield	Conversion
1	DMSO	35 °C	3 days	78%	1 to 2.4 SM:P
2	DMSO	40 °C via microwave irradiation	90 min	75%	1 to 1.1 SM:P
3	H_2O	60 °C	3 days	95%	1 to 1.4 diglycosylamine : P

Table 1: Method Validation for Optimized Kochetkov Amination Procedure

Characterization of Compound 1 from Developed Method

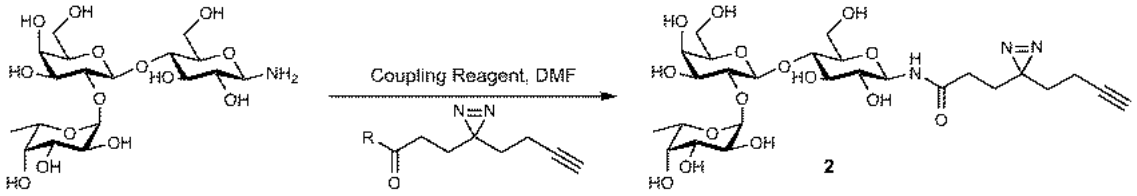


1

(2S,3S,4R,5S,6S)-2-(((2S,3R,4S,5R,6R)-2-(((2R,3S,4R,5R,6R)-6-amino-4,5-dihydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)oxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)oxy)-6-methyltetrahydro-2H-pyran-3,4,5-triol (1): 78%, 2.4:1 SM:P; ^{1}H NMR (600 MHz, D_2O) δ 5.32 (d, *J* = 3.9 Hz, 1H), 4.53 (d, *J* = 7.8 Hz, 1H), 4.25 (m, 1H), 4.10 (d, *J* = 8.8 Hz, 1H), 3.94 (dd, *J* = 11.9, 2.2 Hz, 1H), 3.94 – 3.85 (m, 3H), 3.86 – 3.65 (m, 10H), 3.63 – 3.55 (m, 1H), 3.45 (ddd, *J* = 10.0, 5.8, 2.1 Hz, 1H), 3.22 (t, *J* = 9.0 Hz, 1H), 1.24 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (151 MHz, D_2O) δ 100.2, 99.3, 85.1, 76.2, 76.0, 75.2, 75.0, 74.0, 73.6, 71.6, 70.4, 69.6, 69.1, 68.1, 66.9, 61.1, 60.3, 15.2; HR-ESI-MS (m/z): calcd for $C_{18}H_{32}NO_{14}^-$ $(M-H)^-$ 486.1823, found 486.1816.

Synthesis of 2'-fucosyllactose bioorthogonal probe (2)

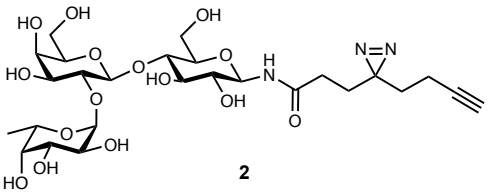
The procedure described above was used to generated the bioorthogonal probe **2** in 47% yield (**Table 2, Entry 1**). The purification technique was amenable for recovery of the unreacted/hydrolyzed oligosaccharide, preventing unwanted loss of precious complex materials. Alternative amide coupling conditions performed similarly in yield but required more specialized reagents (**Table 2, Entries 2-3**). Conversion of the starting carboxylic acid into a *N*-hydroxysuccinimide ester was also conducted, whereby direct basic treatment of the glycosyl amine yielded the desired products in comparable yield (**Entry 4**).[2] the amide coupling conditions developed above proved superior in this case.



Entry	Coupling Reagent	Coupling Partner (R)	Yield
1	EDC/HOBT	Carboxylic acid	47%
2	HATU	Carboxylic acid	17%
3	PyClock	Carboxylic acid	50%
4	K_2CO_3	*N*-hydroxysuccinimide ester	19%

Table 2: Method Validation for Optimized Amide Bond Formation

Characterization of Compound 2 from Developed Method



2

3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-((2R,3R,4R,5S,6R)-5-(((2S,3R,4S,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-(((2S,3S,4R,5S,6S)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)propenamide (2): 47%; ^{1}H NMR (600 MHz, D_2O) δ 5.34 (d, *J* = 3.6 Hz, 1H), 4.98 (d, *J* = 9.2 Hz, 1H), 4.56 (d, *J* = 7.8 Hz, 1H), 4.26 (d, *J* = 6.7 Hz, 1H), 3.96 (dd, *J* = 12.2, 2.1 Hz, 1H), 3.94 – 3.86 (m, 2H), 3.86 – 3.57 (m, 13H), 3.46 (t, *J* = 9.3 Hz, 1H), 2.28 – 2.18 (m, 2H), 2.10 – 2.04 (m, 2H), 1.84 (td, *J* = 7.5, 1.6 Hz, 2H), 1.71 (t, *J* = 7.2 Hz, 2H), 1.27 (d, *J* = 6.6 Hz, 3H);^{13}C NMR (151 MHz, D_2O) δ 176.5, 100.2, 99.3, 79.2, 76.8, 76.2, 75.2, 75.1, 73.6, 71.6, 71.5, 69.6, 69.1, 68.1, 66.9, 61.1, 59.9, 30.9, 29.7, 28.6, 27.8, 15.2, 12.4; HR-ESI-MS (m/z): calcd for $C_{26}H_{40}N_3O_{15}^-$ $(M-H)^-$ 634.2459, found 634.2443.

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Declaration of interests:

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional Information

Background

As carbohydrate structure varies greatly across biological source and isolation, the synthesis of chemoproteomic tools for the study of their biological interactions remain a significant challenge.[3] The highly diverse nature of oligosaccharide branching patterns, monosaccharide composition, and conformational considerations are just a few reasons that synthetic efforts in this field are usually monumental endeavors, rarely amenable to broad-spectrum applicability or derivatization. As the scientific community becomes more interested in carbohydrate interactions at the cellular level, access to a variety of bioorthogonal oligosaccharide variants becomes more necessary. These bioorthogonal probes will need to range in potential use from fluorescence assays to protein-target identification. As such, methods for facile generation of these biological tools need to be developed, focusing on broad-spectrum applicability to complex oligosaccharide scaffolds. Herein, we have developed a two-step procedure in which an unprotected oligosaccharide scaffold can be converted into a bioorthogonal tool compound.[4] First, an optimized Kochetkov amination converts the reducing end of the carbohydrate to the corresponding glycosyl amine in a stereoselective fashion. Secondly, a carbodiimide-mediated amide coupling with a carboxylic acid containing functional tag generates a functional carbohydrate probe. This is briefly demonstrated by the use of this two-step sequence in generating a bioorthogonal probe of 2'-fucosyllactose.

References

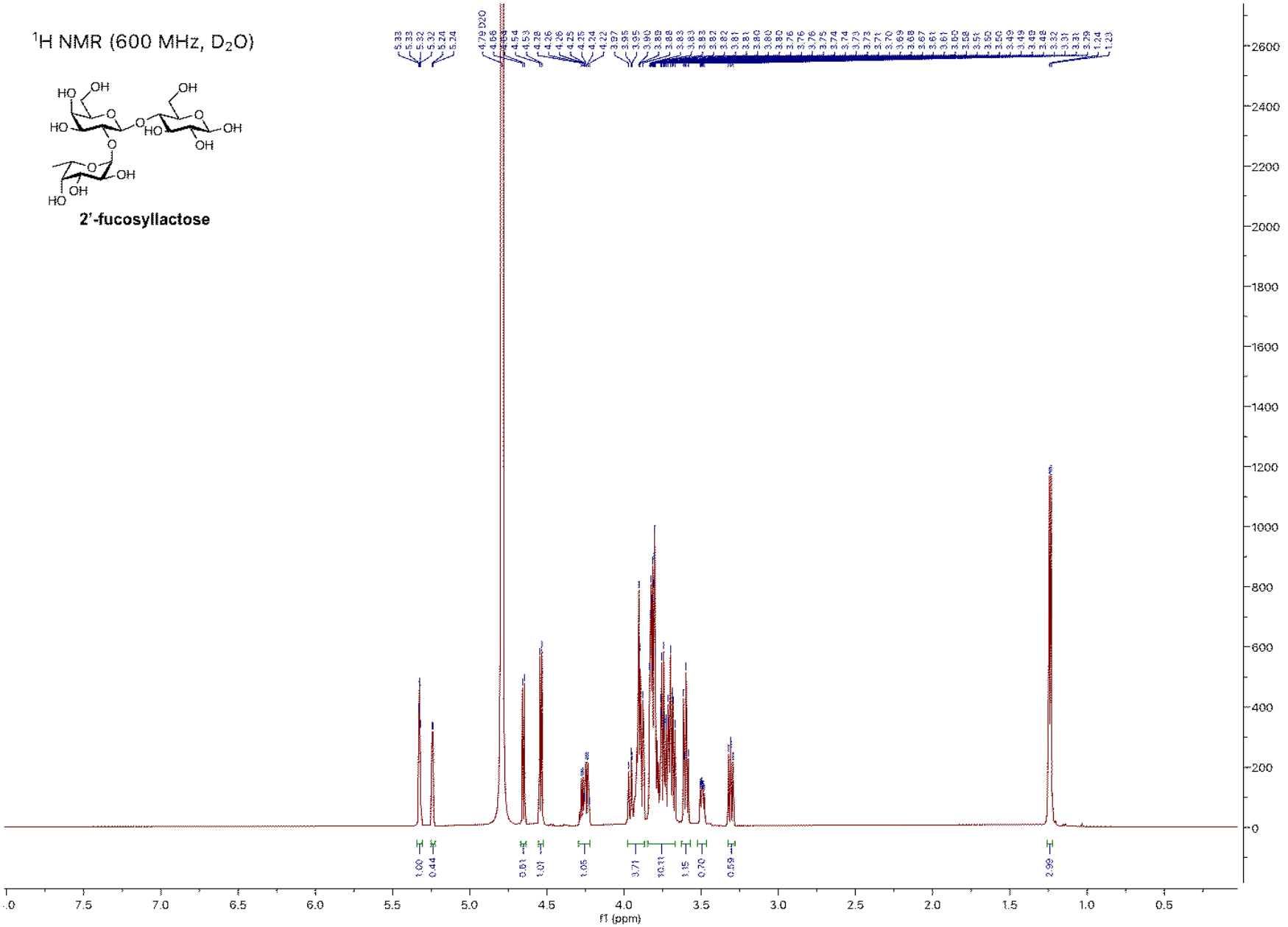
[1] Likhosherstov, L. M., Novikova, O. S., Derevitskaja, V. A., and Kochetkov, N. K. (1986) A new simple synthesis of amino sugar β-d-glycosylamines, *Carb Res 146*, C1-C5.

[2] DeMeester, K. E., Liang, H., Jensen, M. R., Jones, Z. S., D'Ambrosio, E. A., Scinto, S. L., Zhou, J., and Grimes, C. L. (2018) Synthesis of Functionalized N-Acetyl Muramic Acids To Probe Bacterial Cell Wall Recycling and Biosynthesis, *Journal of the American Chemical Society 140*, 9458-9465.

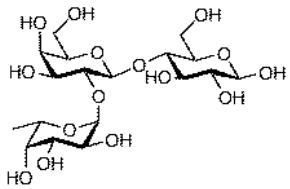
[3] Bertozzi, C. R., Kiessling, and Laura, L. (2001) Chemical Glycobiology, *Science 291*, 2357.

[4] Chambers, S. A., Townsend, S. D. *Carb Res :* CAR_2019_532

Supplementary Information – ^{1}H and ^{13}C NMR Characterization



^{13}C NMR (151 MHz, D_2O)



2'-fucosyllactose

^{1}H NMR (600 MHz, D_2O)

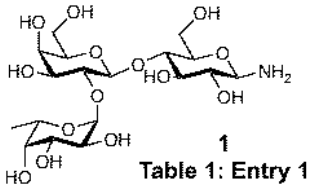


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Table 1: Entry 1

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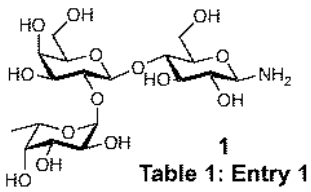

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Table 1: Entry 1

^{1}H NMR (600 MHz, D_2O)

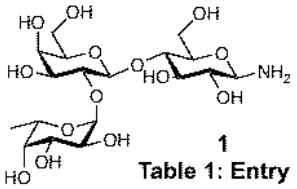

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Table 1: Entry 2

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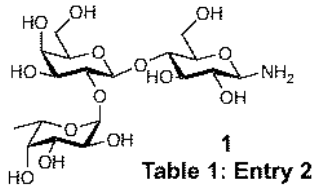

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Table 1: Entry 2

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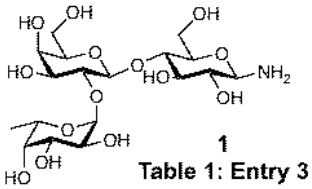

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Table 1: Entry 3

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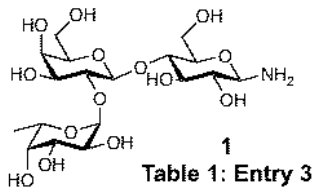

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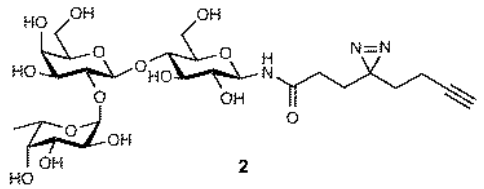
Table 1: Entry 3

^{1}H NMR (600 MHz, D_2O)



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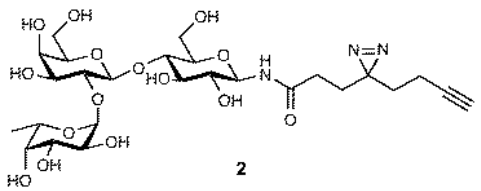
^{13}C NMR (151 MHz, D_2O)



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