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Synthesis of Sugar Arrays in Microtiter Plate

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Abstract: 1,3-Dipolar cycloadditions between azides and alkynes were exploited to attach oligosaccharides to a C_{14} hydrocarbon chain that noncovalently binds to the microtiter well surface. Synthesis of sugar arrays was performed on a micromolar scale in situ in the microtiter plate. As a model study, the β-galactosyllipid **5** was displayed on a 4-μmol scale. Formation of product was confirmed via ESI−MS, and the yield was determined via chemical and biological assays. Several complex carbohydrates (**6**−**16**) were also displayed in microtiter plates and successfully screened with various lectins. Moreover, sialyl Lewis x (**17**) was synthesized via the enzymatic fucosylation of a precursor displayed in the plate. Studies on inhibition of this biotransformation have been carried out, and the IC_{50} value found for the known inhibitor **20** was consistent with previous studies in solution.

Introduction

Cells universally carry a sugar coating formed by glycoproteins and glycolipids, which are involved in highly specific recognition events between cells and proteins, hormones, antibodies, and toxins. Understanding the mechanism of these processes may lead to the development of new anti-infective, anticancer, and anti-inflammatory strategies.[1−3]

Due to the increasing need to decipher the information contained in complex carbohydrates, simple and readily accessible methods for high-throughput analysis must be developed. Microarrays have been reported to be one of the most frequently used approaches because large libraries of compounds can be quickly screened and only small quantities of material are required,[4] an important consideration due to the low availability of some complex carbohydrates. Few approaches have been developed, thus far, for the fabrication of carbohydrate microarrays. Wang et al.[5] have found that nitrocellulose-coated glass slides can be used to immobilize microspots of carbohydrate polymers without covalent conjugation. Mrksich et al.[6] exploited the Diels−Alder mediated immobilization of carbohydrate−cyclopentadiene conjugates to a monolayer that presents benzoquinone groups displayed on a gold surface. Recently, Shin et al.[7] reported on the attachment of maleimide-linked carbohydrates to a glass slide coated by thiol groups, and Feizi et al.[8] described microarrays of oligosaccharides displayed as neoglycolipids on nitrocellulose.

Our goal is to develop a simple and efficient system to attach complex sugars to a microtiter plate to screen for their specific interactions with proteins.

One major difficulty with regard to this subject is to identify the appropriate functional groups and the generally useful chemistry for attaching saccharides to various types of surfaces, either covalently or noncovalently. We have chosen cycloaddition reactions, as they are often simple, reagent-free, and very selective, but we have avoided the use of olefins, as sustaining the reductive conditions often used in the final deprotection of synthetic oligosaccharides is difficult in the olefin group. In our effort to develop a simple, noncovalent method for the preparation of saccharide arrays in microtiter plates, we have found that a saturated hydrocarbon that is 13 to 15 carbons in length can stick to the surface of polystyrene microtiter plates, and the glycolipids displayed are stable under repeated aqueous washings and are functional in biological screens.[9] Because the preparation of glycolipids for microarrays

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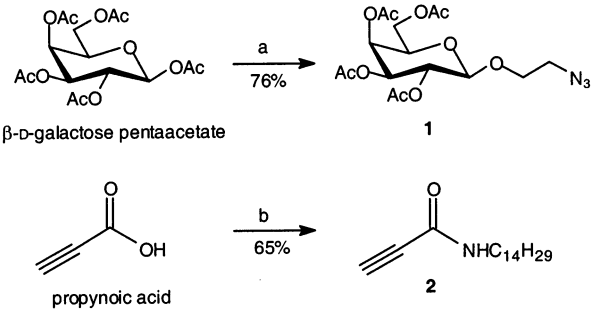
Scheme 1



Reagents and Conditions: (a) 2-azidoethanol, BF_3-Et_2O, CH_2Cl_2, rt, overnight; (b) $C_{14}H_{29}NH_2$, DCC, CH_2Cl_2, 0 °C-rt, 2 h.

can be difficult due to interference of the lipid moiety in isolation and manipulation, we are looking for alternative methods for the synthesis and attachment in situ of sugars to the microtiter plate.

One reaction of interest is the 1,3-dipolar cycloaddition between alkynes and azides, a well-known reaction for the construction of [1,2,3]-triazole rings.[10] The chemical property of the azido group renders this approach suitable for reaction in microtiter plates and applicable to carbohydrate chemistry. Indeed, azides are stable toward a majority of organic synthesis conditions, and syntheses of complex carbohydrates, routinely performed in our laboratory, employ azido-sugars as an ideal "masked form" of more reactive and more difficult to handle amino-sugars. Moreover, enzymatic synthesis of complex carbohydrates has also been performed using azido-sugars as substrates,[11] and, if necessary, conversion of amines to azides using the metal-catalyzed diazotransfer reaction[12] is relatively straightforward, showing therefore the general utility of this approach for microarray fabrication of carbohydrates.

Results and Discussion

Our first attempts to investigate this reaction were performed in a model study using 2-azidoethyl-2,3,4,6-tetraacetyl-β-D-galactopyranoside (**1**), which was prepared from β-D-galactose penta-acetate in 76% yield (Scheme 1). The alkyne counterpart was chosen as the side of attachment to the microtiter plate and therefore needed to contain a long aliphatic chain (C_{14}). Furthermore, alkynes with an electron-withdrawing neighboring group favor 1,3-dipolar cycloaddition.[13] To combine these requirements, we prepared the alkyne **2** from propynoic acid and tetradecylamine via DCC coupling.

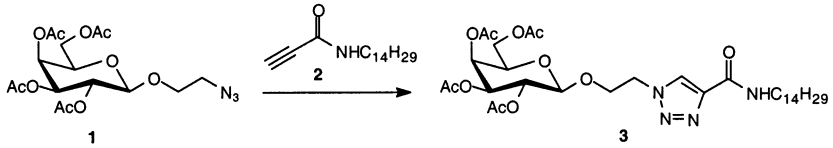
Standard conditions for the construction of the triazole ring involve refluxing the two components in an organic solvent (often toluene). When run neat, this reaction afforded compound **3** in 89% yield and as expected, as a mixture of regioisomers (4:1 mixture of the 1,4- and 1,5-regioisomers, Scheme 2). However, the necessity of running this reaction at high temperature even with the use of an electron-poor alkyne (at room temperature, only traces of **3** could be detected after 7 d) does not make it useful for synthesis in microtiter plates.

Recently, the copper(I)-catalyzed regiospecific 1,3-cycloaddition between terminal alkynes and azides was reported. Sharpless et al.[14] found that Cu(II)/sodium ascorbate catalyzes the regiospecific formation of 1,4-triazoles from a broad range of substrates. Meldal et al.[15] utilized CuI/base as a catalyst and a conjugated terminal alkyne as the dipolarophile linked to a solid support. Both procedures form the triazole moiety regiospecifically (only the 1,4-regioisomer is formed), but more interestingly for our propose, these reactions were run at room temperature. We decided therefore to investigate the reactivity of the acyl-protected azido-sugar **1** with alkyne **2**. This transformation was found to be extremely sensitive to the solvent and base employed, as side reactions for the alkyne **2** can compete with triazole formation.[15] For instance, acetonitrile/Et_3N did not form **3**, whereas simply changing the base from Et_3N to DIPEA formed **3** in 38% yield. We found that the system alkyne/azide/DIPEA/CuI (1:1:1:0.1) in toluene afforded regiospecifically the 1,4-triazole **3** in 85% yield (Scheme 2). The optimized formation of triazole **3** thus represents a general synthesis of glycolipid analogues.

With this knowledge, the triazole formation of free azido-sugars was then undertaken, using 2-azidoethyl-β-D-galactopyranoside (**4**) as a model obtained from **1** in 95% yield. The thermal 1,3-dipolar cycloaddition between **4** and alkyne **2** afforded the triazole derivative **5** in 75% yield as a 5.6:1 mixture of the 1,4- and 1,5-regioisomers.

When the copper(I)-catalyzed cycloaddition (alkyne/azide/DIPEA/CuI, 1:1:5:5) was employed, **5** was isolated in 80% yield

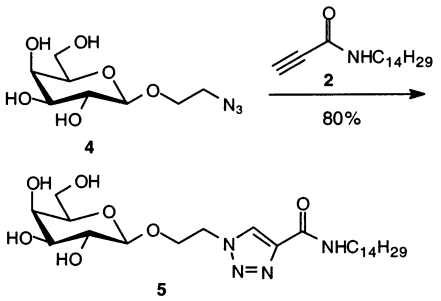
Scheme 2



entry	2	base (1 eq)	CuI	solvent	T	time	yield[a]
1	5eq	none	none	none	80	24h	89%[b]
2	1eq	Et_3N	2eq	CH_3CN	rt	18h	trace
3	1eq	DIPEA	2eq	CH_3CN	rt	18h	38%
4	1eq	Et_3N	0.1eq	toluene	rt	18h	65%
5	1eq	DIPEA	0.1eq	toluene	rt	18h	85%
6	1eq	none	0.1eq	toluene	rt	3days	52%
7	1eq	none	0.1eq	toluene	rt	7days	61%

Reaction Conditions for the Formation of **3**; (a) Isolated Yields; (b) Mixture 4:1 of 1,4- and 1,5-regioisomers.

Scheme 3



Reagents and Conditions: (a) DIPEA, CuI, MeOH, rt, 8 h.

as the single 1,4-regioisomer (Scheme 3). Altering the solvent systems from MeOH to H_2O/CH_3CN (4:1) afforded **5** in comparable yield.

This chemistry was then carried out on microscales to develop new micro-fabrication methods for applications in the biological screening of carbohydrates. To achieve this goal, two crucial points had to be resolved. First, the purification procedures, such as column chromatography, that were used in all previous cases needed to be avoided in order for the reaction to be applicable to high-throughput synthesis and screening. Second, due to the low availability of many natural carbohydrates, the reactions needed to be carried out on a very small scale (possibly micromolar). Therefore, we developed two different approaches, which exploit both the thermal and the copper(I)-catalyzed formation of the triazole ring.

Thermal formation has the advantage of employing only the alkyne and azide, which renders purification simple. The alkyne was used in 5-fold excess. It possesses physical properties completely different from those of the product. Therefore, unreacted alkyne was simply washed away with hot Et_2O, giving **5** in very high purity without further purifications at very low concentration (4 μmol scale). The only problem associated with this transformation is the lack of regiocontrol as described above. However, when we ran this thermal conversion (80 °C, 24 h, neat) in the presence of only traces of CuI, the triazole derivative **5** was obtained regioselectively and in high purity (Figure 1). These results show that if terminal alkynes such as **2** are employed, a complete regiochemical control can be achieved in the thermal 1,3-dipolar cycloaddition reaction with azides.

The copper(I)-catalyzed reaction has the advantage of forming the triazole at room temperature. When this reaction was run in MeOH in the 100 μL microtiter plate well, the crude product could be purified in situ, allowing the reaction to be carried out on a micromolar scale (Figure 2).

As an example, the reaction mixture (80 μL, Figure 2) was incubated at room temperature. After 8 h, ESI−MS analysis showed disappearance of starting material and formation of product **5** (Figure 3B). The plate was then placed in a ventilated fume hood for ~3 h to allow evaporation of the solvent and then washed with water to remove salts and the base. The

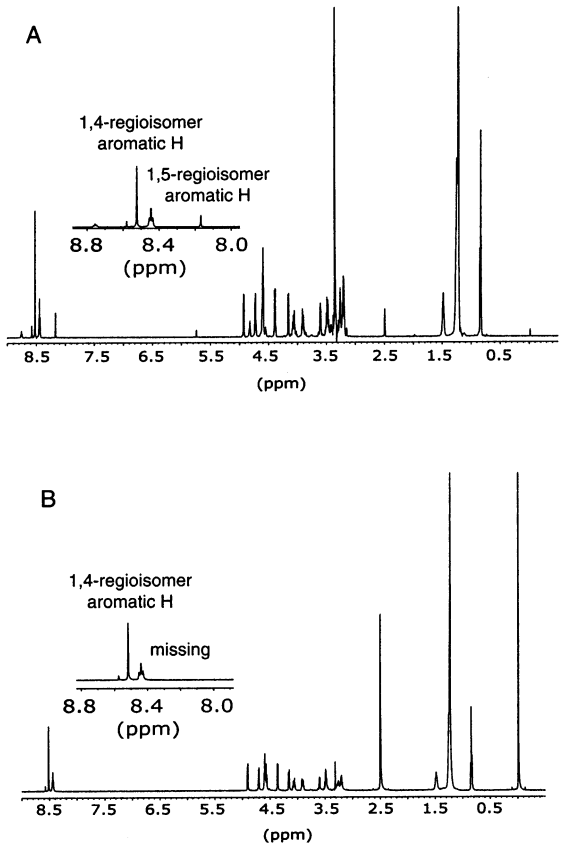


Figure 1. ^{1}H NMR of (A) purified **5** obtained by thermal 1,3-cycloaddition (1,4/1,5-regioisomers 5.6:1). (B) crude **5** (4 μmol scale) obtained by thermal 1,3-dipolar cycloaddition in the presence of traces of CuI (single 1,4-regioisomer).

product is retained in the well due to its hydrophobic interaction with the well surface. Purified **5** was redissolved in MeOH and analyzed by ESI−MS (Figure 3C).

To determine the yield of this transformation, chemical and biological assay methods were undertaken.[9] Following the Sulfuric Acid−Phenol (SAP)[16] assay, an aqueous phenol solution was added to the wells, followed by concentrated H_2SO_4. Absorbance of the phenol adduct was measured at 490 nm. The average absorbance of reaction wells was divided by the average absorbance of wells incubated with pure **5**, which was synthesized independently. This percentage was taken as the yield (92%).

An additional biological assay was also undertaken exploiting the recognition between the lectin Ricin B and β-D-galactose.[17] Wells were incubated with TBS buffer containing bovine serum albumin (BSA) to prevent protein binding to the well surface. Ricin B chain from *Ricinus communis* (castor bean) was then incubated in the well, followed by the specific anti-lectin for *Ricinus communis* from rabbit. Wells were then incubated with monoclonal anti-rabbit immunoglobulin conjugated with an alkaline phosphatase. After the addition of *p*-nitrophenyl phosphate, the absorbance was read at 405 nm. As before, comparison with absorbance of pure **5** allowed yield determination (91%).

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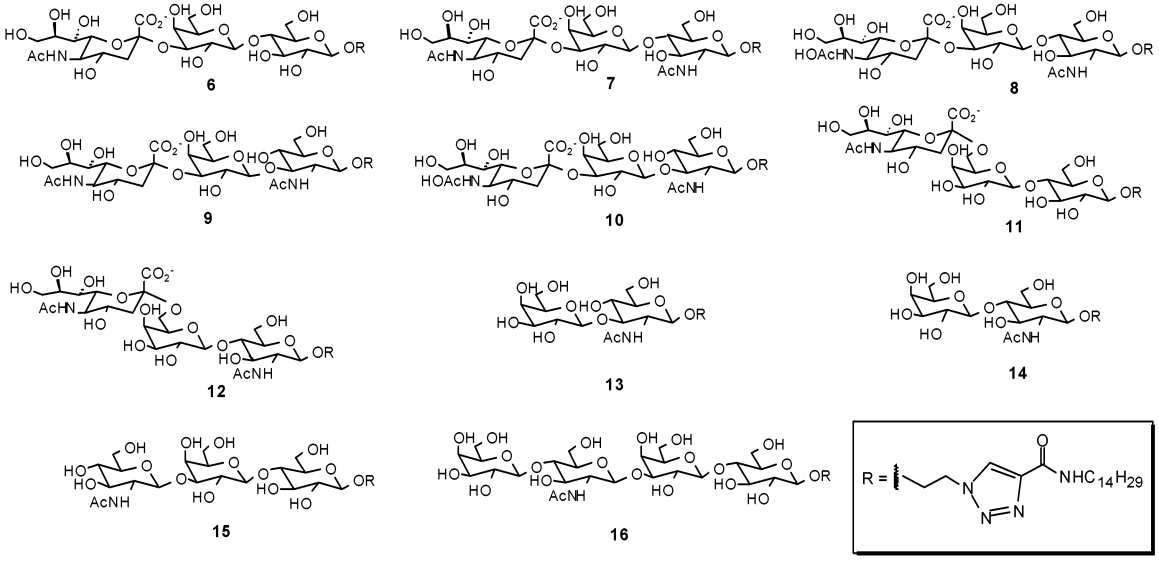
Chart 1. . Oligosaccharides Displayed in the Microtiter Plate via Copper(I)-catalyzed 1,3-dipolar Cycloaddition Reaction



This copper(I)-catalyzed triazole formation has been applied to a series of oligosaccharides. The corresponding triazole derivatives **6**−**16** (Chart 1) were synthesized in the microtiter plate wells from the corresponding azides on a 0.6−1.1 μmol scale which corresponds to a density of 4.1−7.6 μmol/cm^2 (total area per well 0.16 cm^2) for the saccharide. Formation of products and disappearance of starting materials was confirmed via ESI−MS.

Lectin-binding assays were also performed on these oligosaccharides bound to the well surface. Carbohydrates **6**−**12** display various types and linkages of a terminal sialic acid. Fluorescein-labeled *Sambucus nigra* lectin (SNA) was able to bind all sialic acid-containing carbohydrates with preferential binding to the α-2,6 linked over α-2,3 linked sialic acid, as expected.[18] Carbohydrates **13**−**16** were recognized by Ricin B assays as previously described.[9]

To further explore the biological applications, enzymatic transformations of compounds **7** and **9** attached to the microtiter plate were also investigated. Fucosylation of the *N*-acetyl-glucosamine moiety generates tetrasaccharides sialyl Lewis x (from **7**) or sialyl Lewis a (from **9**). Sialyl Lewis x (**17**) is a crucial mediator in inflammation and often found in the sera of gastrointestinal, pancreatic, and breast cancer patients.[19] The fucosylation of **7** and **9** with α-1,3-fucosyltransferase and GDP−Fucose, followed by washings, was monitored with a fucose-specific lectin from *Tetragonolobus purpureas*, and it was observed that compound **7** but not **9** was fucosylated, consistent with the previous study in solution[19] (Figure 4). Conversion of **7** to sialyl Lewis x (**17**) was further confirmed using mass spectrometry.

To establish that *T. purpureas* was indeed recognizing the fucose and not binding nonspecifically to the microtiter plate, varying levels of fucosyllipid **19**, prepared from 2-azidoethyl-α,β−L-fucopyranoside (**18**, Scheme 4), were attached to the microtiter plate and analyzed against the lectin.

Illustrated in Figure 5 is the absorbance curve for varying concentrations of fucosyllipid **19**. The background absorbance, that is, the absorbance of wells that were blocked with bovine

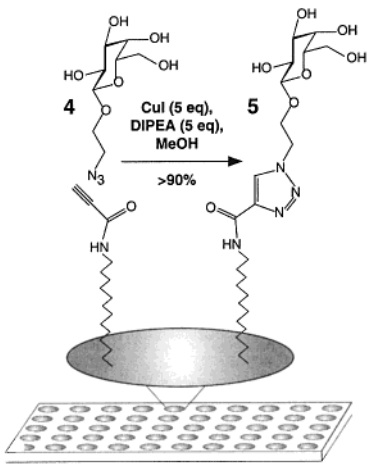


Figure 2. Formation of **5** in microtiter plate well.

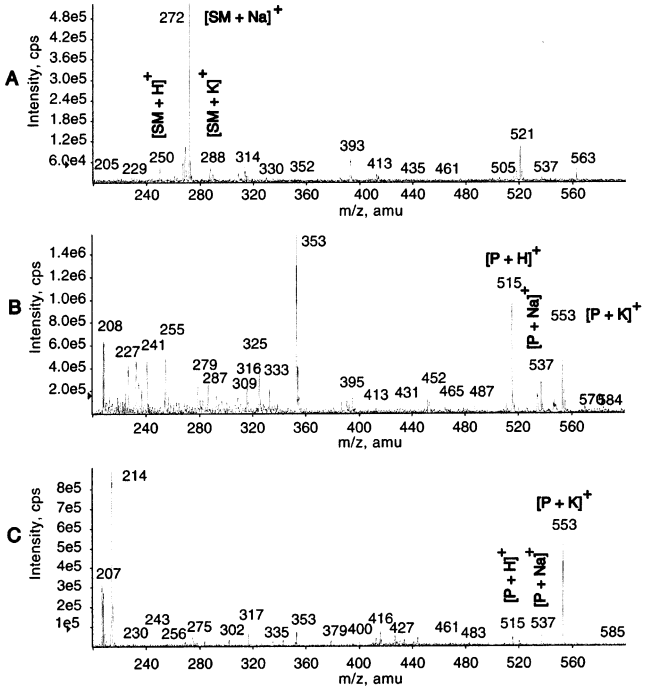


Figure 3. ESI−MS analyses of (A) starting material **4**; (B) reaction after 8 h; (C) purified **5**.

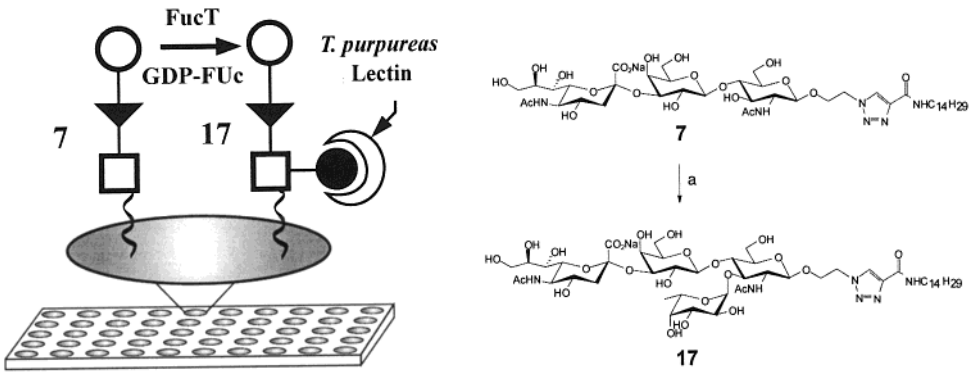


Figure 4. Synthesis of Sialyl Lewis x (**17**) in microtiter plate via enzyme assisted fucosylation of precursor **7**; (a) GDP-fucose, fucosyltransferase, 37 °C, 1 h.

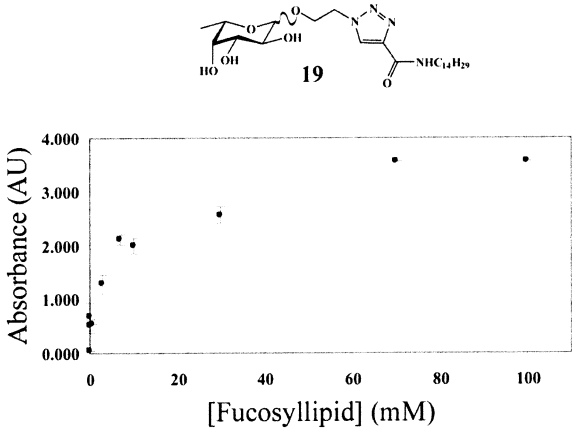
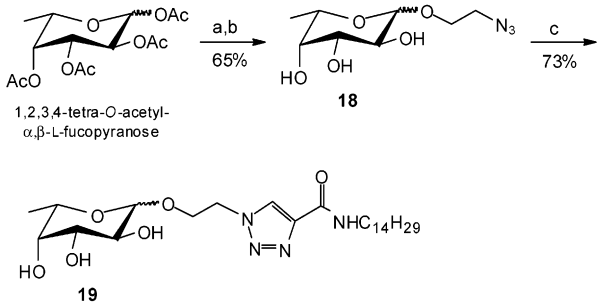


Figure 5. Absorbance of peroxidase-conjugated *T. purpureas* lectin as a function of fucosyllipid **19** concentrations.

Scheme 4



(a) **2**, CuI, DIPEA, MeOH, rt, 8 h.

serum albumin (BSA) but contained no **19**, was measured at 0.056AU (±0.0007). Increasing concentrations of **19** where the wells were blocked with BSA showed increasing absorbance that plateaus at 3.553AU (±0.0000) at 100 mM. With the establishment that varying concentrations of L-fucose bound to the surface of the microtiter plate could be observed and quantitated, we applied this lectin-binding strategy to the analysis of an inhibitor of a fucosyltransferase (FucT).

Prior to treatment of wells containing **7** with the enzyme, α1,3FucT was incubated with compound **20** shown in Figure 6, which was previously found in our laboratory to be a potent inhibitor of this enzymatic reaction.[20] After the wells were

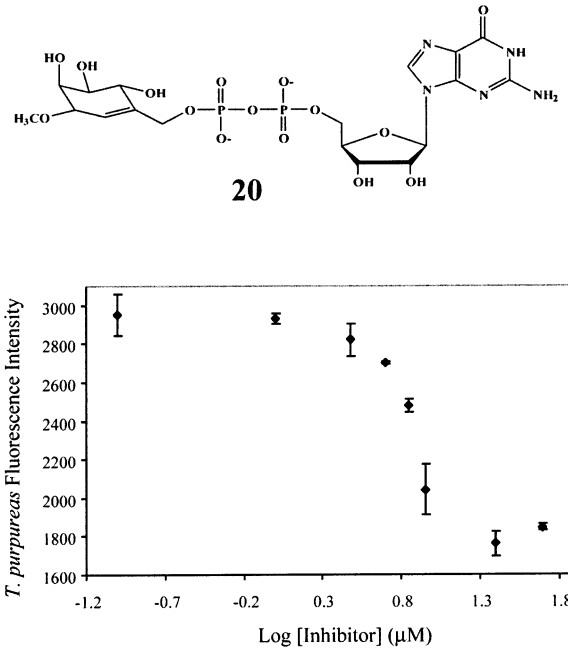


Figure 6. Observation of inhibition of the fucosylation of NeuAcα2,-3Galβ1,4GlcNAc (**7**) to gernerate **17**.

incubated with α1,3FucT, they could be washed and then incubated with the *T. purpureas* lectin and observed as before. In this assay, the FITC-labeled lectin was utilized and only 20 nmol of **7** was necessary to analyze fucosylation through generation of **17**. An IC_{50} value of 6.212 μM was determined for this method, which correlates well with the previously determined $K_i = 5$ μM in solution[20] (Figure 6).

Conclusion

This paper describes the use of Cu(I)-catalyzed triazole formation for the synthesis and in situ attachment of saccharides to the microtiter plate. The reaction was carried out at room temperature in methanol in microtiter wells on micromole scale, followed by evaporation and washing with buffer solution. The glycolipids noncovalently displayed are stable under repeated aqueous washings and are functional in biological screens. The saccharide arrays also act as substrates for glycosyltransferases and are suitable for high throughput specificity studies of sugar-

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protein interaction, enzyme inhibitor screening, and diversity-oriented synthesis.

We believe that with the strategy described here and the methods available for rapid synthesis of oligosaccharides,[21] the preparation of saccharide arrays for high-throughput screening and drug discovery is attainable.

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Supporting Information Available: Experimental procedures, biological assays and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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