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Metal-Free Sequential [3 + 2]-Dipolar Cycloadditions using Cyclooctynes and 1,3-Dipoles of Different Reactivity

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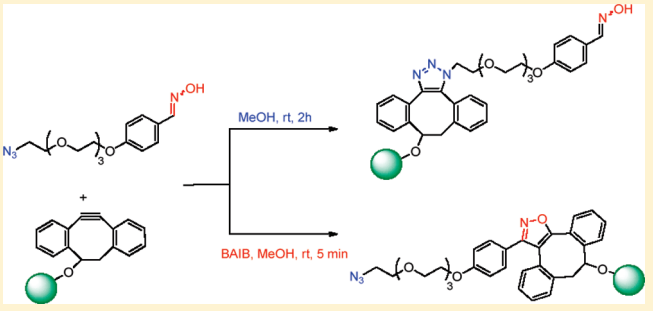
Supporting Information

ABSTRACT: Although metal-free cycloadditions of cyclooctynes and azides to give stable 1,2,3-triazoles have found wide utility in chemical biology and material sciences, there is an urgent need for faster and more versatile bioorthogonal reactions. We have found that nitrile oxides and diazocarbonyl derivatives undergo facile 1, 3-dipolar cycloadditions with cyclooctynes. Cycloadditions with diazocarbonyl derivatives exhibited similar kinetics as compared to azides, whereas the reaction rates of cycloadditions with nitrile oxides were much faster. Nitrile oxides could conveniently be prepared by direct oxidation of the corresponding oximes with BAIB, and these conditions made it possible to perform oxime formation, oxidation, and cycloaddition as a one-pot procedure. The methodology was employed to functionalize the anomeric center of carbohydrates with various tags. Furthermore, oximes and azides provide an orthogonal pair of functional groups for sequential metal-free click reactions, and this feature makes it possible to multifunctionalize biomolecules and materials by a simple synthetic procedure that does not require toxic metal catalysts.



INTRODUCTION

Metal-free click cycloadditions of cyclooctynes with azides[1] to give stable 1,2,3-triazoles have found wide utility in labeling glycans,[2] proteins,[3] and lipids[4] of living cells, glycoprotein enrichment for proteomics,[5] protein,[6] and oligonucleotide modification,[7] and tissue reengineering.[8] These reactions, which have been coined "strain-promoted alkyne−azide cycloadditions (SPAAC)", have also made entry in material sciences and have for example been employed for the assembly, cross-linking,[9] and surface modification of dendrimers,[10] derivatization of polymeric nanostructures,[11] and patterning of surfaces.[12]

Density functional theory (B3LYP) calculations of the transition states of cycloadditions of phenyl azide with acetylene and cyclooctyne indicate that the fast rate of the "strain promoted" cycloaddition is actually due to a lower energy required for distorting the 1,3-dipole and alkyne into the transition-state geometry.[13] The first generation of cyclooctynes proceeded with relatively slow rates of reaction; however, it has been found that significant increases in the rate of strain-promoted cycloaddition can be accomplished by appending electron-withdrawing groups to the propargylic position of cyclooctyne.[2] For example, difluorinated cyclooctyne (DIFO, **1**, Figure 1)[14] reacts with azides approximately 60 times faster than similar cycloadditions with an unsubsituted cyclooctyne. We have reported that derivatives of 4-dibenzocyclooctynol (DIBO, **2**) react fast with azido-containing saccharides and amino acids and can be employed for visualizing metabolically labeled glycans of living cells.[15] Attractive features of DIBO include easy access to the compound by a simple synthetic approach, nontoxicity, and the possibility of straightforward attachment of a variety of probes. Furthermore, the structure of DIBO is amenable to analogue synthesis, and derivatives (**3** and **4**) have been introduced that exhibit even higher rates of reaction than the parent compound.[16]

Our finding that cyclooctynes can undergo fast cycloadditions with nitrones has further expanded the scope of metal-free click reactions,[17] and the usefulness of this approach has been demonstrated by site-specific protein modification by a three-step protocol entailing periodate oxidation of an N-terminal serine to give an aldehyde, which could easily be converted into a nitrone and then reacted with probe-modified dibenzocyclooctynes.

As part of a program to develop metal-free click reactions, we report here that, in addition to azides and nitrones, nitrile oxides and diazocarbonyl derivatives readily undergo cycloadditions with dibenzocyclooctyne to give stable isoxazoles and pyrazoles, respectively. It has been found that the various 1,3-dipoles exhibit distinct levels of reactivity, making it possible to perform sequential cycloadditions. In addition, we have shown, for the first time, that an oxime can function as a latent 1,3-dipole for a nitrile

Received: September 15, 2010
Published: December 23, 2010

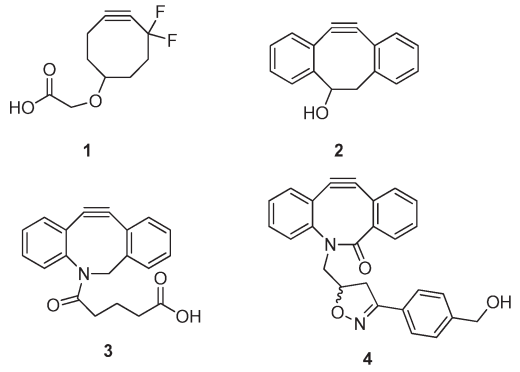
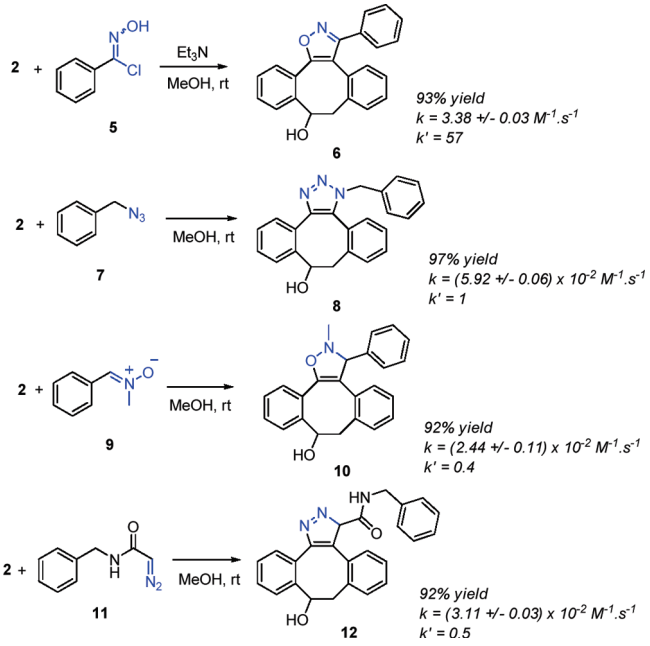


Figure 1. Cyclooctynes for metal-free click reactions.

Scheme 1. Rate Constants of Cycloadditions of DIBO (2) with Various 1,3-Dipoles: Nitrile Oxide, Azide, Nitrone, and Diazocarbonyl Derivatives[a]



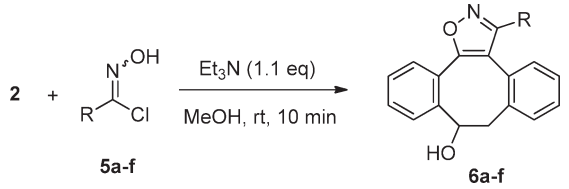
[a] k' is the relative rate with benzyl azide set at 1. The second-order rate constant for nitrone **9** was determined by using equimolar mixture of reagents due to a strong absorbance at 305 nm.

oxide, which is fully orthogonal with cycloadditions of azides. These findings make it possible to employ strain-promoted cycloadditions for the assembly of complex multifunctional and bioinspired materials without the need of employing a toxic metal catalyst.

RESULTS AND DISCUSSION

Nitrile oxides can undergo cycloadditions with terminal alkynes to give 3,5-isoxazoles;[18] however, the success of these reactions is often compromised by a slow rate of reaction and competing dimerization of nitrile oxides.[19] 3,5-Disubstituted isoxazoles have been prepared in high yield by intramolecular cycloadditions,[20] the use of activated dipolarophiles[21] such as benzyne and norbornenes, or by employing a Cu(I) catalyst.[22] Furthermore, diazocarbonyl reagents, which are sufficiently stable for use in chemical synthesis, have been employed in 1,3-dipolar cycloadditions with substituted

Table 1. Rate Constants and Yields for the Cycloadditions of DIBO (2) with Various Nitrile Oxides



entry	R	k (M^{-1} s^{-1})	yield (%)[a]
1	C_6H_5[b,c,d] (**5a**)	3.38 ± 0.03[g]	93
2	C_6H_5[b,c,d] (**5a**)	2.46 ± 0.03[h]	ND
3	4-MeO−C_6H_4[b,e] (**5b**)	2.15 ± 0.02[g]	89
4	4-O_2N−C_6H_4[b,f] (**5c**)	8.47 ± 0.03[g]	93
5	4-F−C_6H_4[b,c,d] (**5d**)	3.99 ± 0.05[g]	90
6	4-Cl−C_6H_4[b,c,d] (**5e**)	3.42 ± 0.03[g]	90
7	4-Br−C_6H_4[b,c,d] (**5f**)	3.31 ± 0.06[g]	93

[a] Isolated yields of combined isomers. [b] Second-order rate constants were determined from pseudo first-order rate constants at various concentrations of in situ formed nitrile oxides at 25 ± 0.1 °C. [c] Pseudo first-order kinetics were determined using UV−vis spectroscopy by following the decay of the absorbance of compound **2** at 305 nm. [d] [**2**] = 6.0 × 10^{-5} M; for details on the concentrations of nitrile oxides, see the Supporting Information. [e] [**2**]=3.0×10^{-5} M; [**5b**] = (2.5−5.0)× 10^{-4} M. [f] Pseudo first-order kinetics were determined by UV−vis spectroscopy following the decay of the absorbance of **5c** at 325 nm; [**5c**] = 6.0 × 10^{-5} M, [**2**] = (7.0−17.5) × 10^{-4} M. [g] Reaction was performed in methanol. [h] Reaction was performed in acetonitrile.

alkynes and benzynes to give pyrazoles and indazoles, respectively.[23] These findings inspired us to explore strain-promoted alkyne−nitrile oxide cycloadditions (SPANOC) and alkyne−diazocarbonyl (SPADC) with DIBO (**2**) and compare the rates of reactions with similar cycloadditions with azides (SPAAC) and nitrones (SPANC) (Scheme 1).

A range of imidoyl chlorides (**5a**−**f**), which can easily be converted into nitrile oxides by treatment with a mild base, was prepared by reactions of the corresponding aldehydes with hydroxylamine[24] followed by chlorination of the resulting oximes with *N*-chlorosuccinimide.[25] Addition of the imidoyl chlorides **5a**−**f** to a solution of DIBO in methanol in the presence of triethylamine led, within several minutes, to the quantitative formation of isoxazoles **6a**−**f** (Table 1).

Accurate rate measurements of the cycloaddition reactions were conducted by UV spectroscopy following the growth of the decay of the characteristic absorbance of the acetylene of DIBO (**2**) at 305 nm. The rates were measured in methanol or acetonitrile solutions at 25 ± 0.1 °C. The kinetics of the cycloadditions was studied under pseudo first-order conditions by maintaining a fixed concentration of DIBO (**2**), while the concentration of the dipoles was varied. Consumption of starting material followed a first-order equation, and the pseudo first-order rate constants were obtained by least-squares fitting of the data to a single exponential equation. The observed rate constants were linearly dependent on the concentration of dipoles,[26] and second-order cycloaddition rate constants calculated from the concentration dependencies of observed rates are listed in Table 1. As can be seen, the cycloadditions with the nitrile oxides are exceptionally fast, and the substituent exerts only small influence. It appears that strongly electron-withdrawing substituents, such as a nitro group (entry 4), somewhat increase the rate of reaction.

dx.doi.org/10.1021/ja1081519 |J. Am. Chem. Soc. 2011, 133, 949–957

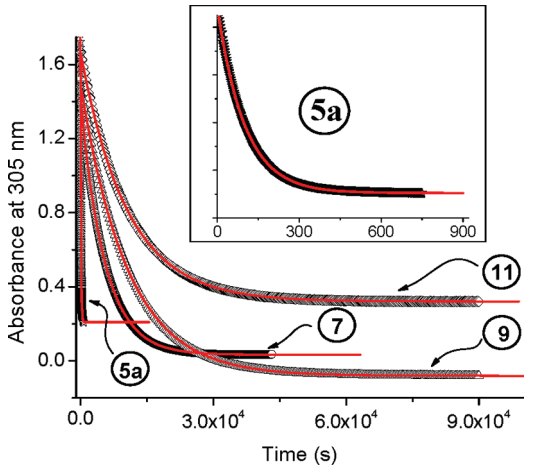


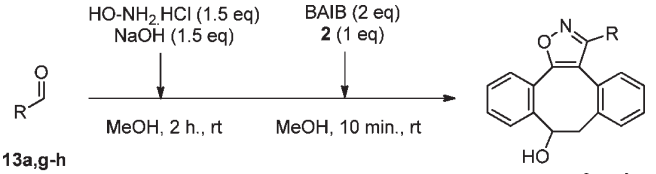
Figure 2. Consumption of DIBO **2** (6.0×10^{-5} M in methanol at 25 °C) in the presence of various dipoles (3 mM). The lines shown were drawn using parameters obtained by least-squares fitting of single exponential equation. The inset shows reaction of DIBO with **5a** at a different time scale.

Furthermore, the use of methanol or acetonitrile had only a marginal influence on the reaction rate (entries 1 and 2).

Next, we compared the reaction rates of 1,3-dipolar cycloadditions of DIBO (**2**) with a nitrile oxide derived from imidoyl chlorides **5a**, benzyl derived azide **7**, nitrone **9**, and diazocarbonyl derivative **11** to give isoxazole **6a**, triazole **8**, *N*-methyl isoxazoline **10**, and pyrazole **12**, respectively (Scheme 1 and Figure 2). It was found that the azide, nitrone, and diazocarbonyl exhibit similar rates of reaction. However, the rate of cycloaddition of the nitrile oxide was 57 times faster than a similar reaction with benzyl azide.

Having established that nitrile oxides react exceptionally fast with DIBO (**2**), attention was focused on streamlining the process of nitrile oxide formation and cycloaddition. It was expected that the number of reaction steps could be reduced by a direct oxidation of oximes to nitrile oxides by using a mild oxidant such as [bis(acetoxy)iodo]benzene (BAIB).[27] Furthermore, a one-pot multistep sequence in which oxime formation, oxidation, and cycloaddition are performed by sequential addition of reagents was expected to reduce the number of workup and purification steps, thereby increasing the efficiency and overall yield of the transformation. Thus, a reaction of benzaldehyde (**13a**) with hydroxylamine in methanol gave, after a reaction time of 2 h, an intermediate benzaldehyde oxime, which was treated with DIBO and BAIB, and after an additional reaction time of 10 min, TLC and MS analysis indicated complete conversion of the oxime into isoxazole **6a**, highlighting that the oxidation and cycloaddition steps proceed with exceptionally high reaction rates (Table 2, entry 1). Additional experiments demonstrated that DIBO (**2**) is stable when exposed to BAIB alone, and thus the oxidation and cycloaddition could be performed as a tandem reaction sequence. However, hydroxylamine decomposes DIBO (**2**) probably by a nucleophilic attack at the strained alkyne. Thus, the success of the transformation required the use of either an equimolar quantity of aldehyde and hydroxylamine or more conveniently the addition of acetone prior to cycloaddition to convert the excess hydroxylamine into ketoxime, which can react with BAIB but does not provide a 1,3-dipole. Alternatively, the addition of an excess of BAIB before administering DIBO (**2**) also led to complete consumption of the remaining excess of hydroxylamine and resulted in smooth formation of the desired isoxazoles **6a**,**g**−**h**. Furthermore, this experimental approach made it possible to prepare isoxazoles in high yield, which have unstable corresponding imidoyl chlorides, notably in the aliphatic series (Table 2, entry 3).

Table 2. One-Pot Oxime Formation and SPANOC with DIBO (2)



entry	R	k (M^{-1} s^{-1})[a,b,c]	yield (%)[d]
1	C_6H_5 (**6a**)	3.44 ± 0.03	55
2	2-Me−C_6H_4 (**6g**)	3.20 ± 0.03	51
3	C_6H_5−CH_2CH_2 (**6h**)	1.38 ± 0.01	90

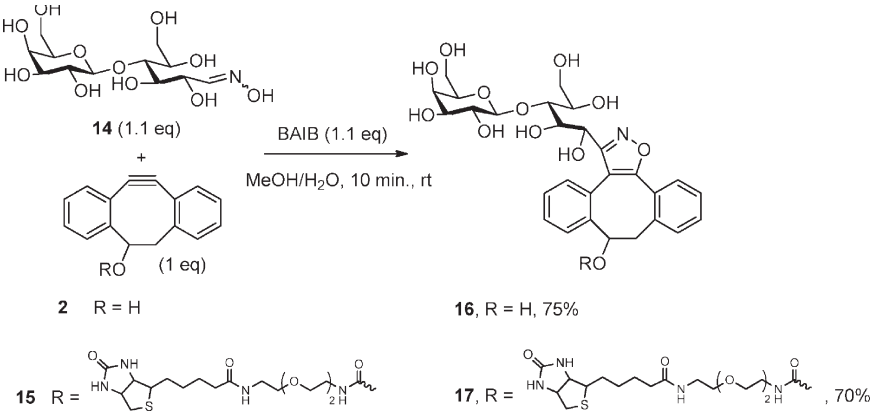
[a] Rate constant was determined from isolated oximes. [b] Second-order rate constants were determined from pseudo first-order rate constants at various concentrations of nitrile oxides at 25 ± 0.1 °C. [c] Pseudo first-order kinetics were determined using UV−vis spectroscopy following the decay of the absorbance of **2** at 305 nm; [**2**] = 6.0×10^{-5} M; for details on the concentrations of nitrile oxides, see the Supporting Information. [d] Isolated yields of combined isomers.

Rate constants were measured for the tandem sequence of oxidation of oximes to nitrile oxides followed by 1,3-dipolar cycloaddition with **2** establishing that the cycloaddition is the rate-limiting step and highlighting that oxidation with BAIB is exceptionally fast. For example, when benzaldehyde oxime was employed, the rate constant of the reaction was 3.44 M^{-1} s^{-1}, which is almost the same as the value obtained when benzaldehyde imidoyl chloride was employed (3.38 M^{-1} s^{-1}). Furthermore, the kinetic data for compounds **6g** and **6h** demonstrate further that the nature of the substituent has only a small effect on the rate of the reactions.

Convenient bioorthogonal reactions require that transformations are modular, have a high tolerance for the presence of functional groups, and proceed at ambient temperature using benign solvents and reagents. To determine whether SPANOC complies with these requirements, we examined the tagging of a carbohydrate with a biotin probe. Complex carbohydrates are involved in a wide variety of biological processes,[28] and fluorescent, biotin, multivalent, and immobilized saccharide derivatives are important tools to study the intriguing properties of this class of biomolecules.[29] It was expected that such derivatives can easily be prepared by reaction of sugar oximes by a sequential reaction of an aldose with hydroxylamine to give an oxime, which can then be functionalized by reaction with DIBO derivatives in the presence of BAIB. The attraction of such an approach is that it allows functionalization of the reducing end of complex carbohydrates with various probes using low equivalents of expensive reagents. Thus, reaction of the readily available oxime **14**[30] with **2** or biotin-modified DIBO **15** (equimolar amounts, Scheme 2) in the presence of BAIB for 10 min gave the sugar derivatives **16** and **17**, respectively. It is interesting to note that the use of BAIB did not oxidize primary hydroxyls of lactose or sulfur of biotin.[31] Compounds such as **16** that are modified with a biotin tag can, for example, be employed for immobilization to a surface coated with Streptavidin.

dx.doi.org/10.1021/ja1081519 |*J. Am. Chem. Soc.* 2011, 133, 949–957

Scheme 2. Modification of the Reducing End of Lactose by SPANOC Employing Oxime 14



We envisaged SPANOC can also be used for the installation of tags into sialic acid containing glycoproteins by mild treatment with $NaIO_4$ to form a C-7 aldehyde, which upon treatment with hydroxylamine will give an oxime that can be oxidized to a nitrile oxide for reaction with derivatives of DIBO. The attraction of such a strategy is that tags can be installed into glycoproteins by stable isoxazoles linkages.[32] To examine the usefulness of such a strategy, the glycoprotein fetuin was treated with a 1 mM solution of $NaIO_4$ for 5 min, after which the excess of oxidizing reagent was removed by spin filtration. The resulting aldehyde containing glycoprotein was treated with hydroxyl amine to install an oxime, which was immediately oxidized to a nitrile oxide by short treatment with BAIB and then reacted with **15** for 15 min to give a biotin containing sialic acid. As a control, BSA, which does not contain sugar moieties, was subjected to the same sequence of reactions. The presence of biotin was examined by Western blotting using antibiotin antibody conjugated to HRP. As can be seen in Figure 3, fetuin showed strong reaction when subjected to the sequential three-step procedure, whereas BSA was not detected. Furthermore, exclusion of one of the reaction steps abolished detection, confirming the selectivity of the procedure. Quantitative protein and biotin determination indicated that two biotin moieties were installed in each fetuin molecule.

The large difference in reactivity of the cycloaddition of DIBO with the various 1,3-dipoles should make it possible to perform sequential click reactions, which may provide opportunities to prepare multifunctional compounds or materials by a simple synthetic procedure. In particular, it was expected that a highly reactive nitrile oxide can selectively undergo a cycloaddition in the presence of an azide. Furthermore, we envisaged that oximes can function as latent 1,3-dipoles, and therefore, a cyclooctyne should react with an azide without affecting an oxime. However, in the presence of BAIB, an oxime is rapidly converted into a nitrile oxide, which can then be reacted with another functionalized cyclooctyne. Thus, by careful selection of appropriate reagents, it should be possible to selectively modify a bifunctional linker (or complex compound) containing an azide and oxime moiety.

As expected, the addition of monosaccharide-modified DIBO **19** to bifunctional azido-oxime linker **18** in methanol resulted in selective cycloaddition at the azide moiety to provide the triazole **20** in high yield (Scheme 3). However, when linker **18** was treated with DIBO derivative **19** in the presence of BAIB, the oxime moiety was rapidly oxidized to a highly reactive nitrile oxide, which underwent a fast SPANOC resulting in the selective formation of isoxazole **21**.

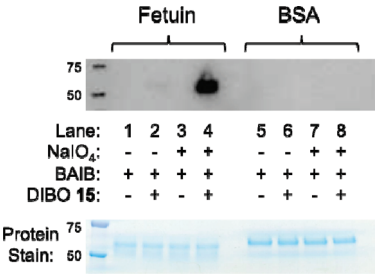


Figure 3. Labeling and detection of sialic acids on the glycoprotein fetuin using SPANOC. Fetuin (samples in lanes 1−4) and BSA (samples in lanes 5−8) were subjected to periodate oxidation using $NaIO_4$ (samples in lanes 3, 4, 7, and 8). Next, the generated C-7 aldehyde (on sialic acid) was reacted with $HONH_2 \cdot HCl$ to form an oxime, which was oxidized by reacting with BAIB to produce nitrile oxide that was reacted with DIBO derivative **15** (samples in lanes 2, 4, 6, and 8). Incorporated biotin was then detected by Western blot using an antibiotin antibody conjugated to HRP. Total protein loading was confirmed by Coomassie staining.

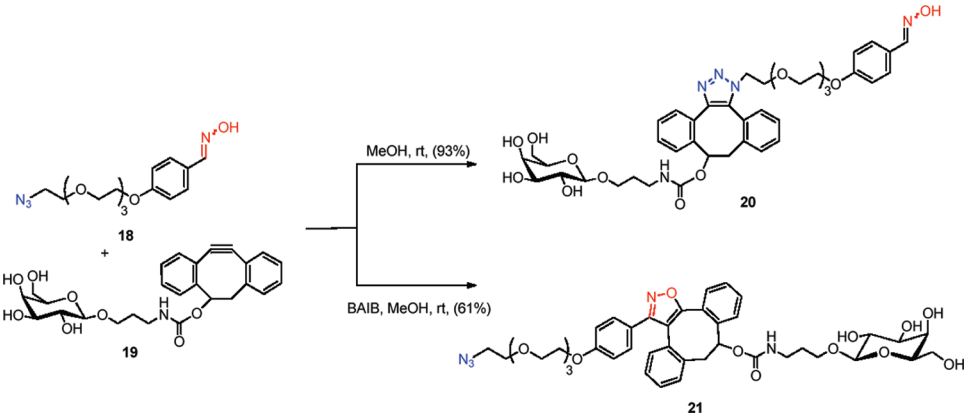
Having established the orthogonality of azides and oximes/nitrile oxides, we examined sequential SPAAC−SPANOC click reactions of bifunctional linker **18** with a biotin (**15**) or a fluorescent probe (**22**) and a cluster of glycosides (**25**)[10b] modified with DIBO (Scheme 4). Thus, treatment of azido-oxime linker **18** with DIBO-modified biotin (**15**) or DIBO-modified coumarin (**22**) in methanol or THF, respectively, at ambient temperature for 2 h led to clean formation of monofuntionalized triazoles **23** and **24**, respectively. Next, triazoles **23** and **24** were exposed to a mixture of BAIB to convert the oxime moiety into a highly reactive nitrile oxide, and reaction with DIBO-modified saccharide cluster **25** lead to a fast SPANOC to give bifunctional compounds **26** and **27**, displaying a cluster of galactoses conjugated to biotin or a fluorescent tag, respectively. It is of interest to note that neither oxidation of biotin moiety by BAIB nor cycloaddition of the in situ generated nitrile oxide at the carbon double bond of coumarin[33] was observed, highlighting that SPANOC is perfectly suitable for the conjugation of sensitive compounds.

■ CONCLUSION

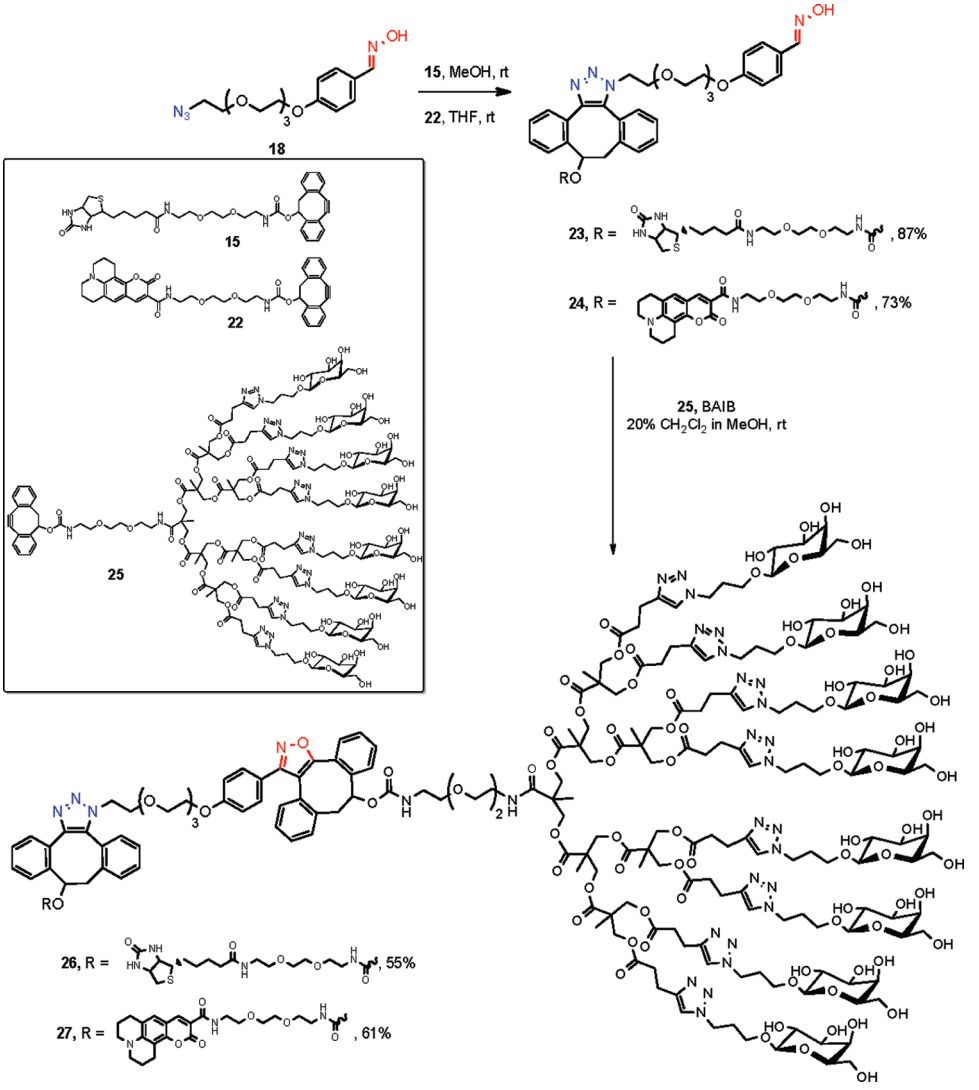
Although metal-free cycloadditions between cyclooctynes and azides to give stable 1,2,3-triazoles have found wide utility in

dx.doi.org/10.1021/ja1081519 |J. Am. Chem. Soc. 2011, 133, 949–957

Scheme 3. Selective Cycloadditions between Galactoside-Modified DIBO 19 with Either the Azide or the Oxime Moiety of Linker 18



Scheme 4. Preparation of a Bifunctional Compound by a Sequential SPAAC and SPANOC



chemical biology[1] and material sciences,[8] there is an urgent need for faster and more versatile bioorthogonal reactions. We have found that 1,3-dipolar cycloadditions of cyclooctynes with nitrile oxides exhibit much faster kinetics than similar reactions with

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azides. The nitrile oxides could easily be prepared by direct oxidation of the corresponding oximes with BAIB, and these reaction conditions made it possible for oxime formation, oxidation, and cycloaddition to be performed as a one-pot procedure. The transformations have a high tolerance for the presence of functional groups, proceed at ambient temperature using benign solvents and reagents, and make it possible to modify compounds by a modular approach. Furthermore, the results presented here demonstrate that oximes and azides provide an orthogonal pair of functional groups for sequential metal-free click reactions. In this respect, sequential click reactions have been reported by Cu(I)-catalyzed alkyne azide cycloaddition[34] (CuAAC) using terminal- and silyl-protected alkynes[35] and by exploiting the differential reactivity of CuAAC with SPAAC and thiol−ene click reactions.[36] The usefulness of these approaches has been demonstrated by the controlled modification of oligonucleotides,[37] proteins,[38] and fullerenes[39] with two or more tags. The results reported here demonstrate, for the first time, that strain-promoted click reactions can be performed in a sequential manner by tuning the reactivity of 1,3-dipoles or by using a latent 1,3-dipole. The attractiveness of the new approach is that it offers chemical flexibility, avoids toxic metal catalysts, and makes it possible to multifunctionalize compounds by simple chemical manipulations.

A variety of methods have been reported for convenient installment of aldehydes in biomolecules,[40] which can easily be converted into oximes. Thus, it is to be expected that a variety of biomolecules can be modified by SPANOC. Metal-free click reactions have found entry into materials science,[8] and it is to be expected that SPANOC will provide an additional tool for the preparation of increasingly complex materials by simple and flexible chemical manipulations. Finally, we anticipate that SPANOC will offer an attractive alternative to the well-established oxime ligation[41] because the synthesis of oximes is simple, the isoxazole products are stable, and a combined use with SPAAC will make it possible to introduce two different functional groups.

EXPERIMENTAL PROCEDURES

Kinetic Measurements. The rate measurements of cycloadditions of dibenzocyclooctynol **2** with various dipoles were conducted by using Cary 50 and Cary 100 UV−vis spectrophotometers at 25.0 ± 0.1 °C. A calculated amount of 0.1 M solutions of a dipole (**5a,b,d−f**, **7**, **9**, **11**, **13a−h**, **14**) required to achieve the desired dipole concentration (2.5×10^{-4} to 2.7×10^{-2} M) was added to a thermally equilibrated solution of dibenzocyclooctynol **2** (3.0×10^{-5} to 6.0×10^{-5} M) in MeOH. In the case of nitrile oxide derivatives of **5a,b,d−f**, the imidoyl chlorides **5a−f** in methanol (6.0×10^{-4} to 1.5×10^{-2} M) were treated with triethylamine and then added to a thermally equilibrated solution of **2**, whereas nitrile oxide derivatives of **13a−h** were generated by the oxidation of oximes **13a−h** using [bis(acetoxy)iodo]benzene. Reactions were monitored by following the decay of the characteristic absorbance of dibenzocyclooctynol **2** at 305 nm. Observed rate constants of the cycloaddition reactions at various concentrations of dipoles are summarized in Tables S1−S14.[26]

In the case of the cycloaddition of dibenzocyclooctynol **2** with the nitrile oxide **5c**, 0.1 M solutions of **2** were required to achieve the desired concentration of **2** (7.0×10^{-4} to 1.75×10^{-3} M), and triethylamine (concentration of triethylamine in the reaction mixture was 1.2×10^{-4} M) was added to a thermally equilibrated solution of **5c** (6.0×10^{-5} M) in methanol. Reaction kinetics of nitrone **9** were monitored by following the second-order growth of the product at 330 nm decay in the equimolar mixture of reagents. Second-order rate constants were determined by fitting the curves with the following equation:

$$y = ((A_o{*}E_{SM}) + (E_P{*}k{*}t{*}[A_0]^2))/(1 + (k{*}t{*}A_o))$$

where y is the observed absorbance at given time "t"; A_0 is the initial concentration of the starting materials in molarity; E_{SM} is the sum of extinction coefficients of starting materials; E_P is the extinction coefficient of the product; and k is the second-order rate constant in $M^{-1}\,s^{-1}$. The obtained second-order rate constants were summarized in Table S15.

1-Benzyl-8,9-dihydro-1*H*-dibenzocycloocta[1,2,3]triazol-8-ol (8). Benzyl azide (**7**) (13 μL, 0.1 mmol) was added dropwise to a solution of 4-dibenzocyclooctynol (**2**) (22 mg, 0.1 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature for 30 min. The solution was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using a mixture of hexane and ethyl acetate to give pure triazole **8** (34 mg, 97%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.90−3.65 (m, 2H, CH_2CH), 4.55−5.10 (m, 1H, C*H*OH), 5.40−5.85 (m, 2H, CH_2N), 6.90−7.70 (m, 13H, aromH). HRMS (MALDI-ToF): 354.1295 ($C_{23}H_{20}N_3O$ (M + H^+) requires 354.1601).

2-Methyl-3-phenyl-2,3,8,9-tetrahydrodibenzo[3,4:7,8]-cycloocta-isoxazol-9-ol (10). Phenyl nitrone **9** (14 mg, 0.1 mmol) was added to a solution of 4-dibenzocyclooctynol (**2**) (22 mg, 0.1 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature for 30 min. The solution was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using a mixture of hexane and ethyl acetate to give pure *N*-methyl dihydroisoxazole **10** (33 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.50−1.95 (m, 1H, OH), 2.95−3.65 (m, 5H, CH_3, CH_2CH), 4.90−5.26 (m, 2H, C*H*N, C*H*OH), 6.75−7.65 (m, 13H, aromH). HRMS (MALDI-ToF): 356.1299 ($C_{24}H_{22}NO_2$ (M + H^+) requires 356.1645).

***N*-Benzyl-9-hydroxy-8,9-dihydro-3*H*-dibenzocycloocta-pyrazole-3-carboxamide (12).** Diazo benzylamide **11** (18 mg, 0.1 mmol) was added to a solution of 4-dibenzocyclooctynol (**2**) (22 mg, 0.1 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature overnight. The solution was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using a mixture of hexane and ethyl acetate to give pure pyrazole **12** (36 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.75−3.60 (m, 2H, CH_2CH), 4.00−4.60 (m, 2H, CH_2NH), 4.70−5.15 (m, 1H, C*H*OH), 6.65−7.90 (m, 15H, aromH, CHN, NH). HRMS (MALDI-ToF): 396.1426 ($C_{25}H_{22}N_3O_2$ (M + H^+) requires 396.1707).

General Procedure for the Formation of Dibenzocyclooctyl-isoxazoles 6a−f from Imidoyl Chlorides 5a−f. Imidoyl chloride **5a−f** (0.11 mmol) was added to a solution of 4-dibenzocyclooctynol (22 mg, 0.1 mmol) and triethylamine (16 μL, 0.11 mmol) in methanol (10 mL). The reaction mixture was stirred at room temperature for 10 min. The solution was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using an appropriate mixture of hexane and ethyl acetate to give pure dibenzocyclooctyl-isoxazoles **6a−f**.

3-Phenyl-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (6a). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.09 (s, 1H, OH), 2.75−3.25 (m, 2H, CH_2), 4.66−5.05 (m, 1H, C*H*OH), 6.55−7.55 (m, 13H, aromH). HRMS (MALDI-ToF): 340.1075 ($C_{23}H_{18}NO_2$ (M + H^+) requires 340.1332).

3-(4′-Methoxyphenyl)-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (6b). ^{1}H NMR (300 MHz, $CDCl_3$): δ 208 (s, 1H, OH), 3.10−3.50 (m, 2H, CH_2), 3.70−3.76 (m, 3H, OMe), 5.00−5.65 (m, 1H, C*H*OH), 6.75−7.55 (m, 12H, aromH). HRMS (MALDI-ToF): 370.1027 ($C_{24}H_{20}NO_3$ (M + H^+) requires 370.1438).

3-(4′-Nitrophenyl)-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (6c). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.08 (brs, 1H, OH), 3.15−3.75 (m, 2H, CH_2), 5.00−5.75 (m, 1H, C*H*OH), 6.70−7.80

(m, 10H, aromH), 8.00−8.20 (m, 2H, aromH). HRMS (MALDI-ToF): 385.0961 ($C_{23}H_{17}N_2O_4$ ($M + H^+$) requires 385.1183).

3-(4′-Fluorophenyl)-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (6d). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.97 (brs, 1H, OH), 3.15−3.75 (m, 2H, CH_2), 5.10−5.70 (m, 1H, C*H*OH), 6.75−7.55 (m, 12H, aromH). HRMS (MALDI-ToF): 358.1016 ($C_{23}H_{17}FNO_2$ ($M + H^+$) requires 358.1238).

3-(4′-Chlorophenyl)-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (6e). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.08 (brs, 1H, OH), 3.15−3.75 (m, 2H, CH_2), 5.00−5.70 (m, 1H, C*H*OH), 6.75−7.65 (m, 12H, aromH). HRMS (MALDI-ToF): 374.0579 ($C_{23}H_{17}{}^{35}ClNO_2$ ($M + H^+$) requires 374.0942).

3-(4′-Bromophenyl)-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (6f). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.07 (brs, 1H, OH), 3.15−3.75 (m, 2H, CH_2), 5.00−5.60 (m, 1H, C*H*OH), 6.70−7.85 (m, 12H, aromH). HRMS (MALDI-ToF): 417.9794 ($C_{23}H_{17}{}^{79}BrNO_2$ ($M + H^+$) requires 418.0437).

General Procedure for the One-Pot Formation of Dibenzocyclooctyl-isoxazoles 6a,g,h from the Corresponding Aldehydes 13a,g,h. Hydroxylamine hydrochloride (10.4 mg, 0.15 mmol) was added to a solution of aldehyde **13a**,**g**,**h** (1.0 mmol) and sodium hydroxide (6 mg, 0.15 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature for 2 h (monitored by TLC). [Bis(acetoxy)iodo]benzene (BAIB) (64 mg, 0.20 mmol) was then added. and the reaction mixture was stirred for 5 min at room temperature. 4-Dibenzocyclooctynol (22 mg, 0.1 mmol) was then added, and the reaction mixture was stirred for an additional 10 min at room temperature. The solution was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using an appropriate mixture of hexane and ethyl acetate to give pure dibenzocyclooctyl-isoxazole **6a**,**g**,**h**.

3-(2′-Toluyl)-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (6g). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.88−2.20 (m, 4H, CH_3, OH), 3.20−3.65 (m, 2H, CH_2), 5.12−5.48 (m, 1H, C*H*OH), 6.60−7.60 (m, 12H, aromH). HRMS (MALDI-ToF): 354.1031 ($C_{24}H_{20}NO_2$ ($M + H^+$) requires 354.1489).

3-(2-Phenylethyl)-8,9-dihydrodibenzocyclooctanyl-isoxazol-9-ol (6h). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.50−1.90 (m, 1H, OH), 2.65−3.65 (m, 6H, 3 × CH_2), 4.90−5.10 (m, 1H, C*H*OH), 6.90−7.70 (m, 13H, aromH). HRMS (MALDI-ToF): 368.1210 ($C_{25}H_{22}NO_2$ ($M + H^+$) requires 368.1645).

General Procedure for the Formation of Lactose Derivatives. 4-Dibenzocyclooctyl-derivative **2** or **15** (0.1 mmol) was added to a solution of [bis(acetoxy)iodo]benzene (35 mg, 0.11 mmol) and lactose oxime **14** (40 mg, 0.11 mmol) in methanol (4 mL), premixed for 1 min. The reaction mixture was then stirred at room temperature for 10 min (TLC monitoring). The solution was concentrated in vacuo, and the residue was purified either by Iatrobeads using a mixture of 10% of water in acetonitrile (for **16**) or by RP-HPLC (0−2 min 0.1% TFA/H_2O, v/v; 2−5 min gradient of 0−20% 0.1% TFA/CH_3CN, v/v; 5−30 min gradient of 20−60% 0.1% TFA/CH_3CN v/v; 30−35 min gradient of 60−100% 0.1% TFA/CH_3CN, v/v; 35−45 min gradient of 100−0% 0.1% TFA/CH_3CN, v/v; t = 21.8 and 23.9 min). Appropriate fractions were combined and lyophilized to give pure dibenzocyclooctyl-isoxazole **16** and **17**, respectively.

3-(Lactose)-8,9-dihydro-dibenzocyclooctanyl-isoxazol-9-ol (16). ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.00−3.85 (m, 11H, 2 × CH_2OH, 4 × CH_{gal}, 3 × CH_{glu}), 3.95−4.30 (m, 2H, CH_2Ar), 4.35−5.45 (m, 3H, ArC*H*OH, OCHO, CHC═N), 7.20−7.80 (m, 8H, aromH). HRMS (MALDI-ToF): 598.1693 ($C_{28}H_{33}NO_{12}Na$ ($M + Na^+$) requires 598.1895).

Lactose-biotin Isoxazole 17. ^{1}H NMR (600 MHz, D_2O): δ 1.00−1.45 (m, 6H, 3 × $CH_{2biotin}$), 2.00−2.10 (m, 2H, $CH_{2biotin}$), 2.40−4.30 (m, 32H, 6 × CH_{2PEG}, $CH_{2biotin}$, 3 × CH_{biotin}, CH_2Ar, 2 × CH_2OH, 5 × CH_{gal}, 4 × CH_{glu}), 5.90−6.00 (m, 1H, ArC*H*OCO), 7.00−7.48 (m, 8H, aromH). HRMS (MALDI-ToF): 998.2939 ($C_{45}H_{61}N_5O_{17}SNa$ ($M + Na^+$) requires 998.3681).

Labeling of Sialic Acid Residues on Glycoproteins. Fetuin (sialylated) and BSA (nonsialylated) as a control were subjected to periodate oxidation (1 mM $NaIO_4$) for 5 min at 4 °C. The protein solution was spin filtered at 14 000*g* for 15 min to remove excess reagent. Next, the generated C-7 aldehyde (on sialic acid) was reacted with $HONH_2 \cdot HCl$ (100 μM in DPBS, pH 6.7) for 1 h at room temperature. The generated oxime was oxidized by reacting with BAIB for 5 min at room temperature to produce nitrile oxide. After removal of excess reagent by centrifugation at 14 000*g* for 15 min, the nitrile oxide was reacted with DIBO **15** by a copper-free cycloaddition reaction for 30 min at room temperature. The samples (25 μg of protein per lane) were resolved on a 4−20% SDS-PAGE gel (Bio-Rad) and transferred to a nitrocellulose membrane. Next, the membrane was blocked in blocking buffer (nonfat dry milk (5%; Bio-Rad) in PBST (PBS containing 0.1% Tween-20 and 0.1% Triton X-100)) for 2 h at room temperature. The blocked membrane was then incubated for 1 h at room temperature with an antibiotin antibody conjugated to horseradish peroxidase (HRP) (1:100 000; Jackson ImmunoResearch Lab, Inc.) in blocking buffer and washed with PBST (4 × 10 min). Final detection of HRP activity was performed using ECL Plus chemiluminescent substrate (Amersham), exposure to film (Kodak), and development using a digital X-ray imaging machine (Kodak). Coomassie Brilliant blue staining was used to confirm total protein loading.

Biotin Quantitation. Incorporation of biotin into the protein was quantified using the Fluorescence Biotin Quantitation Kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, the biotinylated protein was dissolved in PBS, and DyLight Reporter (a premix of fluorescent avidin and 4′-hydroxyazobenzene-2-carboxylic acid (HABA)) was added to the biotinylated samples and a range of biocytin standards. The avidin in this reporter fluoresces when the weakly interacting HABA is displaced by the biotin. A calibration curve of the biocytin standards was used for calculations. The extent of biotinylation is expressed as mol biotin/mol protein.

Triazole 20. Azide **18** (10 mg, 0.03 mmol) was added to a solution of galactose−DIBO derivative **19** (14.3 mg, 0.03 mmol) in methanol (2 mL). The reaction mixture was stirred at room temperature for 2 h. The solution was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using a mixture of 10% methanol in CH_2Cl_2 to give pure triazole **20** (23 mg, 93%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.74 (m, 2H, CH_2), 2.90−3.28 (m, 4H, 2 × CH_2), 3.35−4.24 (m, 23H, 8 × CH_2, CHCH_2, $CH_{2\ gal}$, 3 × CH_{gal}), 4.50−4.62 (m, 2H, 2 × CH_{gal}), 5.85−6.20 (m, 2H, CH_2C*H*O, NH), 6.80−7.70 (m, 12H, aromH), 8.01 (s, 1H, CH═N). HRMS (MALDI-ToF): 844.3492 ($C_{41}H_{51}N_5O_{13}Na$ ($M + Na^+$) requires 844.3376).

Isoxazole 21. A methanolic solution (1 mL) of galactose−DIBO derivative **19** (14.3 mg, 0.03 mmol) was added dropwise to a solution of oxime **18** (12.2 mg, 0.036 mmol) and BAIB (11.6 mg, 0.036 mmol) in methanol (1 mL). The reaction mixture was stirred at room temperature for 10 min. The solution was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using a mixture of 8% methanol in CH_2Cl_2 to give pure isozaxole **21** (14.6 mg, 61%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.70−1.84 (n, 2H, CH_2), 3.30−4.30 (m, 29H, 10 × CH_2, CH_2CHOH, CH_{2gal}, 5 × CH_{gal}), 6.10−6.40 (m, 1H, CH_2C*H*OH), 6.70−7.70 (m, 13H, aromH, NH). HRMS (MALDI-ToF): 842.2192 ($C_{41}H_{49}N_5O_{13}Na$ ($M + Na^+$) requires 842.3219).

General Procedure for SPAAC with Bifunctional Linker 18. Bifunctional linker **18** (0.03 mmol, 10.1 mg) and corresponding DIBO derivative **15** or **22** (0.03 mmol) were dissolved in MeOH or THF (in case of coumarin−DIBO derivative **22**) (2 mL). The reaction mixture was stirred for 3 h, and the solution was concentrated in vacuo. The residue was purified by column chromatography on silica gel.

dx.doi.org/10.1021/ja1081519 |*J. Am. Chem. Soc.* 2011, 133, 949–957

Triazole 23. Purification by silica gel column chromatography (5 then 10% MeOH in CH_2Cl_2) gave **23** as a colorless oil (25.1 mg, 87%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.34−1.45 (m, 2H, $CHCH_2$-CH_2), 1.52−1.76 (m, 4H, $CHCH_2CH_2CH_2$), 2.15−2.21 (m, 2H, CH_2C═O), 2.64−2.69 (m, 1H, CH*H*S), 2.85−3.74 (m, 26H, C*H*HS, 9 × CH_2O, 2 × CH_2NH, CH_2CHO, C*H*S), 3.83−4.06 (m, 4H, 2 × CH_2O), 4.21−4.28 (m, 1H, C*H*NH), 4.41−4.47 (m, 1H, C*H*NH), 4.55−4.61 (m, 2H, CH_2−triazole), 5.89−6.17 (m, 1H, CH_2CHO), 6.83−6.88 (m, 2H, aromH), 7.15−7.65 (m, 10H, aromH), 8.01 (s, 1H, CH═N). MS (MALDI-ToF): 981.4092 ($C_{46}H_{62}N_8O_{11}SNa$ (M + Na^+) requires 981.4157).

Triazole 24. Purification by silica gel column chromatography (3% MeOH in CH_2Cl_2) gave **24** as a yellow amorphous solid (22 mg, 73%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.80−2.02 (m, 4H, 2 × NCH_2-CH_2CH_2), 2.66−2.86 (m, 4H, 2 × $NCH_2CH_2CH_2$), 3.01−4.12 (m, 32H, 2 × $NCH_2CH_2CH_2$, 11 × CH_2O, 2 × CH_2NH, CH_2CHO), 4.37−4.61 (m, 2H, CH_2−triazole), 5.34−6.49 (m, 2H, CH_2CHO, NH), 6.73−6.82 (m, 2H, aromH), 6.93−7.60 (m, 11H, aromH), 7.92−8.10 (m, 1H, NH), 8.56−8.68 (m, 1H, CH═N), 9.01−9.25 (m, 1H, CH−vinyl). MS (MALDI-ToF): 1022.4133 ($C_{54}H_{61}N_7O_{12}Na$ (M + Na^+) requires 1022.4270).

General Procedure for SPANOC between Triazoles 23 or 24 and Glycodendrimer 25. To a stirred solution of DIBO−glycodendrimer **25** (20.5 mg, 5.2 μmol) and oxime **23** or **24** (5.2 μmol) in MeOH/CH_2Cl_2 (4/1, v/v, 1.2 mL) was added a solution of BAIB (1.8 mg, 5.7 μmol) in MeOH (0.18 mL), and the reaction mixture was stirred for 30 min. The solvent was evaporated, and the residue was purified by RP-HPLC. Appropriate fractions were combined and lyophilized.

Glycodendrimer−Biotin Conjugate 26. After RP-HPLC purification (0−5 min 0% B, 5−40 min gradient of 0−100% B, t = 29.4 min) and lyophilization, **26** was obtained as a white powder (14.0 mg, 55%). ^{1}H NMR (500 MHz, D_2O): δ 0.88−1.22 (m, 23H, 7 × CH_3, $CHCH_2CH_2$), 1.32−1.63 (m, 4H, $CHCH_2CH_2CH_2$), 1.95−2.22 (m, 18H, 8 × $CH_2CH_2CH_2$−triazole, CH_2C═O), 2.48−2.80 (m, 17H, CH*H*S, 8 × CH_2CH_2−triazole), 2.80−3.02 (m, 17H, C*H*HS, 8 × CH_2CH_2−triazole), 3.08−3.95 (m, 107H, 2×CH_2CHO, 4 × CH_2NH, 15 × CH_2O, 8 × CH-2_{gal}, 8 × CH-3_{gal}, 8 × CH-5_{gal}, 8 × CH_2-6_{gal}, 8 × CH-4_{gal}, 8 × $CH_2CH_2CH_2$−triazole, CHS), 3.99−4.55 (m, 56H, 9 × CH_2−triazole, 14 × OCH_2, 2 × C*H*NH, 8 × CH-1_{gal}), 5.55−6.15 (m, 2H, 2 × CH_2CHO), 6.33−7.60 (m, 20H, aromH), 7.87 (s, 8H, 8 × $CH_{triazole}$). MS (MALDI-ToF): 4933.4 ($C_{218}H_{310}N_{34}O_{92}SNa$ (M + Na^+) requires 4933.0).

Glycodendrimer−Coumarin Conjugate 27. After RP-HPLC purification (0−5 min 0% B, 5−10 min gradient of 0−40% B, 10−30 min gradient of 40−60% B, t = 25.3 min) and lyophilization, **27** was obtained as a yellow powder (15.1 mg, 61%). ^{1}H NMR (500 MHz, D_2O: CD_3CN, 1:1, v/v) δ 0.99−1.20 (m, 21H, 7 × CH_3), 1.65−1.81 (m, 4H, 2 × $NCH_2CH_2CH_2$), 1.99−2.08 (m, 16H, 8 × $CH_2CH_2CH_2$−triazole), 2.59−2.62 (m, 20H, 2 × $NCH_2CH_2CH_2$, 8 × CH_2CH_2−triazole), 2.84 (t, J = 7.3 Hz, 16H, 8 × CH_2CH_2−triazole), 3.10−3.94 (m, 110H, 2 × CH_2CHO, 4 × CH_2NH, 15 × CH_2O, 2 × $NCH_2CH_2CH_2$, 8 × CH-2_{gal}, 8 × CH-3_{gal}, 8 × CH-5_{gal}, 8 × CH_2-6_{gal}, 8 × CH-4_{gal}, 8 × CH_2CH_2-CH_2−triazole), 3.94−4.25 (m, 36H, 14 × OCH_2, 8 × CH-1_{gal}), 4.25−4.45 (m, 18H, 9 × CH_2-triazole), 5.41−6.19 (m, 2H, 2 × CH_2CHO), 6.58−7.51 (m, 21H, aromH), 7.64 (s, 8H, 8 × $CH_{triazole}$), 8.36−9.12 (m, 1H, CH−vinyl). MS (MALDI-ToF): 4972.8 ($C_{224}H_{309}N_{33}O_{93}Na$ (M + Na^+) requires 4974.0).

■ ASSOCIATED CONTENT

Supporting Information. Synthesis of oximes **14a−f**, imidoyl chlorides **5a−f**, azido-oxime linker **18**, DIBO−galactose **19**, amino-PEG−coumarin **33**, and DIBO−glycodendron **25**. Spectral data of new compounds, and detailed reaction profiles in kinetic studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This research was supported by the National Cancer Institute of the U.S. National Institutes of Health (R01 CA88986, G.-J.B.), the National Science Foundation Plant Genome Program (IOS-0923992, G.-J.B.), the National Science Foundation (CHE-0449478, V.V.P.), and the Georgia Cancer Coalition (V.V.P.).

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