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A Potentially Valuable Advance in the Synthesis of Carbohydrate-Based Anticancer Vaccines through Extended Cycloaddition Chemistry

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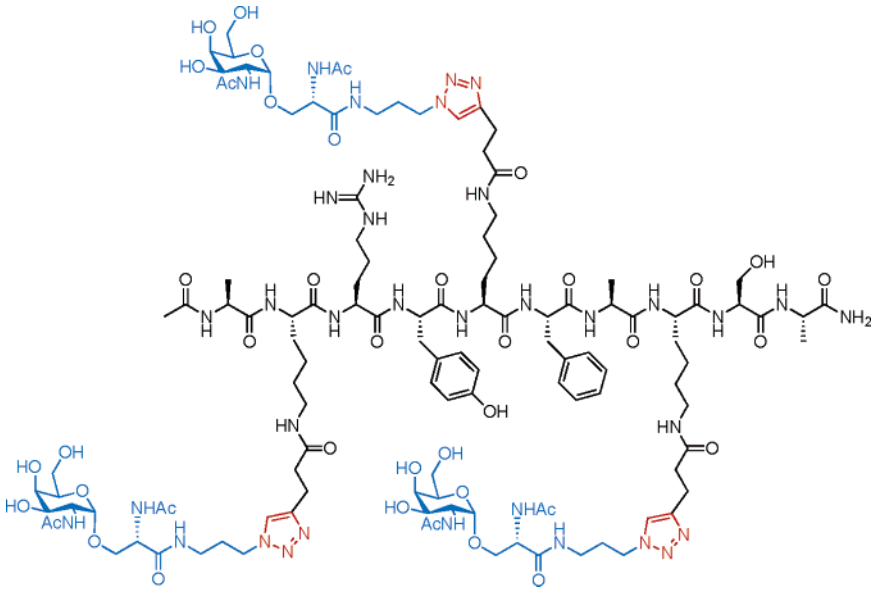
Received July 6, 2006



An advance in the long-term problem of joining complex oligosaccharides to polypeptides and even proteins is described herein. The key method involves equipping the oligosaccharide sector with an azide and the polypeptide with pendant alkynyl functionality. The two sectors are joined through a "click-like" cycloaddition. The method encompasses oligosaccharide constructs with several azide linkages which undergo concurrent cycloaddition to peptide-based acetylenes. This technology could well prove to be useful in the construction of fully synthetic vaccines.

Introduction

Our laboratory has been pursuing the development of clinically useful, carbohydrate-based antitumor vaccines based on extensive findings that cancer cells typically display aberrant levels and patterns of cell surface glycosylation.[1] Conceivably, by exposing a host system to molecules containing cancer-associated cell surface carbohydrate antigens, it could be possible to evoke an exploitable antitumor immune response. It has been found that one of the most effective ways to induce antibodies against a carbohydrate antigen is through conjugation of the antigen to large biomolecules. To date, we have focused on the particular carrier protein, keyhole limpet hemocyanin (KLH).[2] Our preoccupation with KLH did not arise from a confidence that it is optimally structured to serve as the ideal antigen carrier. However, not withstanding issues of homogeneity criteria, lack of structural definition, solubility, and incorporation of trace

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10.1021/jo061406i CCC: $33.50
Published on Web 09/09/2006

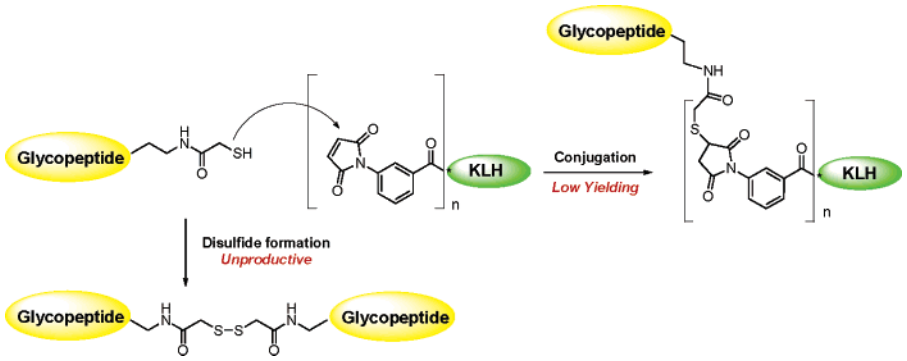

FIGURE 1. Current conjugation protocol.

metals, KLH is, at this stage of development, the most broadly employed carrier in preclinical and clinical evaluation.

Over the course of many years, our laboratory has registered a series of advances in carbohydrate and glycopeptide assembly techniques. These have enabled the de novo synthesis and Phase I clinical evaluation of increasingly sophisticated carbohydrate-based antitumor vaccine constructs. Several of our monomeric and clustered carbohydrate antigen-KLH constructs have been evaluated in preclinical and early clinical settings. One particularly promising candidate, Globo-H-KLH, is scheduled for advancement to Phase II/III clinical trials for breast cancer.[3]

More recently, we have focused our efforts on the synthesis of more complex, multiantigenic vaccine constructs, designed to be responsive to the heterogeneity of tumor cell surface glycosylation.[4] We reasoned that, by combining several different tumor-associated carbohydrate antigens on a single molecule, it should be possible to target and eliminate a greater percentage of malignantly transformed cells. Accordingly, we prepared a number of unimolecular, multiantigenic vaccine constructs, each composed of a polypeptide backbone displaying anywhere from three to six different tumor-associated carbohydrate antigens.[5] These multivalent constructs have been shown, in preclinical settings, to effectively induce antibodies against the component antigens at levels roughly equivalent to those achieved with the corresponding monovalent constructs.

The syntheses of these constructs commence with the preparation of the component glycosylamino acids. These are then iteratively coupled to form the fully glycosylated polypeptide backbone. The glycopeptide is conjugated to the carrier, for the moment, KLH (Figure 1). Typically, a sulfhydryl handle installed at the *N*-terminus participates in a Michael-type addition to a maleimide functionality on the surface of the modified KLH. This conjugation step had occurred, albeit in disappointingly low yields, possibly as a result of a nonproductive disulfide dimerization of the glycopeptide. Given the complexity of building the multiantigenic glycopeptide fragments, the substantial loss of material associated with this conjugation protocol is particularly egregious.

In targeting increasingly complex glycopeptide fragments for conjugation to various protein-based carriers, we sought a protocol which would allow for the reliable functionalization of each component in advance of the conjugation event. Furthermore, the chemistry used in the conjugation must be compatible with the chemistry of the components. Finally, the conjugation should proceed with a fairly rapid reaction rate, to allow for the introduction of a high percentage of vaccine molecules to the surface of the carrier protein.[6]

With these considerations in mind, we were drawn to the Cu^{I}-catalyzed version of the Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes, developed independently by the Sharpless and Meldal groups.[7] Since the pioneering investigations at Scripps, this commendably straightforward chemistry (which can be conducted in aqueous media!) has been widely applied as a powerful tool for the selective modifications of enzymes,[8] viruses,[9] and cells.[10] Notably, as practiced by Sharpless and associates, the cycloaddition reaction is highly chemoselective and tolerates a variety of functionality. Furthermore, such reactions can be conducted under aqueous conditions, such as would be necessary to preserve the nature of the carrier protein.[11] The installation of the requisite azido and acetylenic functional handles on glycopeptides and carrier protein seemed likely to be a straightforward matter. Indeed, herein, we describe the development of an efficient 1,3-dipolar cycloaddition protocol which results in the merger of the carbohydrate and polypeptide domains. This logic is likely to prove applicable

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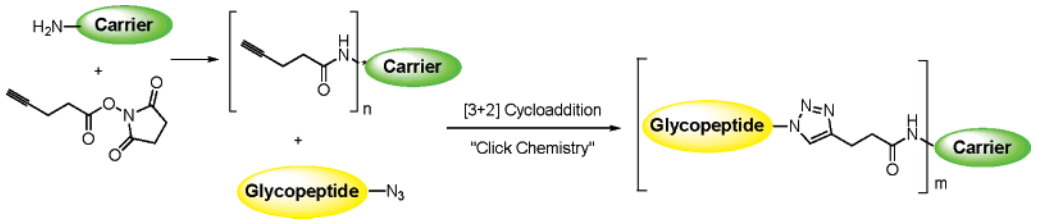
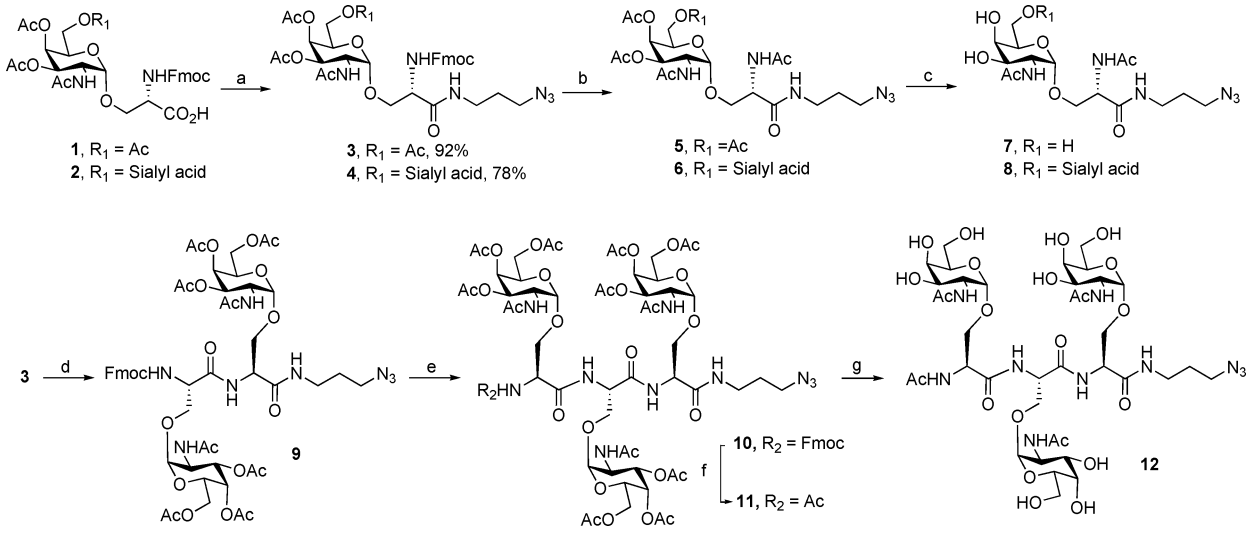


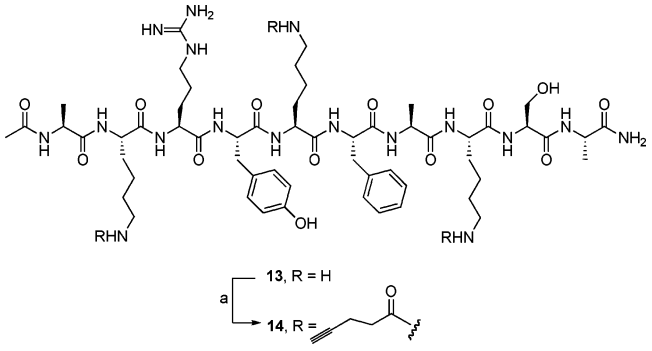
FIGURE 2. Strategy based on azide−acetylenic cycloaddition.

SCHEME 1. Preparation of 7, 8, and 12[a]



[a] Reagents and conditions: (a) HOBt, EDCI, $H_2N(CH_2)_3N_3$; (b) (i) 5% Et_2NH/DMF, (ii) Ac_2O; (c) 0.1 N NaOH in MeOH; (d) (i) 1% Et_2NH/DMF, (ii) HOBt, EDCI, **1**, 88%; (e) (i) 1% Et_2NH/DMF, (ii) HOBt, EDCI, **1**, 90%; (f) (i) 2% Et_2NH/DMF, (ii) Ac_2O, 96%; (g) 7 N NH_3 in MeOH, 76%.

SCHEME 2. Synthesis of Modified Polypeptide (14)[a]



[a] Reagents and conditions: (a) *N*-succinimidyl-4-pentynoate, DMF, $NaHCO_3$, H_2O, 68%.

to the formation of glycopeptide−carrier protein conjugates, though application to the particular KLH carrier will be challenging for the reasons discussed above.

Under our modified conjugation strategy, an azido "handle" would be mounted on the carbohydrate, while the lysine residues of the carrier protein would be equipped with acetylene units (Figure 2). A 1,3-dipolar cycloaddition between the azide and alkyne would fashion the carbohydrate−carrier protein conjugate.

Results and Discussion

To evaluate in a preliminary way the feasibility of the proposed conjugation strategy, we prepared two monomeric glycosylamino acids displaying the Tn (**7**) and STn (**8**) antigens, as well as a trimeric cluster composed of three Tn antigens (**12**) (Scheme 1). The synthesis of **7** commenced with the readily available carboxylic acid **1**.[12] Amide formation, as shown, allowed for the introduction of the azido group. Next, removal of the Fmoc group and capping of the amine with acetic anhydride, followed by global deprotection, produced coupling precursor **7**. The STn glycosylamino acid, **8**, was accessed from **2**[13] in a similar fashion.

The Tn cluster, **12**, was prepared from **3** through iterative Fmoc removal and coupling reactions with the monomeric unit, **1**. A series of standard manipulations provided **12**.

The surfaces of carrier proteins possess abundant lysine residues. To simulate the environment of the carrier protein, we designed a 10-mer polypeptide, **13**, possessing three lysine residues (Scheme 2). The acetylenic functional groups were introduced through exposure of **13** to *N*-succinimidyl-4-pentynoate in the presence of sodium bicarbonate.[14] Amide formation at the ϵ-amino groups of the lysine residues provided the desired polypeptide alkynes of **14** in good yield.

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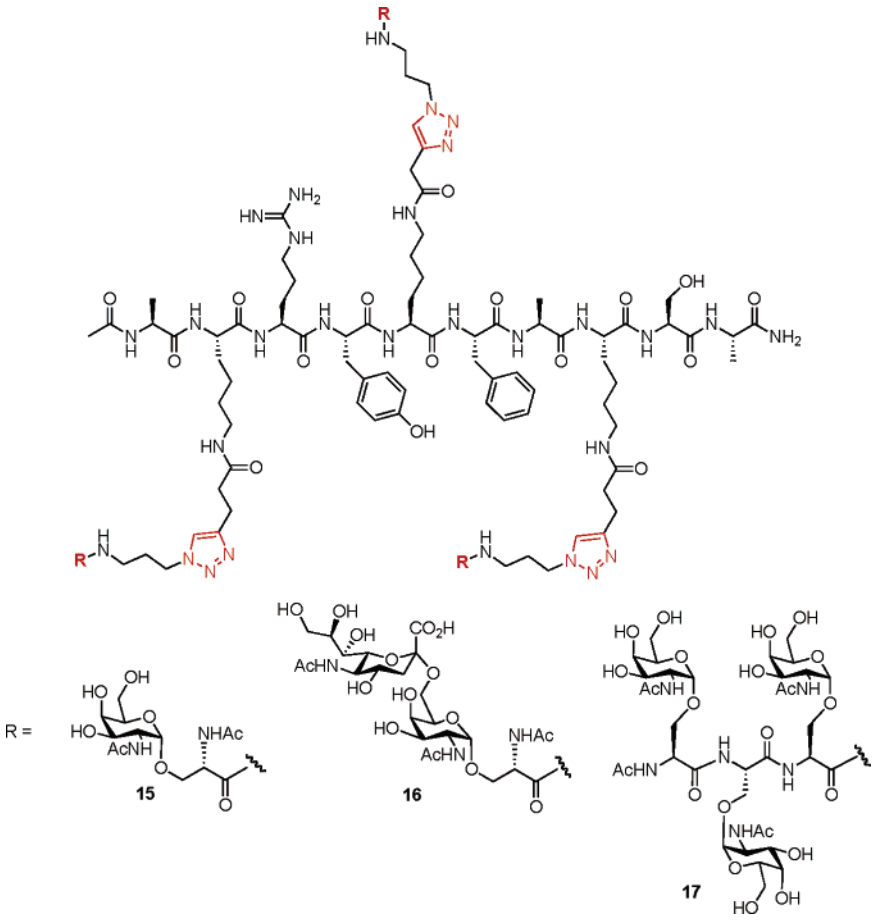
SCHEME 3. Conjugation Adducts



With the azido-carbohydrate and the alkynyl polypeptide components in hand, we investigated conditions to accomplish their merger. A variety of reaction conditions had already been reported for the generation of the active Cu^{I} species.[15] For instance, the catalytic Cu(I) species could be introduced directly from the Cu(I) salt, most commonly from copper halide. However, in this case, prior exclusion of oxygen from the reaction is required in order to prevent the formation of undesired byproducts. Alternatively, Cu(I) can be generated through in situ reduction of Cu(II) with a variety of reducing agents, such as copper wire,[8] sodium ascorbate,[7a] and phosphine derivatives.[16] Several additives, such as tris-triazoles[17] and triethylamine hydrochloride,[18a] have been observed to significantly accelerate the cycloaddition event, presumably by stabilizing the catalytic Cu(I) species. An additional constraint imposed by our system was the need to operate in proper aqueous medium in order to ensure protein stability.

A number of ligation conditions were explored, with varying degrees of success. In our initial attempts, a combination of copper wire and $CuSO_4$ was utilized. Thus, to a solution of **7** and **14** in water and acetonitrile (1:1, v/v) was added polished copper wire and a solution of $CuSO_4$ in water. The reaction was monitored by LC-MS, which indicated that the tris-triazole adduct (**15**, Scheme 3) was formed quantitatively after approximately 40 h. When sodium ascorbate was used as the reducing agent instead of copper wire, the reaction was found to be markedly less efficient.

Although we had identified conditions appropriate for the conjugation of the glycosylamino acid (**7**) with the polypeptide (**14**), we were not unmindful of the potential difficulties involved in translating this particular protocol to a protein setting. Thus, conjugation of the glycosylamino acid (or glycopeptide) to a carrier protein would require aqueous conditions and would be difficult to achieve in the presence of acetonitrile. Unfortunately, recourse to a purely aqueous solution from 50% acetonitrile resulted in a prohibitive decrease in reaction rate.

We next explored alternate means by which to gain access to Cu(I)—namely through oxidation of copper metal. In this regard, we found that, upon exposure to nanosized copper powder in water and *t*-BuOH (4:1 v/v), azide **7** and alkynyl peptide **14** underwent cycloaddition to provide the desired tris-triazole adduct (**11**) after 3 d (Figure 3a).[18b] We were surprised to find that addition of $CuSO_4$ to the reaction mixture actually had the effect of decreasing the reaction rate. We suspect that cycloaddition may actually occur on the surface of the nanosized copper,[19] which could be modified by the presence of $CuSO_4$.

To accelerate the conjugation rate and minimize the potential damage to the carrier protein caused by organic solvents, we

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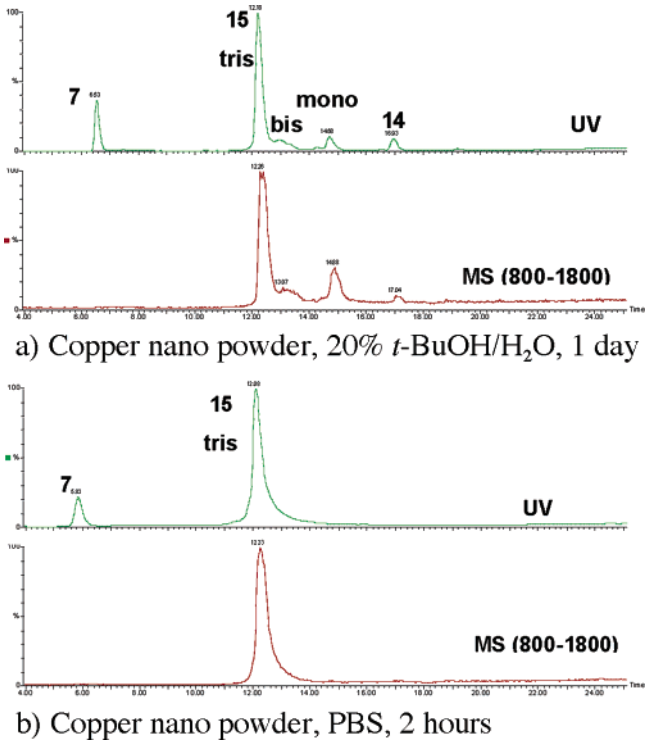


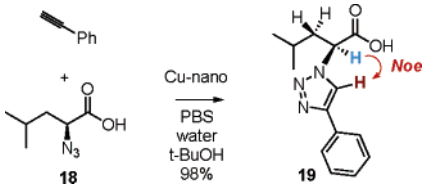
FIGURE 3. LC−MS traces of the [3 + 2] cycloaddition of **7** and **14**.

performed the cycloaddition of **7** and **14** in water in the presence of Et_3N·HCl as an additive. Remarkably, it was found to greatly accelerate the cycloaddition reaction in aqueous *t*-BuOH.[18a] We were pleased to observe that each of three alkynyl moieties of **14** was transformed to a triazole over 3 h, and that the desired adduct was isolated in excellent yield. With the concern that Et_3N·HCl may have some detrimental effect on the carrier protein, the conjugation was carried out with nanosized Cu in the PBS (pH 7.2) without addition of Et_3N·HCl. We were pleased to find that the desired cycloaddition proceeded smoothly, at a rate comparable to that observed when the reaction was performed in the presence of Et_3N·HCl (Figure 3b). Although successful bioconjugations had been reported, additional ligands were employed in such conjugations.[8−10] To our knowledge, this is the first time that PBS alone has been reported to greatly enhance the reaction rate of the nanosized Cu-catalyzed Huisgen cyclization.

With these results in hand, we successfully applied the reaction conditions to the conjugation of the STn azide, **8**, and the clustered Tn azide, **12**. In each case, the appropriate tris-triazoles were formed cleanly[20] and in good yield in approximately 2 h.

In summary, we have reported herein the application of Sharpless-inspired protocols to the conjugation of fully synthetic glycopeptide fragments to appropriately modified lysine residues. These mild, aqueous reaction conditions should be readily extendable to the conjugation of glycopeptide fragments to carrier proteins. This newly developed protocol represents a potentially powerful addition to the collection of conjugation techniques currently practiced, although admittedly application to the particular KLH case could remain problematic. While this application to the azide acetylene cycloaddition reaction is not yet a fully mature process, we expect that it will ease the path for the total synthesis and clinical evaluation of new vaccines directed to the enlistment of the formidable resources of the immune system in countering diseases. More broadly, this work is an exciting example of the potentiality in merging the teachings of chemistry and biology with a view to clinical application.

(20) The nanosized-Cu-catalyzed 1,3-dipolar cycloaddition of alkynes to azides in PBS buffer is a 1,4-regiospecific reaction. The regioselectivity was confirmed by NOE study. The synthesis of compound **18** followed a known literature precedent: Lundquist, J. T., IV; Pelletier, J. C. *Org. Lett.* **2001**, 3, 781−783.



Experimental Section

AcHN-Tn(OAc)$_3$-CONH-(CH$_2$)$_3$-N$_3$ (5). To a solution of Tn-acid (**1**, 0.12 g, 0.18 mmol) in DMF−CH_2Cl_2 (1:1, 2.0 mL) at −20 °C was added HOBt (39.0 mg, 0.29 mmol) followed by EDCI (45.5 mg, 0.24 mmol). The reaction mixture was stirred at 0 °C for 30 min, and then $H_2N-(CH_2)_3-N_3$ (18.3 mg, 19.3 μL, 0.18 mmol) was added. After the mixture was stirred for 11 h, Et_2NH (100.0 μL) was added. The reaction mixture was stirred at room temperature for 10 h, and then Ac_2O (50.0 μL) was added. After being stirred overnight, the reaction mixture was diluted with EtOAc and washed with brine. The organic phase was dried with $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography (2%, 5%, 10% gradient MeOH in CH_2Cl_2) to give the desired azide (**5**, 92.0 mg, 0.17 mmol, 90% over three steps) as a white foam: $[\alpha]^{22}_D$ = 70.6 (*c* 1.0, $CHCl_3$); IR 3299, 3079, 2939, 2100, 1748, 1660, 1544, 1440, 1373, 1238, 1133, 1050, 916 and 732 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.94 (t, 1 H, J = 5.7 Hz), 6.66 (d, 1 H, J = 8.0 Hz), 6.24 (d, 1 H, J = 9.6 Hz), 5.33 (d, 1 H, J = 2.8 Hz), 5.06 (dd, 1 H, J = 3.2, 11.3 Hz), 4.83 (d, 1 H, J = 3.6 Hz), 4.66 (ddd, 1 H, J = 4.0, 4.5, 8.5 Hz**),** 4.54 (ddd, 1 H, J = 3.6, 9.6, 11.3 Hz), 4.10 (m, 2 H), 4.02 (dd, 1 H, J = 10.7, 6.5 Hz), 3.86 (dd, 1 H, J = 4.5, 10.1 Hz), 3.75 (dd, 1H, J = 4.5, 10.1 Hz), 3.35 (m, 4 H), 2.13 (s, 3 H), 2.05 (s, 3 H), 2.02 (s, 3 H), 1.96 (s, 6 H), 1.77 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9, 170.8, 170.6, 170.5, 170.3, 169.5, 99.2, 69.2, 68.5, 67.2, 67.1, 61.8, 52.8, 49.3, 47.5, 37.4, 28.5, 23.2, 23.1, 20.73, 20.70; MS (EI) *m*/*z* 581.3 [M + Na]$^+$; HRMS calcd for $C_{22}H_{35}N_6O_{11}$ [M + H]$^+$ 559.2364, found 559.2358.

AcHN-Tn-CONH-(CH$_2$)$_3$-N$_3$ (7). To a solution of compound **5** (26.0 mg, 46.5 μmol) in MeOH (1.1 mL) was added aqueous NaOH (0.1 N, 1.1 mL) at 0 °C. The reaction mixture was stirred at room temperature overnight before it was neutralized with Dowex and filtered. The residue was purified by HPLC (0−25% solvent B (CH_3CN with 0.04% TFA) in solvent A (H_2O with 0.05% TFA) over 20 min) to give the desired compound (**7**, 19.0 mg, 95%) as a white foam: ^{1}H NMR (500 MHz, D_2O) δ 4.87 (d, 1 H, J = 3.7 Hz), 4.50 (t, 1 H, J = 5.2 Hz), 4.13 (dd, 1 H, J = 3.7, 11.0 Hz), 3.96 (d, 1 H, J = 3.0 Hz), 3.90 (dd, 1 H, J = 4.6, 10.8 Hz), 3.85 (m, 2 H), 3.75 (dd, 1 H, J = 5.9, 10.8 Hz), 3.72 (m, 2 H), 3.34 (t, 2 H, J = 6.6 Hz), 3.28 (m, 2 H), 2.05 (s, 3 H), 2.02 (s, 3 H), 1.76 (t, 1 H, J = 6.6 Hz), 1.73 (t, 1 H, J = 6.6 Hz); ^{13}C NMR (125 MHz, D_2O + trace of acetone) δ 175.0, 174.8, 171.9, 98.4, 71.9, 68.9, 68.2, 67.8, 61.7, 54.6, 50.3, 49.1, 30.7, 28.2, 22.5, 22.2; MS (EI) *m*/*z* 455.3 [M + Na]$^+$, 467.2 [M + Cl]$^-$.

Compound 15. To a mixture of compound **7** (2.4 mg, 5.58 μmol) and compound **14** (1.8 mg, 1.24 μmol) were added PBS buffer (300.0 μL) and Cu(0) nanosize powder (1.0 mg) at room temperature. The reaction mixture was stirred for 2 h and then centrifuged for 5 min. The aqueous phase was removed, and the Cu(0) powder was washed with 500 μL of water and centrifuged. The aqueous

phases were combined and passed through a 0.2 μm filter. The light blue solution was purified by HPLC (5−35% solvent B in solvent A over 30 min, semi prep C18 column) to give the desired compound (**15**, 2.2 mg, 65%) as a white powder: selected ^{1}H NMR (500 MHz, D_2O) δ 7.76 (s, 2 H), 7.74 (s, 1 H), 7.31 (m, 2 H), 7.22 (m, 3 H), 7.05 (d, 2 H, J = 8.5 Hz), 6.79 (d, 1 H, J = 8.4 Hz), 6.77 (d, 1 H, J = 8.4 Hz), 4.87 (d, 3 H, J = 3.7 Hz), 2.09 (s, 3 H), 2.08 (s, 9 H), 2.06 (s, 3 H), 2.05 (s, 3 H), 2.02 (s, 3 H), 2.00 (s, 9 H); MS (EI) m/z 1375.2 $[M + 2H]^{2+}$.

Acknowledgment. Support for this work was provided by the National Institutes of Health (CA28824). A postdoctoral fellowship is gratefully acknowledged by Q.W. and J.C. (Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research, and the Experimental Therapeutics Center, SKI). We thank Dr. George Sukenick, Ms. Sylvi Rusli, and Ms. Hui Fang of the Sloan-Kettering Institute's NMR core facility for mass spectral and NMR spectroscopic analysis (SKI core grant no. CA02848).

Supporting Information Available: Experimental procedures, NMR spectra, and characterization for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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