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# Modified Bleomycin Disaccharides Exhibiting Improved Tumor Cell Targeting

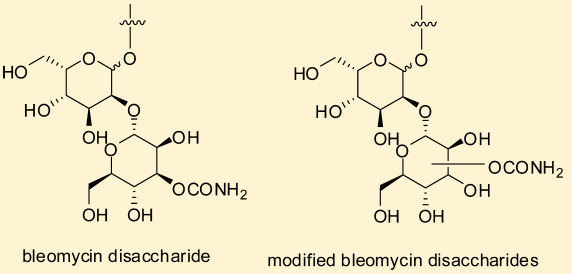
Manikandadas M. Madathil,[†] Chandrabali Bhattacharya,[†] Zhiqiang Yu,[†] Rakesh Paul,[†] Michael J. Rishel,[‡] and Sidney M. Hecht*[,†]

[†]Center for Bioenergetics, Biodesign Institute, and Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287, United States

[‡]GE Global Research, 1 Research Circle, Niskayuna, New York 12309, United States

Supporting Information

**ABSTRACT:** The bleomycins (BLMs) are a family of antitumor antibiotics used clinically for anticancer chemotherapy. Their antitumor selectivity derives at least in part from their ability to target tumor cells, a property that resides in the carbohydrate moiety of the antitumor agent. In earlier studies, we have demonstrated that the tumor cell selectivity resides in the mannose carbamoyl moiety of the BLM saccharide and that both the BLM disaccharide and monosaccharide containing the carbamoyl moiety were capable of the delivery/uptake of a conjugated cyanine dye into cultured cancer cell lines. Presently, the nature of the participation of the carbamoyl moiety has been explored further to provide compounds of utility for defining the nature of the mechanism of tumor cell recognition and uptake by BLM saccharides and in the hope that more efficient compounds could be identified. A library of seven disaccharide−Cy5** dye conjugates was prepared that are structural analogues of the BLM disaccharide. These differed from the natural BLM disaccharide in the position, orientation, and substitution of the carbamoyl group. Studies of these compounds in four matched sets of tumor and normal cell lines revealed a few that were both tumor cell selective and internalized 2−4-fold more efficiently than the natural BLM disaccharide.




The bleomycins are a family of glycopeptide antitumor antibiotics originally isolated from *Streptomyces verticillus* in 1966 by Umezawa and his colleagues.[1] The selective cytotoxicity of bleomycins toward tumor cells has led to their clinical use in the treatment of squamous cell carcinomas and malignant lymphomas.[2−4] The cytotoxic activity of bleomycin has often been attributed to its ability to mediate double-strand cleavage of DNA.[5,6]

The therapeutic utility of bleomycin is enhanced by its low administered dose, which consists of ∼5 μmoles of BLM. The very low dose implies that bleomycin must target tumor cells selectively to achieve its therapeutic effects, and numerous reports employing radionuclide complexes of BLM have documented that the complexes selectively target a variety of types of tumors.[7−14] The importance of the carbohydrate moiety to the tumor selectivity of BLM (Figure 1) was suggested by imaging studies carried out using microbubbles to which multiple copies of BLM derivatives had been attached covalently.[15] Microbubbles containing attached bleomycins adhered selectively to monolayers of cultured tumor cells; those containing the BLM aglycone (deglycoBLM) did not.[15]

Furthermore, carbohydrates are known to play a pivotal role in mediating a number of biological processes. Glycopeptides, glycolipids, and other glycoconjugates participate in cell−cell interactions, inflammation, fertility and development, and signal transduction.[16−19] The recognition and internalization of carbohydrate residues by specific cell surface carbohydrate-binding proteins play a crucial role in mediating the cellular

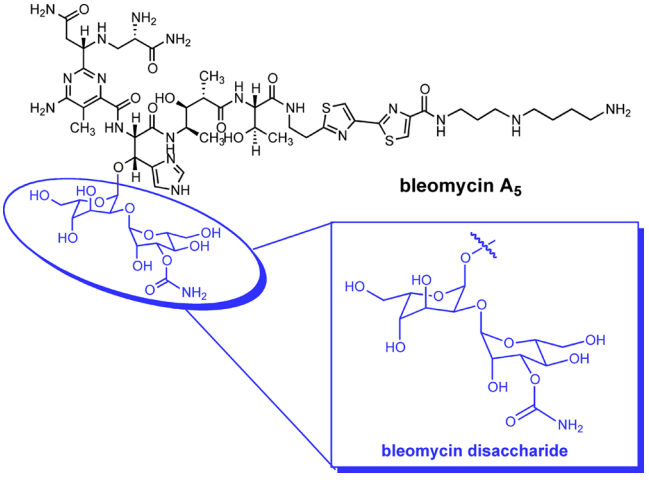



**Figure 1.** Structure of bleomycin $A_5$ with the disaccharide moiety highlighted in blue.

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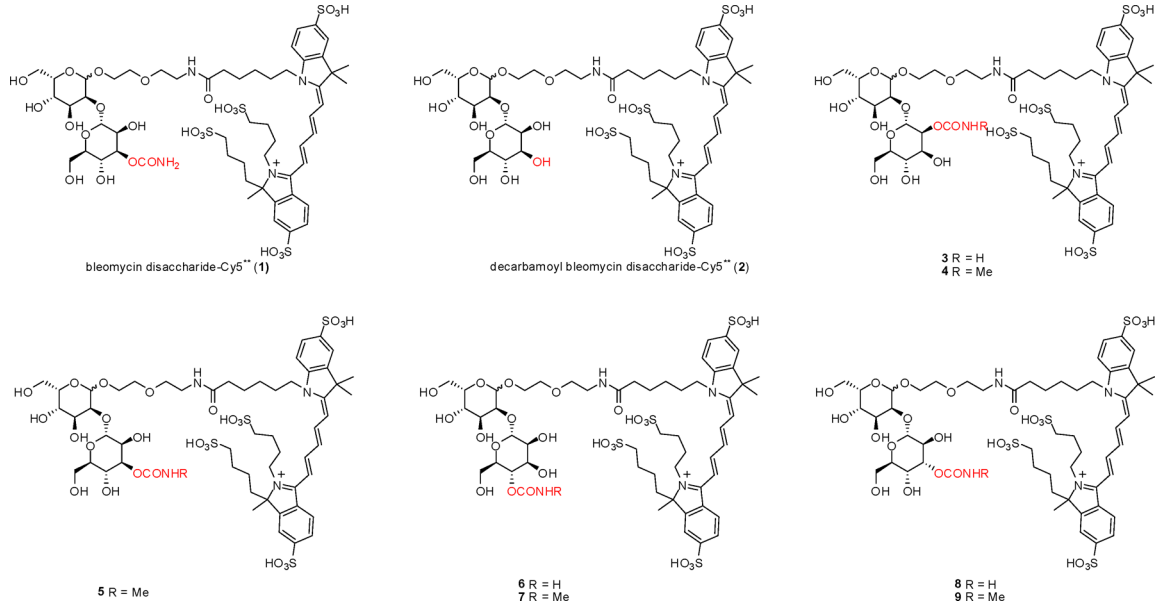


**Figure 2.** Structures of disaccharide−dye conjugates **3**−**9** prepared for evaluation.

uptake of many glycosylated natural products and control their biological activity.[20]

In view of the documented importance of carbohydrates in cellular recognition, the possible role of the carbohydrate moiety of bleomycin in cancer cell selectivity has been explored more directly. In previous studies, it was shown that BLM disaccharide[21] and BLM monosaccharide,[22] both of which contain the carbamoylmannose moiety, could recapitulate the effects of BLM itself in mediating the delivery of an attached dye selectively to cancer cells. In both cases, the carbamoyl moiety of carbamoylmannose was required to support tumor cell targeting. In order to begin to develop a more complete understanding of the structural factors in the carbohydrate domain that conduce to selective, potent binding and uptake by tumor cells, a focused library of structural analogues of the natural bleomycin disaccharide has been synthesized. Given the importance of the carbamoyl group, this functionality was modified and its position was altered systematically to afford a library of seven disaccharide−dye conjugates (Figure 2). The cellular targeting and uptake of the conjugates were studied by fluorescence microscopy. These studies have better defined the required positioning and modification of the carbamoyl group for effective tumor cell targeting and identified specific disaccharides having 2−4-fold improved binding/uptake in human tumor cell lines, relative to that for the natural BLM disaccharide.

## MATERIALS AND METHODS

**Cell Growth Conditions.** A498 kidney cancer cells (ATCC HTB-41) and A549 lung cancer cells (ATCC CCL-185) were grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin−streptomycin mix antibiotic supplement (Cellgro, Manassas, VA). DU-145 (ATCC HTB-81) prostate cancer cells, BxPC-3 (ATCC CRL-1687) pancreatic cancer cells, SVR A221a (CRL-2386) normal pancreatic cells, PZ-HPV-7 (ATCC CRL-2221) normal prostate cells, WI-38 (ATCC CCL-75) normal lung cells, and CCD-1105 KIDTr (CRL- 2305) normal kidney cells were grown in MEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin−streptomycin mix antibiotic supplement. Cell lines were maintained at 37 °C under a humidified atmosphere of 5% $CO_2$ and 95% air.

**Fluorescence Microscopy.** The cells were grown on 16-well Lab-Tek glass chamber slides at a cell density of 5000 cells/well (Thermo Scientific, Waltham, MA) at 37 °C for 48 h. When the cell confluency reached about 70%, the cells were rinsed twice with phosphate buffered saline (PBS), and the old medium was replaced with RPMI 1640 (no phenol red). Subsequently, the dye-labeled conjugates were added to the final desired concentration (25 $\mu$M), and incubation of the cells was carried out at 37 °C for 1 h. Thereafter, the cells were washed with PBS and fixed with 4% paraformaldehyde at 37 °C for 5 min. Finally, the slide was mounted with Prolong Gold Antifade reagent (with DAPI) (Invitrogen). The fluorescent images were obtained using a Zeiss Axiovert 200M inverted fluorescence microscope fitted with an AxioCam MRm camera equipped with a 300 W xenon lamp (Sutter, Novato, CA) and a Cy5 cyanine filter (Chroma, Bellows Falls, VT). The cells were imaged using a Zeiss EC Plan Neofluor 40×/1.3 DIC M27 oil objective, and the target cells were counted for quantification. For comparative studies, the exposure time and source intensity were kept identical for accurate measurements. Three different viewing fields each containing at least 10 individual cells were analyzed from each experiment. Numerical values representing mean pixel intensities (arbitrary units) [per unit area of the cells] in the viewing fields were measured to give the normalized fluorescence, and quantification values were generated by using AxioVision 4 v 4.7.1.0 software in conjunction with the interactive measurement tool. Briefly, regions of interest within each cell were manually outlined, and the mean pixel intensity within the region of interest was calculated. The mean value of pixels in a region of comparable area outside the cells was subtracted from each measurement to account for background intensity. The data were then collated, and final results are expressed as mean pixel intensity ± standard error of mean per area unit.

**Synthesis of Disaccharide−Dye Conjugates.** The general synthetic strategy adopted for the synthesis of the disaccharide−dye conjugates is outlined in Scheme 1. The


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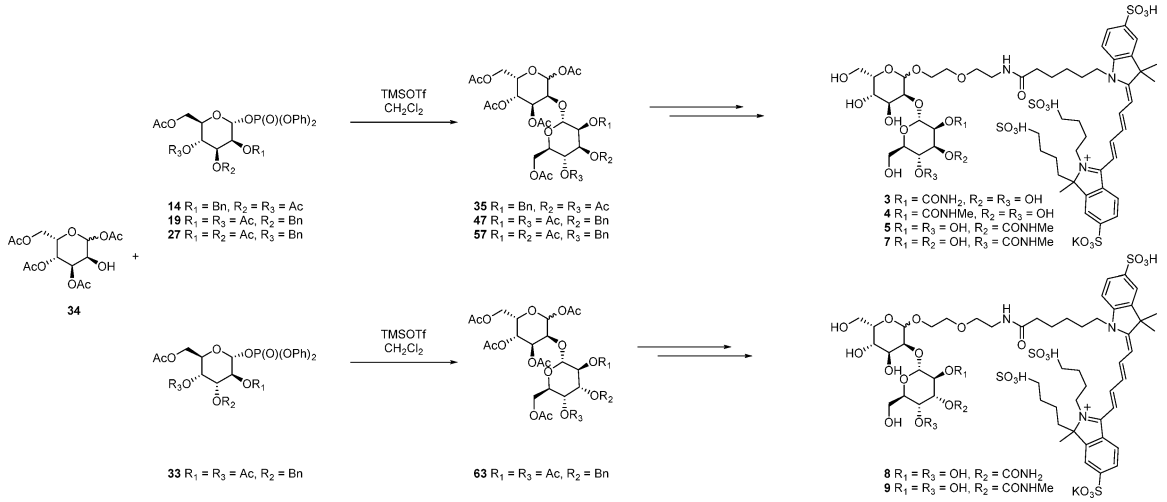
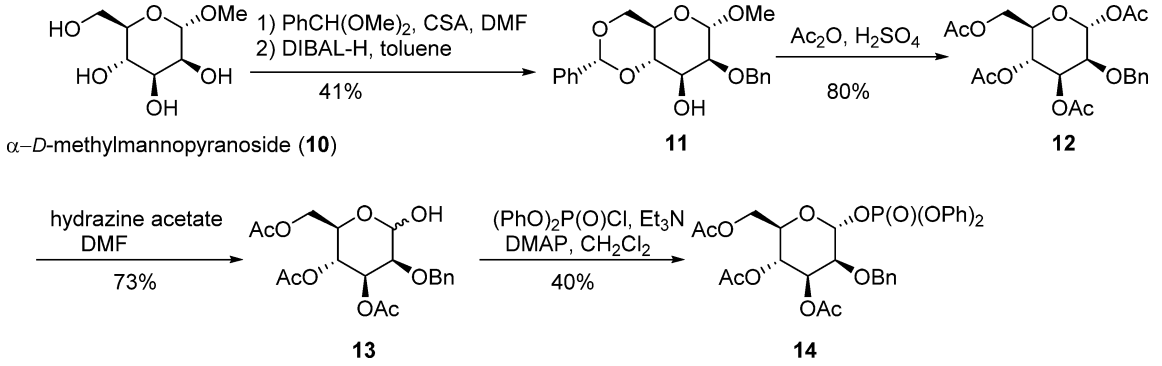
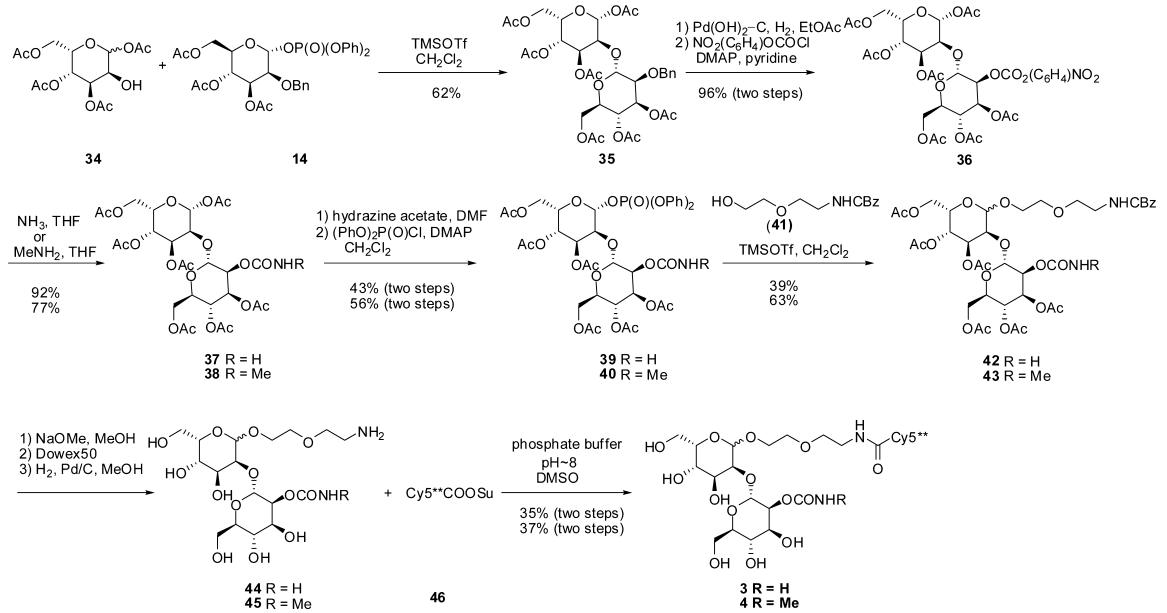
**Scheme 1. General Strategy for the Synthesis of Disaccharide−Dye Conjugates**




**Scheme 2. Synthesis of 2-*O*-Benzylated Mannosyl Donor 14**




**Scheme 3. Synthesis of Disaccharide−Dye Conjugates 3 and 4 Containing Carbamoyl Moieties at the 2-Position of Mannose**




syntheses of the different disaccharide−dye conjugates are described below and outlined in Schemes 2, 3, and S1−S8. The experimental procedures and compound characterizations are given below and in the Supporting Information.

Compounds **11**, **12**, **34**, **41**, and **46** were synthesized according to published procedures.[21−27]

*3,4,6-Tri-O-acetyl-2-O-benzyl-α-D-mannopyranoside (**13**).* To a solution containing 1.13 g (2.58 mmol) of compound **12** in 21 mL of anh DMF was added 286 mg (3.10 mmol) of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hydrazine acetate. The reaction mixture was stirred at room temperature for 2.5 h and quenched by the addition of 100 mL of ethyl acetate. The organic layer was washed with three 50 mL portions of brine and dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (20 × 3 cm). Elution with 1:2 ethyl acetate−hexanes afforded pyranoside **13** as a colorless oil: yield 793 mg (73%); silica gel TLC $R_f$ 0.23 (1:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.97 (s, 3H), 2.00 (s, 3H), 2.02 (s, 3H), 3.81−3.87 (m, 1H), 4.05−4.17 (m, 2H), 4.20 (dt, 1H, $J$ = 9.3 and 4.7 Hz), 4.56−4.63 (m, 3H), 5.21−5.33 (m, 2H), 5.40 (t, 1H, $J$ = 9.9 Hz), 7.21−7.36 (m, 5H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.57, 20.58, 20.7, 62.7, 66.6, 68.2, 70.9, 72.8, 75.6, 92.2, 127.7, 128.2, 137.6, 169.8, 170.2, 171.1; mass spectrum (APCI), $m/z$ 397.1498 $(M + H)^+$ ($C_{19}H_{25}O_9$ requires 397.1498).

*3,4,6-Tri-O-acetyl-2-O-benzyl-α-D-mannopyranosyl Diphenyl Phosphate (**14**).* To a stirred solution containing 793 mg (2.00 mmol) of **13** in 120 mL of anh dichloromethane were added 305 mg (2.50 mmol) of *N*,*N*-dimethylaminopyridine (DMAP), 3.00 mL (2.17 g, 21.6 mmol) of $Et_3N$, and 4.00 mL (5.20 g, 19.2 mmol) of diphenylphosphoryl chloride. The reaction mixture was stirred at 0 °C for 1.5 h and then poured into a stirred mixture of 300 mL of ethyl acetate and 150 mL of satd aq $NaHCO_3$. The aqueous and organic layers were separated, and the organic layer was washed with three 50 mL portions of water and brine and then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (20 × 3 cm). Elution with 1:2 ethyl acetate−hexanes afforded **14** as a colorless oil: yield 508 mg (40%); silica gel TLC $R_f$ 0.44 (1:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 2.17 (s, 3H), 2.20 (s, 3H), 2.23 (s, 3H), 4.10−4.25 (m, 3H), 4.42 (dd, 1H, $J$ = 12.2 and 3.9 Hz), 4.76−4.88 (m, 2H), 5.49 (d, 1H, $J$ = 8.0 Hz), 5.73 (t, 1H, $J$ = 10.1 Hz), 6.21 (d, 1H, $J$ = 5.7 Hz), 7.33−7.62 (m, 15H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.39, 20.46, 20.53, 61.7, 65.3, 69.8, 70.8, 73.1, 74.4, 96.6, 119.9, 120.05, 120.09, 120.14, 124.59, 125.63, 127.8, 127.9, 128.3, 129.3, 129.8, 136.8, 149.9, 150.1, 150.8, 169.3, 169.8, 170.53; mass spectrum (APCI), $m/z$ 629.1788 $(M + H)^+$ ($C_{31}H_{34}O_{12}P$ requires 629.1788).

*1,3,4,6-Tetra-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-benzyl-α-D-mannopyranosyl)-β-L-gulopyranose (**35**).* To a stirred solution containing 234 mg (0.67 mmol) of glycosyl acceptor **34** and 508 mg (1.17 mmol) of glycosyl donor **14** in 4.8 mL of anh dichloromethane at 0 °C was added 244 μL (300 mg, 1.35 mmol) of TMSOTf. The reaction mixture was stirred at 0 °C for 10 min, at which time it was poured into a two-phase mixture of 30 mL of ethyl acetate and 30 mL of satd aq $NaHCO_3$. The organic and aqueous layers were separated, and the organic layer was washed with two 20 mL portions of brine and dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (30 × 3 cm). Elution with 2:1 ethyl acetate−hexanes afforded compound **35** as a colorless oil: yield 302 mg (62%); silica gel TLC $R_f$ 0.2 (1:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.84 (s, 3H), 1.94 (s, 3H), 1.99 (s, 3H), 2.04 (s, 3H), 2.08 (s, 3H), 2.09 (m, 6H), 3.51−3.61 (m, 1H), 3.87−4.23 (m, 5H), 4.31 (t, 1H, $J$ = 6.3 Hz,), 4.44−4.47 (m, 1H), 4.56−4.69 (m, 1H), 4.80−4.97 (m, 2H), 5.02−5.07 (m, 2H), 5.27−5.47 (m, 2H), 5.78 (d, 1H, $J$ = 8.5 Hz), 7.16−7.36 (m, 5H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.61, 20.63, 20.67, 20.72, 61.3, 62.2, 65.3, 66.0, 67.7, 68.8, 69.2, 70.4, 71.3, 72.2, 73.9, 90.6, 94.2, 127.7, 128.1, 128.2, 137.6, 168.7, 169.36, 169.37, 169.4, 170.0, 170.3, 170.6; mass spectrum (APCI), $m/z$ 727.2453 $(M + H)^+$ ($C_{33}H_{43}O_{18}$ requires 727.2450).

*1,3,4,6-Tetra-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-((p-nitrophenyloxy)carbonyloxy)-α-D-mannopyranosyl)-β-L-gulopyranose (**36**).* To a solution containing 200 mg (0.27 mmol) of disaccharide **35** in 38 mL of ethyl acetate was added a catalytic amount of $Pd(OH)_2$/C, and the reaction mixture was stirred overnight under 1 atm of $H_2$. The solvent was filtered through a pad of Celite, and the filtrate was concentrated under diminished pressure to afford a crude residue. The residue was used for the next reaction; silica gel TLC $R_f$ 0.08 (1:1 ethyl acetate−hexanes).

To a solution containing 198 mg (0.31 mmol) of the crude residue in 1.2 mL of anh pyridine were added 151 mg (1.24 mmol) of DMAP and 276 mg (1.24 mmol) of *p*-nitrophenyl chloroformate. The reaction mixture was stirred at 40 °C overnight, at which time it was poured into a mixture of 30 mL ethyl acetate and 10 mL of $H_2O$. The organic and aqueous layers were separated, and the organic layer was washed successively with three 10 mL portions of 1 N HCl, 10 mL of satd aq $NaHCO_3$, and then brine. The solution was dried ($MgSO_4$) and filtered, and the filtrate was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (25 × 3 cm). Elution with 1:1 ethyl acetate−hexanes afforded **36** as a colorless foam: yield 211 mg (96% over two steps); silica gel TLC $R_f$ 0.30 (1:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.98 (m, 3H), 2.03 (s, 6H), 2.10 (s, 3H), 2.12 (s, 3H), 2.14 (s, 3H), 2.17 (s, 3H), 3.96−4.18 (m, 2H), 4.19−4.29 (m, 2H), 4.35 (t, 1H, $J$ = 6.5 Hz), 4.96−5.03 (m, 2H), 5.06−5.23 (m, 3H), 5.27−5.40 (m, 2H), 5.44 (t, 1H, $J$ = 3.0 Hz), 5.88 (d, 1H, $J$ = 8.4 Hz), 7.39 (d, 2H, $J$ = 8.0 Hz), 8.26 (d, 2H, $J$ = 9.1 Hz); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.70, 20.72, 20.75, 20.76, 20.9, 61.3, 62.0, 65.5, 65.7, 67.8, 68.8, 69.4, 70.1, 71.4, 73.5, 90.6, 94.5, 121.7, 125.4, 145.6, 149.8, 151.6, 155.3, 168.7, 169.3, 169.5, 169.7, 169.7, 170.5, 170.6; mass spectrum (APCI), $m/z$ 802.2053 $(M + H)^+$ ($C_{33}H_{40}NO_{22}$ requires $m/z$ 802.2042).

*1,3,4,6-Tetra-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-(carbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranose (**37**).* To a solution containing 94.0 mg (0.12 mmol) of **36** in 5 mL of dichloromethane was added 2.2 mL of THF saturated with $NH_3$. The reaction mixture was stirred at room temperature for 3 h. The solvent was concentrated under diminished pressure to afford a crude residue. The residue was purified by flash chromatography on a silica gel column (15 × 2.5 cm). Elution with 3:1 ethyl acetate−hexanes afforded **37** as a colorless foam: yield 73 mg (92%); silica gel TLC $R_f$ 0.13 (3:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.98 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H); 2.11 (s, 3H), 2.13 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 3.98 (dd, 1H, $J$ = 8.4 and 3.3 Hz), 4.06−4.11 (m, 2H), 4.14−4.15 (m, 1H), 4.17−4.19 (m, 1H), 4.22−4.27 (m, 1H), 4.33−4.37 (m, 1H), 4.85 (br s, 2H), 4.95−4.97 (m, 1H), 5.00−5.02 (m, 2H), 5.08−5.14 (m, 1H), 5.22−5.27 (m, 1H), 5.44 (t, 1H, $J$ = 3.6 Hz), 5.89 (d,1H, $J$ = 8.4 Hz); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.62, 20.65, 20.68, 20.71, 20.8, 61.4, 62.1, 65.6, 65.7, 67.6, 68.9, 69.1, 69.4, 69.8, 71.3, 90.7, 95.3, 154.9, 168.7, 169.2, 169.6, 169.9, 170.4, 170.5; mass spectrum (APCI), $m/z$ 680.2026 $(M + H)^+$ ($C_{27}H_{38}O_{19}$ requires $m/z$ 680.2038).

*1,3,4,6-Tetra-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-(N-methylcarbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranose (**38**).* To a solution containing 201 mg (0.25 mmol) of nitrophenyl ester **36** in 6 mL of anh THF was added dropwise at 0 °C 125 μL (2 M solution in THF, 0.25 mmol) of $CH_3NH_2$.


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The reaction mixture was stirred at room temperature for 15 h, at which time silica gel TLC analysis indicated that the reaction was complete. The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (25 × 3 cm). Elution with 1:1 ethyl acetate−hexanes afforded disaccharide **38** as a colorless oil: yield 134 mg (77%); silica gel TLC $R_f$ 0.14 (1:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.94 (s, 3H), 1.98−2.15 (m, 18H), 2.75 (d, 3H, $J$ = 3.7 Hz), 3.93−4.13 (m, 4H), 4.18−4.22 (m, 2H), 4.30−4.33 (m, 1H), 4.87−5.10 (m, 4H), 5.17−5.21 (m, 2H), 5.33 (m, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.62, 20.63, 20.68, 20.72, 20.75, 20.77, 20.85, 27.6, 61.4, 62.0, 65.9, 67.6, 68.0, 70.5, 71.4, 90.7, 93.2, 155.38, 155.40, 155.49, 169.24, 169.27, 169.30, 170.50, 170.51, 170.6, 170.9; mass spectrum (APCI), $m/z$ 694.2169 $(M + H)^+$ ($C_{28}H_{40}NO_{19}$ requires $m/z$ 694.2195).

*3,4,6-Tri-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-(carbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranosyl Diphenyl Phosphate (**39**).* To a solution containing 66 mg (0.10 mmol) of disaccharide **37** in 1 mL of anh DMF was added 13.0 mg (0.14 mmol) of hydrazine acetate. The reaction mixture was stirred at room temperature for 3 h and quenched by the addition of 14 mL of ethyl acetate. The organic solution was washed with 12 mL of water, 12 mL of satd aq $NaHCO_3$, and 12 mL of brine and then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford the crude product as light yellow oil: yield 56 mg (90%); silica gel TLC $R_f$ 0.23 (1:4 hexanes−ethyl acetate). The residue was used immediately in the next reaction. To a stirred solution containing 56.0 mg (0.09 mmol) of the crude residue in 3.3 mL of anh dichloromethane were added 13.0 mg (0.11 mmol) of DMAP, 133 μL (96 mg, 0.95 mmol) of $Et_3N$, and 176 μL (229 mg, 0.85 mmol) of diphenylphosphoryl chloride. The reaction mixture was stirred at 0 °C for 2 h and then poured into a mixture of 5 mL of ethyl acetate and 5 mL of satd aq $NaHCO_3$. The organic layer was separated, washed with three 10 mL portions of water and brine, and then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (20 × 2 cm). Elution with 2:1 ethyl acetate−hexanes afforded phosphate ester **39** as a colorless oil: yield 36 mg (43% over two steps); silica gel TLC $R_f$ 0.18 (2:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.77 (s, 3H), 2.03 (s, 3H), 2.05 (s, 3H), 2.13 (s, 3H), 2.21 (s, 3H), 2.27 (s, 3H), 4.03−4.10 (m, 2H), 4.14−4.21 (m, 2H), 4.24−4.28 (m, 1H), 4.36−4.42 (m, 2H), 4.87 (br s, 2H), 5.05−5.10 (m, 3H), 5.24−5.27 (m, 1H), 5.29−5.35 (m, 1H), 5.51−5.53 (m, 1H), 5.75−5.79 (m, 1H), 7.22−7.28 (m, 2H), 7.32−7.43 (m, 8H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.3, 20.63, 20.64, 20.68, 20.71, 61.2, 61.8, 65.3, 65.6, 67.4, 69.0, 69.1, 69.5, 71.1, 71.2, 71.6, 95.6, 96.17, 96.22, 120.19, 120.24, 125.57, 125.70, 125.71, 129.6, 129.9, 150.05, 150.10, 154.9, 169.2, 169.6, 169.7, 170.4, 170.6; mass spectrum (APCI), $m/z$ 870.2224 $(M + H)^+$ ($C_{37}H_{45}NO_{21}P$ requires $m/z$ 870.2222).

*3,4,6-Tri-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-(N-methylcarbamoyl)-α-D-mannopyranosyl)-β-L-gulopyranosyl Diphenyl Phosphate (**40**).* To a solution containing 108 mg (0.16 mmol) of disaccharide **38** in 1.2 mL of anh DMF was added 17.0 mg (0.19 mmol) of hydrazine acetate. The reaction mixture was stirred at room temperature for 1.5 h and quenched by the addition of 20 mL of ethyl acetate. The organic solution was washed with three 10 mL portions of brine and dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was used for the next reaction.

To a stirred solution containing 90.0 mg (0.14 mmol) of the crude residue in 8.2 mL of anh dichloromethane were added 21.0 mg (0.17 mmol) of DMAP, 210 μL (152 mg, 1.49 mmol) of $Et_3N$, and 270 μL (351 mg, 1.32 mmol) of diphenylphosphoryl chloride. The reaction mixture was stirred at 0 °C for 2 h and then poured into a mixture of 40 mL of ethyl acetate and 20 mL of satd aq $NaHCO_3$. The organic layer was washed with three 10 mL portions of water and brine and then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (25 × 3 cm). Elution with 2:1 ethyl acetate−hexanes afforded phosphate ester **40** as a colorless oil: yield 82 mg (56% over two steps); silica gel TLC $R_f$ 0.18 (2:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.67 (s, 3H), 1.94 (d, 6H, $J$ = 7.4 Hz), 2.01 (s, 3H), 2.11 (s, 3H), 2.16 (s, 3H), 2.76 (s, 3H), 3.89−4.39 (m, 7H), 4.75−5.05(m, 4H), 5.10−5.30 (m, 2H), 5.44 (s, 1H), 5.68 (s, 1H), 7.11−7.39 (m, 10H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.4, 20.70, 20.76, 20.8, 27.7, 61.2, 62.0, 65.5, 65.8, 67.5, 69.1, 69.3, 69.4, 71.7, 95.9, 96.3, 120.3, 125.6, 125.8, 129.7, 130.0, 155.4, 169.3, 169.7, 169.8, 170.4, 170.67, 170.68; mass spectrum (APCI), $m/z$ 884.2371 $(M + H)^+$ ($C_{38}H_{47}NO_{19}$ requires $m/z$ 884.2378).

*2-[2-(Benzyloxycarbonylamino)ethyloxy]ethyl 3,4,6-Tri-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-(carbamoyl)-α-D-mannopyranosyl)-α,β-L-gulopyranose (**42**).* To a stirred solution containing 31.0 mg (0.04 mmol) of phosphate ester **39** in 3.9 mL of anh dichloromethane was added a solution of 9.40 mg (0.04 mmol) of CBz linker **41** in 4.5 mL of anh dichloromethane at 0 °C. To the cooled reaction mixture was added 41.0 μL (51.0 mg, 0.23 mmol) of TMSOTf, and the reaction mixture was stirred at 0 °C for 15 min, at which time it was poured into a mixture of 20 mL of ethyl acetate and 20 mL of satd aq $NaHCO_3$. The aqueous and organic layers were separated, and the organic layer was washed successively with three 10 mL portions of water and brine and then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (12 × 2 cm). Elution with 3:1 ethyl acetate−hexanes afforded disaccharide−linker conjugate **42** as a colorless oil: yield 12 mg (39%); silica gel TLC $R_f$ 0.12 (3:1 ethyl acetate−hexanes); $^1$H NMR ($CDCl_3$) $\delta$ 1.99 (s, 3H), 2.02 (s, 3H), 2.03 (s, 3H), 2.07 (s, 3H), 2.09 (s, 3H), 2.12 (s, 3H), 3.37−3.42 (m, 2H), 3.55−3.65 (m, 3H), 3.67−3.69 (m, 2H), 3.83−3.88 (m, 1H), 3.97 (t, 1H, $J$ = 3.9 Hz), 4.09−4.15 (m, 4H), 4.28 (dd, 1H, $J$ = 11.9 and 5.2 Hz), 4.46 (t, 1H, $J$ = 6.6 Hz), 4.77−4.90 (br s, 2H), 4.93 (d, 1H, $J$ = 3.9 Hz), 5.03−5.06 (m, 4H), 5.23−5.29 (m, 3H), 5.46−5.48 (m, 1H), 7.30−7.35 (m, 5H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.62, 20.65, 20.72, 20.76, 40.9, 62.1, 62.5, 63.8, 65.8, 66.1, 66.6, 67.6, 68.6, 68.7, 69.0, 70.07, 70.16, 70.3, 71.0, 77.2, 97.0, 97.6, 128.10, 128.18, 128.5, 136.5, 155.0, 169.3, 169.72, 169.73, 170.0, 170.56, 170.59; mass spectrum (APCI), $m/z$ 859.2987 $(M + H)^+$ ($C_{37}H_{51}N_2O_{21}$ requires $m/z$ 859.2984).

*2-[2-(Benzyloxycarbonylamino)ethyloxy]ethyl 3,4,6-Tri-O-acetyl-2-O-(3,4,6-tri-O-acetyl-2-O-(N-methylcarbamoyl)-α-D-mannopyranosyl)-α,β-L-gulopyranose (**43**).* To a stirred solution containing 90.0 mg (0.10 mmol) of phosphate ester **40** in 1.1 mL of anh dichloromethane was added a solution of 22.0 mg (0.09 mmol) of CBz linker **41** in 1.1 mL of anh dichloromethane at 0 °C. To the cooled reaction mixture was added 33.0 μL (41.0 mg, 0.18 mmol) of TMSOTf, and the reaction mixture was stirred at 0 °C for 15 min, at which time it was poured into a mixture of 20 mL of ethyl acetate and 20 mL

of satd aq $NaHCO_3$. The aqueous and organic layers were separated, and the organic layer was washed with three 10 mL portions of water and brine and then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (25 × 3 cm). Elution with 12:12:1 ethyl acetate−hexanes−methanol afforded disaccharide−linker conjugate **43** as a colorless oil: yield 56 mg (63%); silica gel TLC $R_f$ 0.20 (12:12:1 ethyl acetate−hexanes−methanol); $^1$H NMR ($CDCl_3$) $\delta$ 1.96 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.05−2.08 (m, 6H), 2.10 (s, 3H), 2.78 (d, 3H, $J$ = 4.6 Hz), 3.38 (d, 2H, $J$ = 4.4 Hz), 3.51−3.70 (m, 4H), 3.78−3.87 (m, 1H), 3.95 (d, 1H, $J$ = 3.50 Hz), 4.00−4.15 (m, 4H), 4.20−4.30 (m, 2H), 4.45 (t, 1H, $J$ = 6.1 Hz), 4.89−5.12 (m, 6H), 5.20−5.30 (m, 3H), 5.42−5.49 (m, 1H), 5.46 (s, 1H), 7.27−7.38 (m, 5H); $^{13}$C NMR ($CDCl_3$) $\delta$ 20.71, 20.73, 20.77, 20.80, 20.84, 20.88, 27.7, 62.3, 62.7, 63.9, 66.0, 66.3, 66.7, 68.7, 68.9, 69.2, 70.1, 70.2, 70.4, 97.2, 97.9, 128.21, 128.23, 128.28, 128.59, 128.61, 136.7, 155.5, 169.4, 169.80, 169.84, 170.0, 170.66, 170.69; mass spectrum (APCI), $m/z$ 873.3166 $(M + H)^+$ ($C_{38}H_{53}N_2O_{21}$ requires $m/z$ 873.3141).

*Disaccharide−Dye Conjugate* ***3***. To a solution of 2.20 mg (2.60 mmol) of compound **42** in 1 mL of anh methanol was added a freshly prepared solution of 500 $\mu$L of 0.4 M sodium methoxide in methanol. The reaction mixture was stirred at room temperature for 3 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, and filtered. To the solution of the crude product in methanol was added a catalytic amount of Pd/C, and $H_2$ gas was bubbled through for 1 h. The complete consumption of starting material was confirmed by MALDI-TOF mass spectral analysis. The reaction mixture was filtered through Celite, and the filtrate was concentrated under diminished pressure to afford **44**, which was used directly in the next reaction; mass spectrum (APCI), $m/z$ 473.1986 $(M + H)^+$ ($C_{17}H_{33}N_2O_{13}$ requires $m/z$ 473.1983).

To 101 $\mu$g (0.21 $\mu$mol) of **44** was added a solution of 106 $\mu$g (0.11 $\mu$mol) of Cy5**COOSu (**46**) in 100 $\mu$L of 0.2 M phosphate buffer, pH 8.0, and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Alltech Alltima $C_{18}$ reversed-phase semipreparative (250 × 10 mm, 5 $\mu$m) HPLC column using aq 0.1% $CF_3COOH$ (TFA) and $CH_3CN$ mobile phases. A linear gradient was employed (99:1 0.1% aq TFA−$CH_3CN$ → 69:31 0.1% aq TFA−$CH_3CN$) over a period of 35 min at a flow rate of 4 mL/min. The fractions containing the desired product eluted at 23.5 min and were collected, frozen, and lyophilized to give **3** as a blue solid: yield 48 $\mu$g (35% over two steps); mass spectrum (APCI), $m/z$ 669.1883 $(M - K - 2H)^{2-}$ ($C_{55}H_{78}N_4O_{26}S_4^{2-}$ requires $m/z$ 669.1899).

*Disaccharide−Dye Conjugate* ***4***. To a solution of 4.40 mg (5.00 mmol) of compound **43** in 2 mL of anh methanol was added a freshly prepared solution of 1.0 mL of 0.4 M sodium methoxide in methanol. The reaction mixture was stirred at room temperature for 3 h, and the complete consumption of starting material was confirmed by MALDI-TOF mass spectrometric analysis. The reaction mixture was then quenched by the addition of 500 mg of Dowex 50x resin, shaken for 15 min, and filtered. To the solution of the crude product in methanol was then added a catalytic amount of Pd/C, and $H_2$ gas was bubbled through for 1 h. The complete consumption of starting material was confirmed by MALDI-TOF mass spectrometric analysis. The reaction mixture was filtered through Celite, and the filtrate was concentrated under diminished pressure to afford **45**, which was used directly for the next reaction; mass spectrum (APCI), $m/z$ 487.2140 $(M + H)^+$ ($C_{18}H_{35}N_2O_{13}$ requires $m/z$ 487.2139).

To 101 $\mu$g (0.21 $\mu$mol) of **45** was added a solution of 106 $\mu$g (0.11 $\mu$mol) of Cy5**COOSu (**46**) in 100 $\mu$L of 0.2 M phosphate buffer, pH 8.0, and the reaction mixture was stirred overnight in the dark. The reaction mixture was purified on an Alltech Alltima $C_{18}$ reversed-phase semipreparative (250 × 10 mm, 5 $\mu$m) HPLC column using aq 0.1% TFA and $CH_3CN$ mobile phases. A linear gradient was employed (99:1 0.1% aq TFA−$CH_3CN$ → 69:31 0.1% aq TFA−$CH_3CN$) over a period of 35 min at a flow rate of 4 mL/min. The fractions containing the desired product eluted at 23.5 min and were collected, frozen, and lyophilized to give **4** as a blue solid: yield 53 $\mu$g (37% over two steps); mass spectrum (APCI), $m/z$ 676.1996 $(M - K - 2H)^{2-}$ ($C_{56}H_{80}N_4O_{26}S_4^{2-}$ requires $m/z$ 676.1977).

## RESULTS

**Synthesis of Disaccharide−Dye Conjugates.** The preparation of the different disaccharide−dye conjugates began with the synthesis of the corresponding disaccharides with attached linkers. The general synthetic strategy adopted required the syntheses of C-2, C-3, and C-4 *O*-benzylated mannose and C-3 *O*-benzylated altrose pyranosides (Schemes 1 and 2). The benzylated mannose and altrose pyranosides **14**, **19**, **27**, and **33** were coupled to gulose monosaccharide **34**[24] to yield the corresponding *O*-benzylated disaccharides **35**, **47**, **57**, and **63**, respectively. The latter were then debenzylated and activated as the nitrophenyl carbonates to enable the incorporation of a carbamoyl or *N*-methylcarbamoyl moiety (Schemes 3, S2, S7, and S8). Disaccharide **53** was synthesized by a different strategy involving the coupling of gulose monosaccharide **34**[24] and mannose pyranoside **25** (Scheme S6).

The syntheses of the C-2, C-3, C-4 modified mannose and altrose disaccharide−dye conjugates **3**, **4**, **5**, **7**, **8**, and **9** (Schemes 2, 3, S1−S5, and S7−S8) began with the coupling of gulose glycosyl acceptor **34** and corresponding mannose **14**, **19**, and **27** or altrose **33** glycosyl donor to yield C-2, C-3, and C-4 *O*-benzylated disaccharides **35**, **47**, **57**, and **63** in 40−73% yields.[24] The disaccharides were then debenzylated by hydrogenolysis over palladium hydroxide-on-carbon and converted to the corresponding *p*-nitrophenyl carbonates **36**, **48**, **58**, and **64** in 71−96% yields over two steps.[28] The *p*-nitrophenyl carbonate was then subjected to aminolysis with ammonia and methylamine to yield **37**, **38**, **49**, **58**, **65**, and **66** in 42−92% yields. Each of the disaccharides was converted to a glycosyl donor by a selective hydrazine acetate-mediated deacetylation of the anomeric acetate followed by activation with diphenyl chlorophosphate in the presence of DMAP and $Et_3N$ to yield the $\alpha$-glycosyl diphenyl phosphates **39**, **40**, **50**, **60**, **67**, and **68** in 17−76% yields over two steps.[29] The glycosyl donors were then coupled with CBz-protected linker **41**[26] to yield linker coupled disaccharides **42**, **43**, **51**, **61**, **69**, and **70** in 39−73% yields. The disaccharides were subjected to a one-pot deacetylation and hydrogenolysis procedures to afford the fully deprotected linker disaccharide conjugates, which were coupled to the dye Cy5**COOSu (**46**)[21,22,25] to provide the dye−disaccharide conjugates **3**−**5** and **7**−**9** in 23−48% yields over two steps.


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The synthesis of the C-4 modified mannose disaccharide−dye conjugate **6** is outlined in Scheme S6. It began with the coupling of gulose glycosyl acceptor **34** and mannose glycosyl donor **25** to yield the C-4 carbamoyl disaccharide **53** in 51% yield. Compound **53** was then subjected to selective hydrazine acetate-mediated deacetylation of the anomeric acetate followed by activation with diphenyl chlorophosphate in the presence of DMAP and $Et_3N$ to yield the $\alpha$-glycosyl diphenyl phosphate **54** in 52% yield over two steps.[24] Glycosyl donor **54** was then coupled with CBz-protected linker **41** to yield linker coupled disaccharide **55** in 26% yield. The disaccharide **55** was subjected to a one-pot deacetylation and hydrogenolysis to afford the fully deprotected linker disaccharide conjugate **56**, which was then coupled to Cy5**COOSu (**46**) to afford the dye−disaccharide conjugate **6** in 32% yield.

**Cell Binding/Uptake of Disaccharide−Dye Conjugates.** The cell binding/uptake of BLM disaccharide−Cy5** conjugate (**1**), decarbamoyl BLM disaccharide−Cy5** conjugate (**2**), and the newly synthesized disaccharide−Cy5** conjugates **3**−**9** by BxPC-3 pancreatic carcinoma cells and SVR A221a normal pancreatic cells (Figures 3 and S1), by A549 lung carcinoma cells and WI-38 normal lung cells (Figures 4 and S2), by DU-145 prostate carcinoma cells and PZ-HPV-7 normal prostate cells (Figures 5 and S3), and by A498 kidney carcinoma cells and CCD-1105 KIDTr normal kidney cells (Figures 6 and S4) was quantified by fluorescence imaging. As shown (Figures 3−6), in all tumor and normal cell lines tested, the decarbamoyl BLM disaccharide exhibited very low binding and uptake, highlighting the importance of the carbamoyl moiety to effective cell binding and uptake. This is in agreement with earlier studies reported by our laboratory.[15,21,22]

The binding/uptake of the disaccharide−dye conjugates in human kidney and lung cells was lower compared with that in human prostate and pancreatic cancer cells; however, the overall profiles were not dissimilar. Disaccharide conjugates having an *N*-methylated carbamoyl group (**4**, **5**, and **9**) generally exhibited greater binding/uptake as compared to that of the disaccharides with unmodified carbamoyl groups (**3**, **6**, and **8**). Disaccharide conjugates having a carbamoyl or *N*-methylcarbamoyl group at the C-4 position of the D-mannose subunit (**6** and **7**) exhibited relatively poor binding/uptake in all of the tumor cell lines tested, underscoring the importance of the position and orientation of the carbamoyl group in the BLM disaccharide. This was especially true for the BxPC-3 and A498 cancer cell lines (Figures 3 and 6). The binding/uptake was higher in all tested cancer cell lines for disaccharide conjugates **4** and **9** containing the *N*-methylcarbamoyl groups at the C-2 and C-3 positions of the D-mannose and D-altrose subunits, respectively. All of the disaccharide−dye conjugates exhibited negligible binding/uptake in normal cells, highlighting the role of the disaccharide and carbamoyl moiety in the cancer cell specificity of BLM.

One interesting facet of the cellular interaction of the BLM disaccharide−Cy5** conjugate (**1**) with cancer cells was found to be its temperature-dependent binding/uptake by cultured MCF-7 cells.[21] In order to test the generality of that observation, conjugates **1**, **4**, and **5** were studied at 4 and 37 °C for binding/uptake by A549 and DU-145 cancer cell lines. As shown in Figure 7, all three compounds exhibited significant cell uptake only at 37 °C.

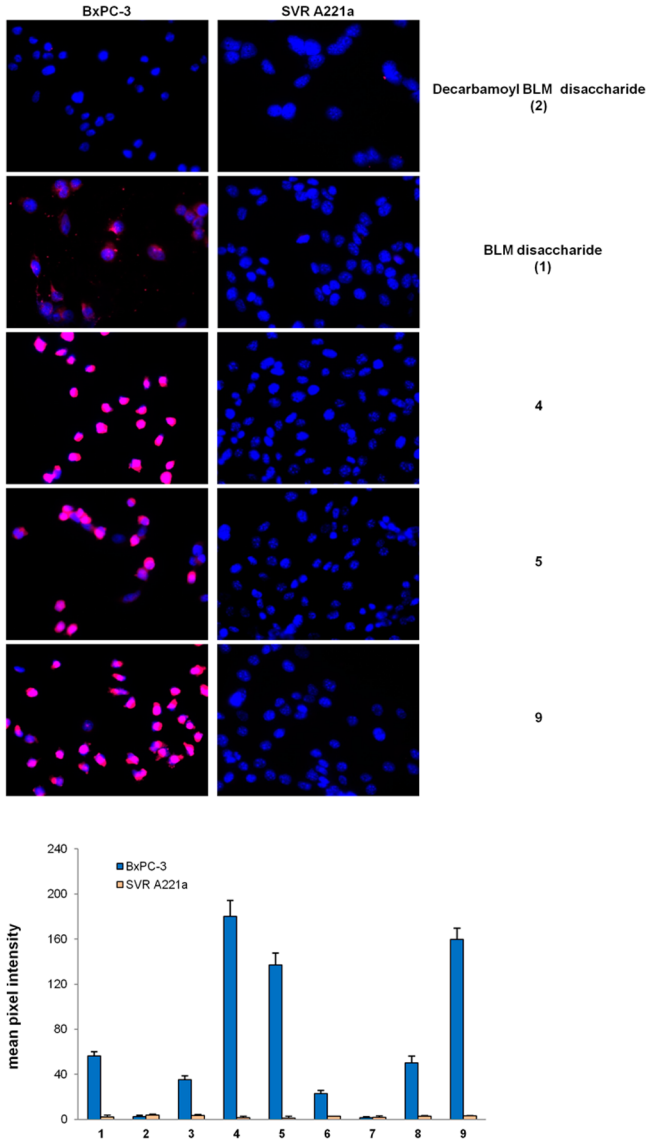


**Figure 3.** (Top) Comparison of binding/uptake of disaccharide−Cy5** conjugates **1**, **2**, **4**, **5**, and **9** in BxPC-3 and SVR A221a cell lines. The cells were treated with 25 $\mu$M disaccharide−Cy5** conjugates at 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. The cell nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI). Fluorescence imaging was carried out with a 2 s exposure time. (Bottom) Quantification of the binding/uptake of disaccharide−Cy5** conjugates **1**−**9** in BxPC-3 and SVR A221a cell lines. The cells were treated with 25 $\mu$M dye conjugates, irradiated for 2 s prior to imaging, and then analyzed using a Zeiss Axiovert 200M inverted microscope with a 40× oil objective.

## ■ DISCUSSION

The natural disaccharide moiety in BLM composed of L-gulose and D-mannose subunits has been found to play an important role in the tumor selectivity and specificity of BLM. Recent reports have highlighted the importance of the carbamoyl group present in the BLM disaccharide for selective tumor cell binding and uptake.[21,22] The present study was undertaken to explore the effects of placement of the carbamoyl moiety in alternative locations within the disaccharide moiety as well as the effect of *N*-methylation of the carbamoyl moiety. Seven new disaccharide−dye conjugates were synthesized for this purpose, as outlined in Schemes 3 and S5−S8.


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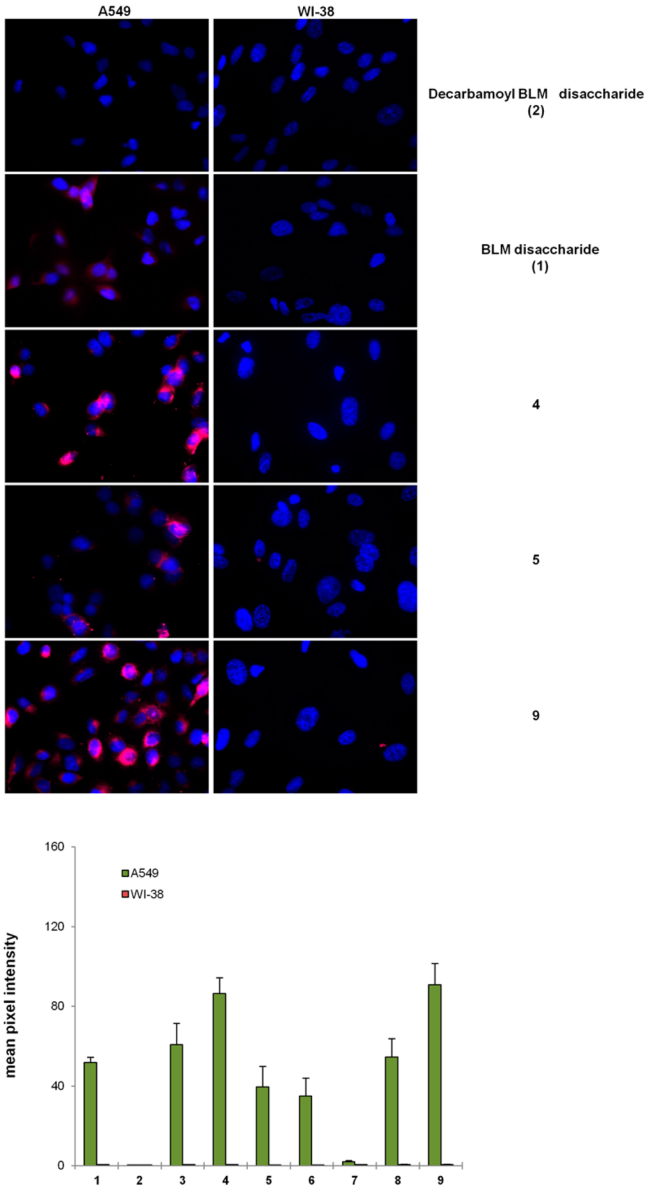


**Figure 4.** (Top) Comparison of binding/uptake of disaccharide–Cy5** conjugates **1**, **2**, **4**, **5**, and **9** in A549 and WI-38 cell lines. The cells were treated with 25 $\mu$M disaccharide–Cy5** conjugates at 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. The cell nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI). Fluorescence imaging was carried out with a 2 s exposure time. (Bottom) Quantification of the binding/uptake of disaccharide–Cy5** conjugates **1**–**9** in A549 and WI-38 cell lines. The cells were treated with 25 $\mu$M dye conjugates, irradiated for 2 s prior to imaging, and then analyzed using a Zeiss Axiovert 200M inverted microscope with a 40× oil objective.

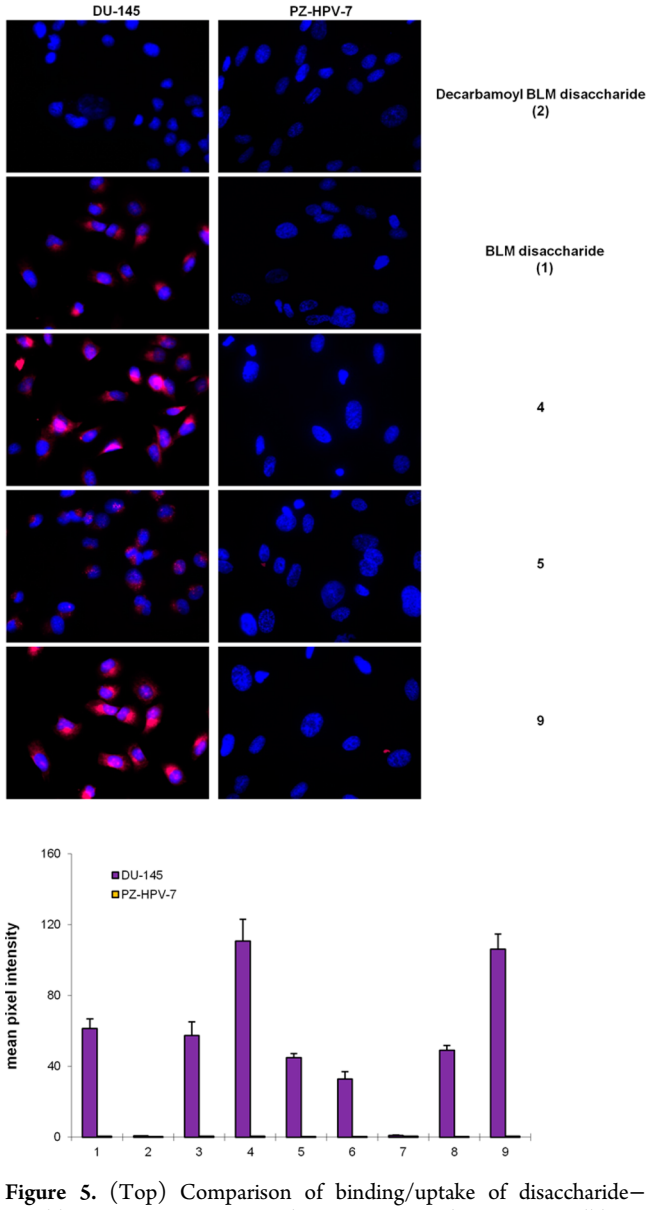


**Figure 5.** (Top) Comparison of binding/uptake of disaccharide–Cy5** conjugates **1**, **2**, **4**, **5**, and **9** in DU-145 and PZ-HPV-7 cell lines. The cells were treated with 25 $\mu$M disaccharide–Cy5** conjugates at 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. The cell nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI). Fluorescence imaging was carried out with a 2 s exposure time. (Bottom) Quantification of the binding/uptake of disaccharide–Cy5** library conjugates **1**–**9** in DU-145 and PZ-HPV-7 cell lines. The cells were treated with 25 $\mu$M dye conjugates, irradiated for 2 s prior to imaging, and then analyzed using a Zeiss Axiovert 200M inverted microscope with a 40× oil objective.

As is also the case for the natural BLM disaccharide, all of the newly synthesized disaccharides had an L-gulose monosaccharide subunit. They differed in the position of the carbamoyl group in the D-mannose moiety. Unlike the natural disaccharide, which has a carbamoyl group at the C-3 position of the mannose monosaccharide, the synthesized disaccharides had (i) a carbamoyl group at the C-2 or C-4 position of the D-mannose moiety (disaccharide–dye conjugates **3** and **6**), (ii) an *N*-methylcarbamoyl group at the C-2, C-3, or C-4 position of the D-mannose moiety (disaccharide–dye conjugates **4**, **5**, and **7**), or (iii) a carbamoyl or *N*-methylcarbamoyl group at the C-3 position of the D-altrose moiety, a C-3 epimer of D-mannose (disaccharide–dye conjugates **8** and **9**).

Initial studies monitoring cell binding and uptake clearly showed that the BLM disaccharide containing its carbamoyl moiety was essential for the selective binding and uptake of BLM in tumor cells.[15,21,22] The dye Cy5** was chosen as the fluorescent probe to circumvent initially encountered problems of autofluorescence and nonspecific cell surface binding. Cys5** is a cyanine dye exhibiting emission wavelengths in the red or near-infrared region. The binding/uptake of the dye itself was found to be extremely low in cancer as well as normal cells. The broad tumor cell specificity of the synthesized


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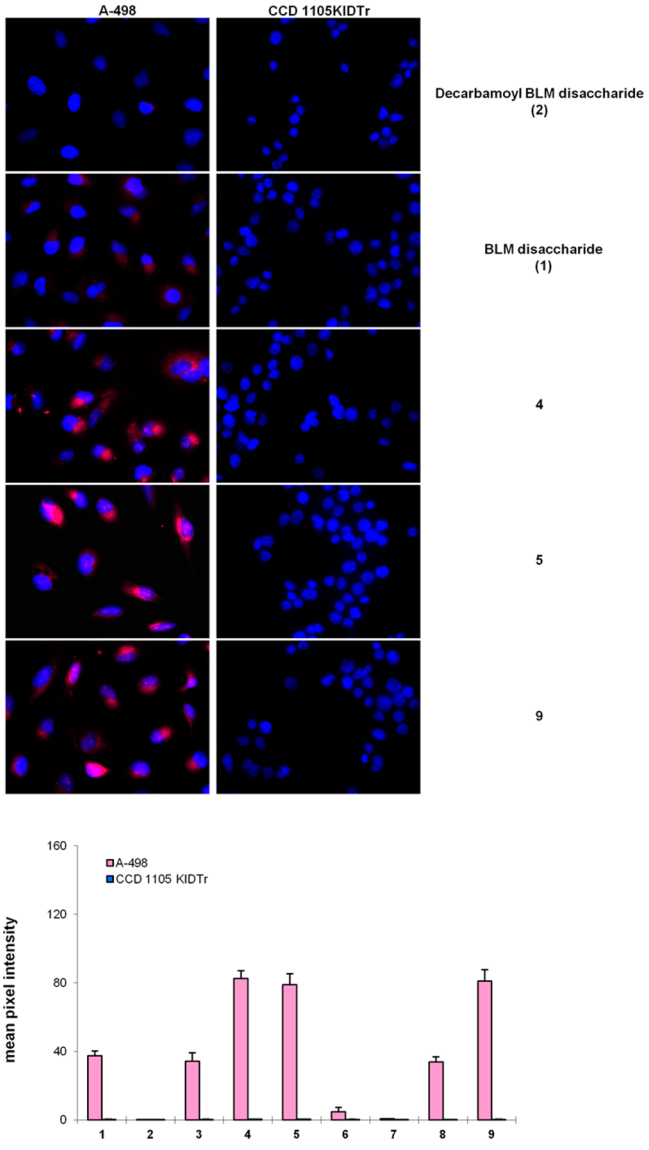


**Figure 6.** (Top) Comparison of binding/uptake of disaccharide–Cy5** conjugates **1**, **2**, **4**, **5**, and **9** in A498 and CCD-1105 KIDTr cell lines. The cells were treated with 25 $\mu$M disaccharide–Cy5** library conjugates at 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. The cell nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI). Fluorescence imaging was carried out with a 2 s exposure time. (Bottom) Quantification of the binding/uptake of disaccharide–Cy5** library conjugates **1**–**9** in A498 and CCD-1105 KIDTr cell lines. The cells were treated with 25 $\mu$M dye conjugates, irradiated for 2 s prior to imaging, and then analyzed using a Zeiss Axiovert 200M inverted microscope with a 40× oil objective.

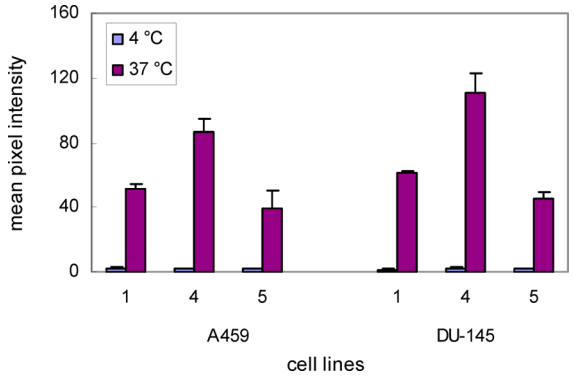


**Figure 7.** Effect of incubation temperature on the internalization of disaccharide–Cy5** conjugates **1**, **4**, and **5** in A549 and DU-145 cancer cell lines. The cells were treated with 25 $\mu$M disaccharide–Cy5** at 4 or 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. Fluorescence imaging was carried out with a 2 s exposure time.

disaccharide–dye conjugates was validated by the similarity in their uptake profiles in spite of the variation in their uptake efficiency for the different cancer and matched normal cell lines studied (Figures 3–6). The decarbamoylated BLM disaccharide conjugate (**2**) exhibited low uptake in all of the cell lines tested, reiterating the importance of the carbamoyl moiety. With the exception of disaccharide conjugate **7**, the disaccharide conjugates having an *N*-methylcarbamoyl group (**4** and **9**) exhibited greater uptake as compared to that of the respective disaccharide conjugates having an unmodified carbamoyl group (**3** and **8**). The disaccharide conjugate related to BLM disaccharide **1** but having an *N*-methylated carbamoyl group (**5**) gave mixed results (Figures 3–6). The role of the disaccharide in selective targeting of cancer cells by BLM was further substantiated by the inability of the synthesized disaccharide–dye conjugates to bind to any of the normal human cells tested. Disaccharides **4** and **9**, having a modified carbamoyl group at the C-2 and C-3 positions of the D-mannose and D-altrose subunits, respectively, exhibited the best binding and uptake profiles in all four cancer cell lines studied.

In common with the results of earlier studies,[21,22] we observed little dye associated with the cell surface of any of the tumor cell lines studied. The lack of cell surface fluorescence is consistent with the interpretation that internalization of the disaccharide–dye conjugate is rapid relative to cell surface binding. This interpretation is supported by the observation that microbubbles whose surface had been modified by the covalent attachment of multiple BLM or BLM disaccharide molecules bound selectively to cultured cancer cells but could not be internalized.[21]

Earlier studies carried by our group with trivalent clusters of BLM monosaccharide indicated that these trivalent saccharide–dye conjugates were bound and internalized by six cancer cell lines (A498, A549, BxPC-3, DU-145, BT-474, and MCF-7 cell lines) more efficiently than that for the simple BLM monosaccharide–dye conjugate.[22] The present study provides another strategy for enhancing the efficiency of targeting and internalization of Cys5** selectively in cancer cells by the use of simple sugars. It seems likely that multivalent clusters of the more promising analogues identified in this study would provide still more efficient delivery of attached cargoes. Clearly, the results obtained with the seven analogues included in the present study also suggest that additional carbamoylated sugars may demonstrate further improvements in the efficiency of tumor cell targeting/uptake.

In addition to their promise as vehicles for the delivery of molecular cargoes, such as cytotoxic agents, selectively to tumor cells the saccharides characterized in the present study may find utility in the characterization of the cell surface receptors responsible for tumor cell binding and uptake of BLM saccharides. Studies aimed at understanding the mechanism of cellular uptake are in progress.

From the perspective of drug discovery, the current findings are of interest for two types of applications. First, it seems likely


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that bleomycin analogues containing disaccharides such as **4** or **9** in lieu of the normal BLM disaccharide may exhibit antitumor properties superior to those of BLM itself. Second, the delivery of cytotoxic agents selectively to tumors using antibodies is currently showing great promise as a strategy for the development of new therapeutic regimens.[30−32] It seems possible that optimized BLM saccharides might be able to serve the role currently assigned to antibodies in antibody−drug conjugates. In fact, the BLM saccharide displays the same tumor targeting properties as those of BLM itself,[21,22] and we have recently shown that the BLM saccharide is responsible for tumor targeting/uptake by BLM.[33]

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Procedures for the synthesis and characterization of the BLM disaccharide−dye conjugates and comparison of binding/uptake of disaccharide−Cy5** conjugates **1**, **3**, **6**, **7**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*E-mail: sid.hecht@asu.edu.. Phone: (480) 965-6625. Fax: (480) 965-0038.

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### Notes

The authors declare the following competing financial interest(s): Sidney Hecht is a consultant to Isis Pharmaceuticals.

## ■ ABBREVIATIONS

APCI, atmospheric pressure chemical ionization; BLM, bleomycin; BLEDTA, bleomycin−EDTA conjugate; CBz, carboxybenzyl; Cy5**, cyanine 5**; DAPI, 4′,6-diamino-2-phenylindole; DMAP, *N*,*N*-dimethylaminopyridine; DMF, dimethylformamide; FAB, fast atom bombardment; h, hour; Hz, Hertz; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization; PBS, phosphate buffered saline; $R_f$, ratio of fronts; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMSOTf, trimethylsilyl triflate

## ■ REFERENCES

(1) Umezawa, H., Suhara, Y., Takita, T., and Maeda, K. (1966) Purification of bleomycins. *J. Antibiot. 19*, 210−215.

(2) Levi, J. A., Raghavan, D., Harvey, V., Thompson, D., Sandeman, T., Gill, G., Stuart-Harris, R., Snyder, R., Byrne, M., and Kerestes, Z. (1993) The importance of bleomycin in combination chemotherapy for good-prognosis germ cell carcinoma. Australasian germ cell trial group. *J. Clin. Oncol. 11*, 1300−1305.

(3) Hecht, S. M. (1999) Bleomycin: new perspectives on the mechanism of action. *J. Nat. Prod. 63*, 158−168.

(4) Hecht, S. M. (2012) Bleomycin group antitumor agents, in *Anticancer Agents from Natural Products* (Cragg, G. M., Kingston, D. G. I., and Newman, D. J., Eds.) 2nd ed., pp 451−478, CRC Press, Boca Raton, FL.

(5) Povirk, L. F., Han, Y. H., and Steighner, R. J. (1989) Structure of bleomycin-induced DNA double-strand breaks: predominance of blunt ends and single-base 5′ extensions. *Biochemistry 28*, 5808−5814.

(6) Roy, B., and Hecht, S. M. (2014) Hairpin DNA sequences bound strongly by bleomycin exhibit enhanced double-strand cleavage. *J. Am. Chem. Soc. 136*, 4382−4393.

(7) Silberstein, E. B., Kornblut, A., Shumrick, D. A., and Saenger, E. L. (1974) $^{67}$Ga as a diagnostic agent for the detection of head and neck tumors and lymphoma. *Radiology 110*, 605−608.

(8) Jones, S. E., Lilien, D. L., O'Mara, R. E., Durie, B. G. M., and Salmon, S. E. (1975) Indium-111 bleomycin tumor scanning in lymphoma. *Med. Pediatr. Oncol. 1*, 11−21.

(9) Silverstein, M. J., Verma, R. C., Greenfield, L., and Morton, D. L. (1976) $^{111}$Indium-bleomycin breast and axilla imaging. *Cancer 37*, 36−42.

(10) Rasker, J. J., Beekhuis, H., van de Wal, A. M., Homan van der Heide, J. N., and Woldring, M. G. (1976) Cobalt-57-bleomycin scanning of hila and mediastinum in patients with bronchial carcinoma: a prospective study. *Thorax 31*, 641−649.

(11) Burton, I. E., Todd, J. H., and Turner, R. L. (1977) Static and dynamic imaging with indium-111 labelled bleomycin in the localization of squamous cell neoplasia. *Br. J. Radiol. 50*, 508−512.

(12) Bekerman, C., Moran, E. M., Hoffer, P. B., Hendrix, R. W., and Gottschalk, A. (1977) Scintigraphic evaluation of lymphoma: a comparative study of $^{67}$Ga-citrate and $^{111}$In-bleomycin. *Radiology 123*, 687−694.

(13) DeRiemer, L. H., Meares, C. F., Goodwin, D. A., and Diamanti, C. I. (1979) BLEDTA: tumor localization by a bleomycin analog containing a metal-chelating group. *J. Med. Chem. 22*, 1019−1023.

(14) Goodwin, D. A., Meares, C. F., DeRiemer, L. H., Diamanti, C. I., Goode, R. L., Baumert, J. E., Sartoris, D. J., Lantieri, R. L., and Fawcett, H. D. (1981) Clinical studies with In-111 BLEDTA, a tumor-imaging conjugate of bleomycin with a bifunctional chelating agent. *J. Nucl. Med. 22*, 787−792.

(15) Chapuis, J.-C., Schmaltz, R. M., Tsosie, K. S., Belohlavek, M., and Hecht, S. M. (2009) Carbohydrate dependent targeting of cancer cells by bleomycin−microbubble conjugates. *J. Am. Chem. Soc. 131*, 2438−2439.

(16) Kansas, G. S. (1996) Selectins and their ligands: current concepts and controversies. *Blood 88*, 3259−3287.

(17) Tiemeyer, M., and Goodman, C. S. (1996) Gilolectin is a novel carbohydrate-binding protein expressed by a subset of gila in the embryonic *Drosphila* nervous system. *Development 122*, 925−936.

(18) Sacchettini, J. C., Baum, L. G., and Brewer, C. F. (2001) Multivalent protein−carbohydrate interactions. A new paradigm for supramolecular assembly and signal transduction. *Biochemistry 40*, 3009−3015.

(19) Geijtenbeek, T. B. H., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C. F., Adema, G. J., van Kooyk, Y., and Figdor, C. G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell 100*, 575−585.

(20) Lis, H., and Sharon, N. (1998) Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chem. Rev. 98*, 637−674.

(21) Yu, Z., Schmaltz, R. M., Bozeman, T. C., Paul, R., Rishel, M. J., Tsosie, K. S., and Hecht, S. M. (2013) Selective tumor cell targeting by the disaccharide moiety of bleomycin. *J. Am. Chem. Soc. 135*, 2883−2886.

(22) Bhattacharya, C., Yu, Z., Rishel, M. J., and Hecht, S. M. (2014) The carbamoylmannose moiety of bleomycin mediates selective tumor cell targeting. *Biochemistry 53*, 3264−3266.

(23) Ogawa, T., and Sasajima, K. (1981) Synthetic studies on cell surface glycans. Part 4. Reconstruction of glycan chains of glycoprotein: branching mannopentaoside and mannohexaoside. *Tetrahedron 37*, 2787−2792.

(24) Dondoni, A., Marra, A., and Massi, A. (1997) Carbohydrate homologation by the use of 2-(trimethylsilyl)thiazole. Preparative scale synthesis of rare sugars: L-gulose, L-idose, and the disaccharide subunit of bleomycin $A_2$. *J. Org. Chem. 62*, 6261−6267.

(25) West, R. M., Bosworth, N., and Mujumdar, R. B. (2005) Cyanine dye labelling reagents. PCT Int. Appl. WO2005044923 A1 20050519.

(26) Aspland, S. E., Ballatore, C., Castillo, R., Desharnais, J., Eustaquio, T., Goelet, P., Guo, Z., Li, Q., Nelson, D., Sun, C., Castellino, A. J., and Newman, M. J. (2006) Kinase-mediated trapping of bi-functional conjugates of paclitaxel or vinblastine with thymidine in cancer cells. *Bioorg. Med. Chem. Lett. 16*, 5194−5198.
(27) Cox, D. J., Smith, M. D., and Fairbanks, A. J. (2010) Glycosylation catalyzed by a chiral Brønsted acid. *Org. Lett. 12*, 1452−1455.
(28) Boger, D. L., and Honda, T. (1994) Total synthesis of bleomycin $A_2$ and related agents. 4. Synthesis of the disaccharide subunit 2-*O*-(3-*O*-carbamoyl-*α*-D-mannopyranosyl)-L-gulopyranose and completion of the total synthesis of bleomycin $A_2$. *J. Am. Chem. Soc. 116*, 5647−5656.
(29) Miyashita, H., Kai, Y., Nohara, T., and Ikeda, T. (2008) Efficient synthesis of *α*- and *β*-chacotriosyl glycosides using appropriate donors, and their cytotoxic activity. *Carbohydr. Res. 343*, 1309−1315.
(30) Carter, P. J., and Senter, P. D. (2008) Antibody−drug conjugates for cancer therapy. *Cancer J. 14*, 154−169.
(31) Ducry, L., and Stump, B. (2010) Antibody−drug conjugates: linking cytotoxic payloads to monoclonal antibodies. *Bioconjugate Chem. 21*, 5−13.
(32) Krop, I. E., Beeram, M., Modi, S., Jones, S. F., Holden, S. N., Yu, W., Girish, S., Tibbitts, J., Yi, J.-H., Sliwkowski, M. X., Jacobson, F., Lutzker, S. G., and Burris, H. A. (2010) Phase I study of trastuzumab-DM1, an HER2 antibody−drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J. Clin. Oncol. 28*, 2698−2704.
(33) Schroeder, B. R., Ghare, M. I., Bhattacharya, C., Paul, R., Yu, Z., Zaleski, P. A., Bozeman, T. C., Rishel, M. J., and Hecht, S. M. (2014) The disaccharide moiety of bleomycin facilitates uptake by cancer cells. *J. Am. Chem. Soc. 136*, 13641−13656.