Design and Synthesis of Substrate and Internal Standard Conjugates for Profiling Enzyme Activity in the Sanfilippo Syndrome by Affinity Chromatography/Electrospray Ionization Mass Spectrometry

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We describe the design and synthesis of substrate and internal standard conjugates for application in profiling enzyme activity of the enzymes α-D-2-deoxy-2-*N*-sulfonamido-glucosamine sulfamidase, α-D-2-deoxy-2-*N*-acetyl-glucosamine hydrolase, acetyl-coenzymeA:α-D-2-deoxy-2-amino-glucosamine transferase, and α-D-2-deoxy-2-*N*-acetyl-glucosamine-6-sulfate sulfatase. Deficiency of any one of these enzymes results in a single clinical phenotype known as Sanfilippo syndrome. Such substrates have been proven effective in the confirmation of enzyme deficiency by a combination of affinity chromatography (AC) and electrospray ionization mass spectrometry (ESIMS), which forms the foundation for a new analytical technology (ACESIMS) of general interest and application to clinical and biomedical research.

INTRODUCTION

Affinity chromatography has become increasingly popular over the past several decades as a simple yet highly selective method for the purification of a wide assortment of biomolecules (*1*, *2*). In particular, the use of biotin and its corresponding receptor proteins avidin and streptavidin is now quite common in laboratories focused on life science research (*3*, *4*). As a result, there is a growing body of biotin-conjugate chemistry in the literature for the preparation of biotinyl derivatives for a host of applications. In our research (*5*), we are developing biotinylated enzyme−substrate conjugates as the foundation of a mass spectrometric method to determine clinically relevant enzyme velocities directly in crude cell lysates from children suspected of suffering from enzyme-deficient disease.

The lack of properly functioning enzyme activity due to genetic aberrations in the coding sequence of such enzymes is a common molecular basis for inherited disease (*6*). Although there are a variety of methods in the clinical laboratory used to biochemically confirm a diagnosis of enzyme deficiency, there exists to date no single, unifying method capable of determining multiple, unique enzyme activities simultaneously, in a single sample (multiplexing). Such a technology could allow for the rapid and prompt screening of newborns for the most common genetic diseases. This would be advantageous and beneficial in the prompt identification and treatment of possible afflictions and would lead to reduced exposure of young patients to repeated biopsy.

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Our recent efforts to establish such a technology have been successfully applied to several enzyme-deficient diseases (*5*, *8*). In our approach, biotinylated substrate conjugates are incubated with fibroblast lysate obtained from cultures of skin biopsies from either apparently normal or putatively affected individuals. Action of the enzyme of interest on the substrate portion of the conjugate compositionally modifies the substrate conjugate, resulting in a change in mass of the substrate. A known amount of internal standard is added that is chemically identical to the product generated by enzyme activity but with deuterium incorporated into the conjugate. The mixture of excess substrate, nondeuterated enzyme product, and internal standard is purified from the biological matrix by addition of streptavidin−agarose to the sample and washing with purified water. The biotinylated conjugates are then selectively eluted from the bound receptor by addition of excess free biotin acid. This eluant is transferred to an electrospray-ionization mass spectrometer for analysis. The absolute amount of product formed during the reaction is determined by calculating the ratio of product to internal standard ion currents observed in the mass spectrum. In addition, an enzyme initial velocity can be calculated by determining the moles of product generated during the time in which the initial velocity condition exists.

The design of our substrate conjugates starts with biotin as the molecular handle with which such conjugates can be simply and selectively purified from the biological sample. Biotin is in turn conjugated to sarcosine to form an *N*-methyl biotinamide motif which is resistant to the enzyme biotinidase (*7*), known to be present in biological samples such as serum. Biotinidase would otherwise cleave our handle from the remainder of the conjugate. Sarcosinyl biotin is further linked to a water-soluble polyether linker. The linker also serves to position the structurally distinct biotin away from the substrate to avoid steric discrimination by the targeted enzyme, avidin or streptavidin. The linker also provides

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a location for facile synthetic incorporation of deuterium for internal standards. The length (i.e., mass) of the linker can also be adjusted to avoid mass redundancy with other conjugate pairs in a multiplex analysis. The amine terminus of the linker is attached to the substrate portion, either by direct conjugation to the substrate or by way of an adapter segment of variable composition for conjugation to a wide variety of substrate substructures.

In the present work, we describe the synthesis of substrate and internal standard conjugates used to profile the enzyme velocities involved in Sanfilippo syndrome. This approach has recently been shown to be successful in the confirmation of diagnoses for several diseases of enzyme deficiency, including the lysosomal storage disorder known as Sanfilippo syndrome (*8*). In this condition, deficiency of the four heparinoid-degrading enzymes α-D-2-deoxy-2-*N*-sulfonamido-glucosamine sulfamidase, α-D-2-deoxy-2-*N*-acetyl-glucosamine hydrolase, acetyl-coenzymeA:α-D-2-deoxy-2-amino-glucosamine transferase, and α-D-2-deoxy-2-*N*-acetyl-glucosamine-6-sulfate sulfatase results in a Sanfilippo subtype A, B, C, or D, respectively (*9*−*12*).

EXPERIMENTAL PROCEDURES

General. Reagent-grade chemicals (including solvents) were obtained from commercial vendors and were generally used without further purification. Anhydrous dimethylformamide and pyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI), and used as received. Triethylamine was frequently redistilled from calcium hydride and stored under argon. HPLC-grade solvents were purchased from Fischer (Fair Lawn, NJ), and water for HPLC was purified at point-of-use by a Milli-Q water system (Millipore, Bedford, MA). Trifluoroacetic acid was purchased from Advanced Chemtech (Louisville, KY), and was added to HPLC solvents as required, followed by 0.2 μm filtration. Triethylene glycol was purchased from Fluka (Buchs, Switzerland). Methyl *N*-methyl-*N*-biotinyl glycinate (*7*), 1-fluoro-2-deoxy-2-(*N*-trifluoroacetyl)-3,4,6-tri-*O*-acetyl-glucosamine (*13*), 1-α-(*p*-nitrophenyl)-2-deoxy-2-(*N*-trifluoroacetyl)-3,4,6-tri-*O*-acetyl-glucosamine (*13*), and 2,3,5,6-tetrafluorophenyl trifluoroacetate (*14*) were prepared according to the literature. All syntheses involving highly toxic materials (sodium cyanide etc.) were performed in a fume hood. Due caution is advised in reproducing these syntheses.

Spectral Analyses. ^{1}H NMR data were obtained on Bruker (Rheinstetten, Germany) spectrometers: AC-200 (200 MHz), AC-300 (300 MHz), or AC-500 (500 MHz). Proton chemical shifts are designated as parts-per-million (ppm) from tetramethylsilane (δ = 0.00 ppm). Mass spectral data were obtained using either a Kratos (Manchester, United Kingdom) HV-4 electrospray ionization-double-focusing (E/B) magnetic sector or Bruker Daltonics (Bremen, Germany) Esquire electrospray-ion trap mass spectrometers with standard, commercial interfaces. Methanol/acetic acid (99.5:0.5) was used as solvent, and contained 5 μM gramicidin S (average, doubly charged molecular weight $(M+2H^+)^{2+} = 571.6$) to verify mass accuracy.

Chromatography. Silica gel chromatography was performed using 230−400 mesh 60 Å silica (Merck, Darmstadt, Germany). Thin-layer chromatography was performed on 60 Å silica with F_{254}, aluminum-backed plates (Merck) and visulized by UV at 254 nm, phosphomolybdic acid, iodine, or ninhydrin, as indicated. Reversed-phase HPLC separations were performed using a Vydac (Hisperia, CA) semipreparative column (up to 5 mg total loading, 10 μm packing, 10 mm × 250 mm, catalog no. 218TP1010) at 3.0 mL/min, or a preparative column (up to 300 mg total loading, 10 μm packing, 22 mm × 250 mm, catalog no. 218TP1022) at 7 mL/min. Solvents were water/0.08% TFA (solvent A) and acetonitrile/0.08% TFA (solvent B), unless otherwise indicated. Common gradient conditions used were: (1) 2% A for 5 min, then a linear gradient from 2% to 35% B for 40 min; and (2) 5% A for 5 min, then a linear gradient from 5% to 55% B for 60 min, unless otherwise specified.

Quantification of Final Conjugates. Quantification of final substrate and internal standard conjugates was performed by ^{1}H NMR analysis in the presence of an internal standard. In a typical procedure, 6 μmol of dimethylformamide (10 μL of a 0.6 μmol/μL anhydrous dimethylformamide in D_2O stock solution) was added to the conjugate of interest dissolved in 490 μL of D_2O in an NMR tube. Analysis by ^{1}H NMR was performed on the AC-500, setting the proton relaxation delay to 4 s to properly integrate a broad range of proton relaxation rates. The mole amount of conjugate was determined using the integrals of the aromatic protons on the conjugate of interest and the internal standard. After analysis, the D_2O and dimethylformamide were removed by lyophilization, and the sample was redissolved to the indicated final concentration with purified water and stored in aliquots at −20 °C until use.

*4,7-Dioxa-1,10-decanedinitrile (**1a**).* To a stirred solution of 2% (w/v) sodium hydroxide (5 mL) and ethylene glycol (5.0 g, 81 mmol) at 0 °C was added acrylonitrile (12.8 g, 243 mmol) over 10 min. The mixture was allowed to stir at 0 °C for 1 h, followed by stirring overnight at room temperature. Dichloromethane (75 mL) was added, and the organic layer was washed 1× (5 mL) with water and 2× (5 mL each) with a dilute brine solution and dried ($MgSO_4$). The solvent was removed by rotary evaporation. The oily residue was treated with 25 mL of anhydrous ethanol, and the solvent was removed by rotary evaporation. This was repeated 2× to remove excess unreacted acrylonitrile, followed by removal of solvent by rotary evaporation and drying in vacuo. The resulting clear, colorless oil (13.5 g, 97%) was used without further purification. R_f 0.59, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.75 (m, 4H), 3.69 (s, 4H), 2.64 (q, 4H). ESI-MS $(M+Na^+)^+$: 191.02

*4,8-Dioxa-1,11-undecanedinitrile (**1b**).* As for **1a**, using 1,3-propanediol. Yield 95%, R_f 0.63, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.75 (m, 4H), 3.69 (s, 4H), 2.64 (q, 4H), 1.80 (m, 2H). ESI-MS $(M+Na^+)^+$: 205.04.

*4,7,10,13-Tetraoxa-1,16-hexadecanedinitrile (**1c**).* As for **1a**, using triethylene glycol. Yield 92%, R_f 0.46, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.75 (m, 4H), 3.70 (s, 16H), 2.65 (q, 4H). ESI-MS $(M+Na^+)^+$: 279.07.

*[3,3,8,8-^{2}H]-4,7-Dioxa-1,10-decanedinitrile (**1a$_d$**).* Ethylene glycol (0.5 g, 8 mmol) was added to 0.1 g of dry potassium hydroxide in 20 mL of acetonitrile, followed by 1.4 g (24 mmol) of d_3-acrylonitrile (Cambridge Isotope Laboratories, Andover, MA) with stirring overnight at room temperature. The reaction was filtered, and the solvent was removed by rotary evaporation to yield an oil. Final product was purified by silica chromatography (R_f 0.59, 9:1 chloroform/methanol) to yield 0.9 g (65%) of a colorless oil. ^{1}H NMR ($CDCl_3$) δ 3.69 (s, 4H), 2.64 (q, 4H). ESI-MS $(M+Na^+)^+$: 195.03.

*4,7-Dioxa-1,10-decanediamine (**2a**).* Raney nickel (50 mg) was washed 5 times with anhydrous methanol by inversion and decantation and placed in 20 mL of

anhydrous methanol in a 50 mL screw-cap vial. A small hole (~3 mm) was drilled in the center of the cap, which was covered from the inside with a Teflon-lined rubber septum (2 mm thickness). **1a** (1 g, 6 mmol) was added, and the vial headspace was purged with H_2 gas via an 16-gauge needle piercing the septum. The cap was screwed on tightly, and the entire assembly was charged to 40 psi with H_2 gas through the needle and placed in a hot water bath (80 °C) for 4 h, after which the solid catalyst was removed by filtration over diatomaceous earth. The filtrate was dried by rotary evaporation to yield an oil which was purified by preparative reversed-phase HPLC (preparative column, gradient 0−10 min, 0% B; 10−50 min, 0−18% B, t_R = 18.4 min) to yield 94 mg (9%) of a colorless oil. ^{1}H NMR (D_2O) δ 3.59 (s, 4H), 3.56 (t, 4H), 1.66 (broad m, 8H). ESI-MS $(M+H^+)^+$: 177.02.

*[3,3,8,8-^{2}H]-4,7-Dioxa-1,10-decanediamine (**2a$_d$**).* As done previously for **2a**, using **1a$_d$**. Yield 11%. ^{1}H NMR (D_2O) δ 3.56 (s, 4H), 2.96 (t, 4H), 1.68 (t, 4H). ESI-MS $(M+H^+)^+$: 181.14.

*Diethyl 4,8-Dioxa-1,11-undecanedioate (**3b**).* Nitrile **1b** (2 g, 12.0 mmol) was dissolved in 7 mL of 95% ethanol with stirring at 0 °C. Then 1.5 mL of concentrated sulfuric acid was added slowly over 5 min. The reaction was heated to reflux, and allowed to stir at reflux for 24 h. The mixture was cooled to room temperature, and the solvent was removed by rotary evaporation. The resulting material was extracted with 50 mL of methylene chloride, washed 2× with 5 mL of water and 3× with 5 mL of brine. The organic layer was dried ($MgSO_4$), and solvent was removed to yield an oil. The oil was dissolved in 95% ethanol with stirring at 0 °C. Concentrated sulfuric acid (1 mL) was added over 5 min, and the mixture was returned to reflux for an additional 24 h. The reaction was cooled, extracted, washed, and dried as done previously, and the final product was purified by silica chromatography (gradient dichloromethane/ethyl acetate/acetone) to yield 2.5 g (80%) of product as a colorless oil. R_f 0.76, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 4.14 (m, 4H), 3.73 (m, 4H), 3.60 (s, 4H), 2.58 (m, 4H), 1.78 (m, 2H), 1.26 (t, 6H). ESI-MS $(M+H^+)^+$: 277.08, $(M+Na^+)^+$: 299.12, $(M+K^+)^+$: 315.11.

*Diethyl 4,7,10,13,-Tetraoxa-1,16-hexadecanedioate (**3c**).* As done previously for **3b**, using nitrile **1c**. Yield 72%, R_f 0.59, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 4.14 (m, 4H), 3.73 (m, 4H), 3.60 (s, 12H), 2.58 (m, 4H), 1.26 (t, 6H). ESI-MS $(M+H^+)^+$: 351.13, $(M+Na^+)^+$: 373.16, $(M+K^+)^+$: 389.19.

*4,8-Dioxa-1,11-undecanediol (**4b**).* Lithium aluminum hydride (0.66 g, 16.5 mmol) was slowly added to 100 mL of distilled [lithium aluminum hydride] tetrahydrofuran under argon with stirring at 0 °C. The suspension was allowed to warm to room temperature and stirred for an additional 15 min. **3b** (1.7 g, 6.5 mmol) (previously dried in vacuo in the presence of P_2O_5 and dissolved in 5 mL of distilled [lithium aluminum hydride] tetrahydrofuran) was added dropwise over 10 min.The mixture was stirred an additional 60 min, followed by cooling to 0 °C and dropwise addition of ethanol to quench excess hydride. Salts were precipitated by dropwise addition of saturated sodium sulfate solution with efficient stirring until a white precipitate formed (ca. 6 mL). The organic solvent was decanted, the white precipitate was triturated 4× with 15 mL of tetrahydrofuran, the combined organic extracts were removed by rotary evaporation to yield an oil which was redissolved in 10 mL of dichloromethane and dried ($MgSO_4$), and solvent was again removed. The final product was purified by silica chromatography (dichloromethane/ethyl acetate/acetone) to yield 0.9 g (79%) of a colorless oil. R_f 0.46, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.74 (m, 4H), 3.66 (t, 4H), 3.59 (s, 4H), 2.90 (bs, 2H), 1.80 (m, 6H). ESI-MS $(M+H^+)^+$: 193.16, $(M+Na^+)^+$: 215.08.

*4,7,10,13-Tetraoxa-1,16-hexadecanediol (**4c**).* As done previously for **4b**, using **3c**. Yield 81%, R_f 0.33, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.72 (m, 4H), 3.60 (t, 12H), 3.59 (s, 4H), 2.65 (bs, 2H), 1.82 (m, 4H). ESI-MS $(M+H^+)^+$: 267.21, $(M+Na^+)^+$: 289.16.

*4,8-Dioxa-1,11-dichloroundecane (**5b**).* **4b** (1.1 g, 4.9 mmol, previously dried in vacuo in the presence of P_2O_5) was dissolved in 25 mL of dry [activated 4A Linde molecular sieves] benzene with stirring under argon. Distilled [CaH_2] pyridine (1.15 g, 14.6 mmol) was added, followed by cooling to 0 °C and dropwise addition of 1.8 g (14.6 mmol) of thionyl chloride in 5 mL of dry benzene. The mixture was warmed to 50 °C and allowed to stir under argon overnight, followed by cooling to 0 °C and addition of 7 mL of 3 M HCl with vigorous stirring. The organic layer was separated, washed 3× with dilute brine, and dried (Na_2SO_4), and solvent was removed by rotary evaporation to yield 1.2 g (86%) of a yellowish oil. The resulting product was used without further purification. R_f 0.82, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.63 (t, 4H), 3.54 (m, 8H), 2.02 (m, 4H), 1.83 (m, 2H). ESI-MS $(M+H^+)^+$: 229.10, $(M+Na^+)^+$: 251.18.

*4,7,10,13-Tetraoxa-1,16-dichlorohexadecane (**5c**).* As done previously for **5b**, using **4c**. Yield 81%, R_f 0.64, 9:1 dichloromethane/methanol.^{1}H NMR ($CDCl_3$) δ 3.65 (t, 4H), 3.54 (m, 16H), 2.01 (m, 4H), 1.83 (m, 2H). ESI-MS $(M+H^+)^+$: 229.10, $(M+Na^+)^+$: 251.18, $(M+K^+)^+$: 267.09.

*4,8-Dioxa-1,11-undecanedinitrile (**6b**).* Sodium cyanide (0.78 g, 15.5 mmol) in 6 mL of dimethyl sulfoxide was warmed to 80 °C with stirring. After 30 min, 1 g (3.9 mmol) of **5b** was added dropwise as a neat oil. After 2 h, the reaction was cooled slightly and treated with 10 mL of saturated sodium chloride solution, 5 mL of deionized water, and 50 mL of ethyl acetate. After extraction, the organic layer was washed 3× with a brine solution as before and dried (Na_2SO_4), followed by removal of solvent by rotary evaporation. The final product was purified by silica chromatography (gradient dichloromethane/ethyl acetate/acetone) to yield 0.6 g (64%) of product as a colorless oil. R_f 0.68, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.56−3.67 (m, 8H), 2.48 (t, 4H), 1.92 (qint, 4H), 1.81 (quint, 2H). ESI-MS $(M+Na^+)^+$: 233.05.

*4,7,10,13-Tetraoxa-1,16-hexadecanedinitrile (**6c**).* As done previously for **6b**, using **5c**. Yield 68%, R_f 0.55, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 3.56−3.67 (m, 16H), 2.44 (t, 4H), 1.92 (qint, 4H). ESI-MS $(M+Na^+)^+$: 307.14.

*5,9-Dioxa-1,13-tridecanediamine (**7b**).* Lithium aluminum hydride (0.87 g, 20.8 mmol) was carefully added to 150 mL of distilled [lithium aluminum hydride] tetrahydrofuran at 0 °C with stirring under an argon atmosphere. The suspension was heated to gentle reflux under argon for 30 min, followed by addition of 0.5 g (2.6 mmol) of **6b** (previously dried in vacuo in the presence of P_2O_5 and dissolved in 7 mL of distilled [lithium aluminum hydride] tetrahydrofuran) dropwise over 20 min while maintaining reflux by additional heat. After complete addition, the reaction was refluxed an additional 2 h, followed by cooling to 0 °C and dropwise addition of ethanol to quench excess hydride. Salts were precipitated by dropwise addition of saturated sodium sulfate solution with efficient stirring until a white precipitate formed (ca. 8 mL). The organic solvent was decanted, the white precipitate was triturated 4× with 15 mL of tetrahydro-

furan, and the combined organic extracts were evaporated by rotary evaporation to yield an oil which was redissolved in 20 mL of dichloromethane and dried ($MgSO_4$), and solvent was again removed. Final product was purified by preparative reversed-phase HPLC (gradient 0−10 min, 0% B; 10−50 min, 0−18% B; t_R = 20.5 min) to yield 0.35 g (71%) of a colorless oil. ^{1}H NMR (D_2O) δ 3.57 (s, 4H), 3.51 (t, 4H), 2.98 (t, 4H), 1.83 (m, 2H), 1.62 (m, 8H). ESI-MS $(M+H^+)^+$: 219.08.

*[1,1,13,13-^{2}H]-5,9-Dioxa-1,13-tridecanediamine (**7b$_d$**).* As done previously for **7b**, except lithium aluminum deuteride was used in place of lithium aluminum hydride. Yield 74%. Preparative reversed-phase HPLC, gradient 0−10 min, 0% B; 10−50 min, 0−18% B; t_R = 20.7 min. ^{1}H NMR (D_2O) δ 3.57 (s, 4H), 3.51 (t, 4H), 1.62 (m, 8H). ESI-MS $(M+H^+)^+$: 223.11.

*5,8,11,14-Tetraoxa-1,18-octadecanediamine (**7c**).* As done previously for **7b** using **6c**. Yield 64%. Preparative reversed-phase HPLC, gradient 0−10 min, 0% B; 10−50 min, 0−18% B; t_R = 22.2 min. ^{1}H NMR (D_2O) δ 3.56 (s, 12H), 3.51 (t, 4H), 2.98 (t, 4H), 1.62 (m, 8H). ESI-MS $(M+H^+)^+$: 293.15.

*[1,1,13,13-^{2}H]-5,8,11,14-Tetraoxa-1,18-octadecanediamine (**7c$_d$**).* As done previously for **7b$_d$** using **6c**. Yield 68%. Preparative reversed-phase HPLC, gradient 0−10 min, 0% B; 10−50 min, 0−18% B; t_R = 22.5 min. ^{1}H NMR (D_2O) δ 3.57 (s, 4H), 3.50 (t, 4H), 1.62 (m, 8H). ESI-MS $(M+H^+)^+$: 297.05.

*7-Acetoxycoumarin-4-acetic Acid (**8**).* To 100 mg (455 μmol) of 7-hydroxycoumarin-4-acetic acid in 2 mL of acetic anhydride with stirring at room temperature as a slurry was added 20 μL of boron trifluoride/diethyl etherate. A clear, homogeneous solution was observed after 2 min. After stirring for an additional 10 min, the mixture was diluted with 10 mL of water and shaken vigorously for 10 min to hydrolyze any mixed anhydride formed (R_f 0.87, dichloromethane/methanol/acetic acid 9:1:0.1). The resulting suspension was diluted with 30 mL of dichloromethane, washed 2× with 10 mL of dilute brine soution, and dried ($MgSO_4$). The solvent was removed by rotary evaporation to yield a semisolid that was further dried in vacuo in the presence of KOH to yield 122 mg (96%) of product as an off-white solid. R_f 0.53, 9:1:0.1 dichloromethane/methanol/acetic acid. ^{1}H NMR (d_6-DMSO) δ 7.74 (d, 1H), 7.29 (d, 1H), 7.17 (dd, 1H), 6.48 (s, 1H), 3.93 (s, 2H), 2.28 (s, 3H).

*7-Acetoxycoumarin-4-acetic Acid tert-Butyl Ester (**9**).* To 183 mg (1.5 mmol) of anhydrous magnesium sulfate in 4 mL of distilled [CaH_2] dichloromethane under argon was added 10 μL of concentrated sulfuric acid with stirring at room temperature. After 60 min, 146 μL (1.5 mmol) of distilled [CaH_2] *tert*-butyl alcohol was added, followed immediately by 50 mg (190 μmol) of **8** in 1 mL of distilled [CaH_2] dichloromethane. The reaction flask was tightly sealed and allowed to stir at room temperature for 15 h. The mixture was diluted with 50 mL of dichloromethane and filtered. Silica gel (1 g) was added to the filtrate, and the solvent was removed by rotary evaporation. The dried silica gel was loaded onto a silica column (dichloromethane), and the product was eluted (5:1 dichloromethane/ethyl acetate). After removal of solvent by rotary evaporation, the resulting product was dried in vacuo to yield 46 mg of a white solid (77%). R_f 0.91, 9:1 dichloromethane/methanol. ^{1}H NMR (d_6-acetone) δ 7.77 (d, 1H), 7.16 (m, 2H), 6.41 (s, 1H), 3.88 (s, 2H), 2.29 (s, 3H), 1.43 (s, 9H).

*7-Hydroxycoumarin-4-acetic Acid tert-Butyl Ester (**10**).* To 470 mg of **9** in 8 mL of tetrahydrofuran with stirring at room temperature was added 3 mL of saturated methanolic ammonia in a tightly sealed flask. The mixture was allowed to stir for an additional 3 h, followed by dilution with 25 mL of dichloromethane, addition of 2.0 g of silica gel, and removal of solvent by rotary evaporation. The resulting silica gel mixture was loaded onto a silica column (dichloromethane), and eluted (5:1 dichloromethane/acetone). Purified fractions were combined, and solvent was removed to yield 390 mg of a white solid (96%). R_f 0.68, 9:1 dichloromethane/methanol. ^{1}H NMR (d_6-acetone) δ 9.42 (s, 1H), 7.58 (d, 1H), 6.87 (dd, 1H), 6.76 (d, 1H), 6.17 (s, 1H), 3.80 (s, 2H), 1.41 (s, 9H).

*7-O-α-D-(2-Deoxy-2-N-trifluoroacetamido-3,4,6-tri-O-acetyl glucopyranos-1-yl)-coumarin-4-acetic Acid tert-Butyl Ester (**11**).* This compound was prepared as described in the literature (*13*). 7-Trimethylsilyloxy-4-*tert*-butoxyacetic acid ester was prepared as described for 7-hydroxycoumarin using **10** that was previously dried in vacuo in the presence of P_2O_5, and used without characterization. R_f 0.89 (α-anomer), 0.44 (β-anomer); 9:1 dichloromethane/methanol; UV. α-Anomer: ^{1}H NMR (d_6-acetone) δ 8.75 (d, 1H), 7.77 (d, 1H), 7.26 (m, 2H), 6.36 (s, 1H), 6.03 (d, J = 3.7 Hz, 1H), 5.61 (t, 1H), 5.24 (t, 1H), 4.68 (td, 1H), 4.32 (t, 1H), 4.24 (dd, 1H), 4.14 (dd, 1H), 3.90 (s, 2H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.47 (s, 9H).

*7-O-α-D-(2-Deoxy-2-N-trifluoroacetamido-3,4,6-tri-O-acetyl glucopyranos-1-yl)-coumarin-4-acetic Acid (**12**).* To 54 mg (82 μmol) of **11** (previously dried in vacuo overnight in the presence of P_2O_5) in 8 mL of distilled [CaH_2] dichloromethane was added 0.5 mL of distilled [P_2O_5] trifluoroacetic acid with stirring under argon. The mixture was warmed to 50 °C and allowed to stir overnight, followed by removal of solvent by rotary evaporation using a water bath at room temperature (∼20 °C) in a rotary evaporator previously flushed with dry nitrogen for 45 min. The resulting semisolid was rediluted with 5 mL of distilled dichoromethane and again dried by rotary evaporation. This was repeated twice, followed by addition of 5 mL of dichloromethane and 0.4 g of silica gel, and the solvent was removed by rotary evaporation. The resulting powder was loaded onto a short silica column (dichloromethane), and the final product was eluted (gradient dichloromethane/acetone/methanol). After removal of solvent by rotary evaporation, the final product was dried in vacuo to yield an off-white solid that was triturated in 1 mL of ether and dried by vacuum centrifugation to yield 41 mg (82%) of product. R_f 0.28, 9:1:0.1 dichloromethane/methanol/acetic acid. ^{1}H NMR (d_6-acetone) δ 8.70 (d, 1H), 7.75 (d, 1H), 7.20 (m, 2H), 6.35 (s, 1H), 5.96 (d, J = 3.6 Hz, 1H), 5.56 (t, 1H), 5.19 (t, 1H), 4.62 (m, 1H), 4.28 (t, 1H), 4.18 (dd, 1H), 4.09 (dd, 1H), 3.93 (s, 2H), 2.00 (s, 3H), 1.99 (s, 3H), 1.96 (s, 3H).

*Conjugate of **12** and **7b** (**13b**).* To 5 mg (8.3 μmol) of **12** (previously dried in vacuo in the presence of P_2O_5) in a dry, 4 mL screw-cap vial was added 300 μL of anhydrous dimethylformamide under argon. The vial was agitated by orbital shaking to effect dissolution, followed by vacuum centrifugation to remove solvent. This was repeated, followed by additional drying in vacuo overnight in the presence of P_2O_5. The film was dissolved in 300 μL of anhydrous dimethylformamide, followed by addition of 7 μL (17 μmol) of anhydrous pyridine and three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C). After orbital shaking for 2 h at room temperature, the solution was treated with 3 μL of tetrafluorophenyl trifluoroacetate and agitated for an additional 45 min. The solution was removed from the vial with a dry syringe and transferred to another 4 mL

screw-cap vial containing 10 mg (50 μmol) of **7b** (previously dried in vacuo in the presence of P_2O_5), three activated molecular sieves, and 10 μL of dry, distilled [CaH_2] triethylamine in 200 μL of anhydrous dimethylformamide under argon. After 2 h of additional agitation, the molecular sieves were washed with 0.5 mL of methanol, and the combined solvents were removed by vacuum centrifugation to yield a crude film which was redissolved in purified water. The pH was adjusted to 3−4 with trifluoroacetic acid, and the product was purified by reversed-phase HPLC [preparative column, gradient (2), t_R = 44.1 min]. The final product was dried by vacuum centrifugation to yield 5.5 mg (85%) of a thin, colorless film. ^{1}H NMR (d_6-acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34−3.65 (broad m, 12H), 3.24 (t, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 6H), 1.45 (m, 4H). ESI-MS $(M+H^+)^+$: 804.70.

*Conjugate of **12** and **7b$_d$** (**13b$_d$**).* As done previously for **13b**, using **7b$_d$**. Yield 82%, preparative column, gradient (2), t_R = 44.4 min. ^{1}H NMR (d_6-acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34−3.65 (broad m, 8H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 6H), 1.45 (m, 4H). ESI-MS $(M+H^+)^+$: 808.73.

*Conjugate of **12** and **7c** (**13c**).* As done previously for **13b**, using **7c** (81%). Preparative column, gradient (2), t_R = 44.7 min. ^{1}H NMR (d_6-acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34−3.65 (broad m, 18H), 3.24 (t, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.45 (m, 4H). ESI-MS $(M+H^+)^+$: 878.59.

*Conjugate of **12** and **7c$_d$** (**13c$_d$**).* As done previously for **13b**, using **7c$_d$**. Yield 84%, preparative column, gradient (2), t_R = 44.9 min. ^{1}H NMR (d_6-acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34−3.65 (broad m, 16H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.45 (m, 4H). ESI-MS $(M+H^+)^+$: 882.61.

*N-Methyl-N-biotinyl Glycine (**14**).* The title compound was prepared by a modification of the method of Wilbur (*7*). Methyl *N*-methyl-*N*-biotinyl glycinate (*7*) was hydrolyzed in a mixture of 31 mL of methanol and 10 mL of 1 N sodium hydroxide at room temperature with stirring for 1 h. The mixture was diluted with 50 mL of 50% methanol/water and neutralized with cation-exchange resin, hydrogen form (BioRad). The solution was filtered, the resin was washed (3 × 50 mL) with 50% methanol/water, and the solvents were removed by rotary evaporation to yield 1.6 g (90%) of *N*-methyl-*N*-biotinyl glycine as an off-white solid. ^{1}H NMR (CD_3OD) δ 4.49 (m, 1H), 4.32 (m, 1H), 4.11 (d, 2H), 3.22 (m, 1H), 3.04 (d, 3H), 2.93 (dd, 1H), 2.71 (d, 1H), 2.39 (dt, 2H), 1.69 (m, 4H), 1.49 (m, 2H). ESI-MS $(M+H^+)^+$: 316.2

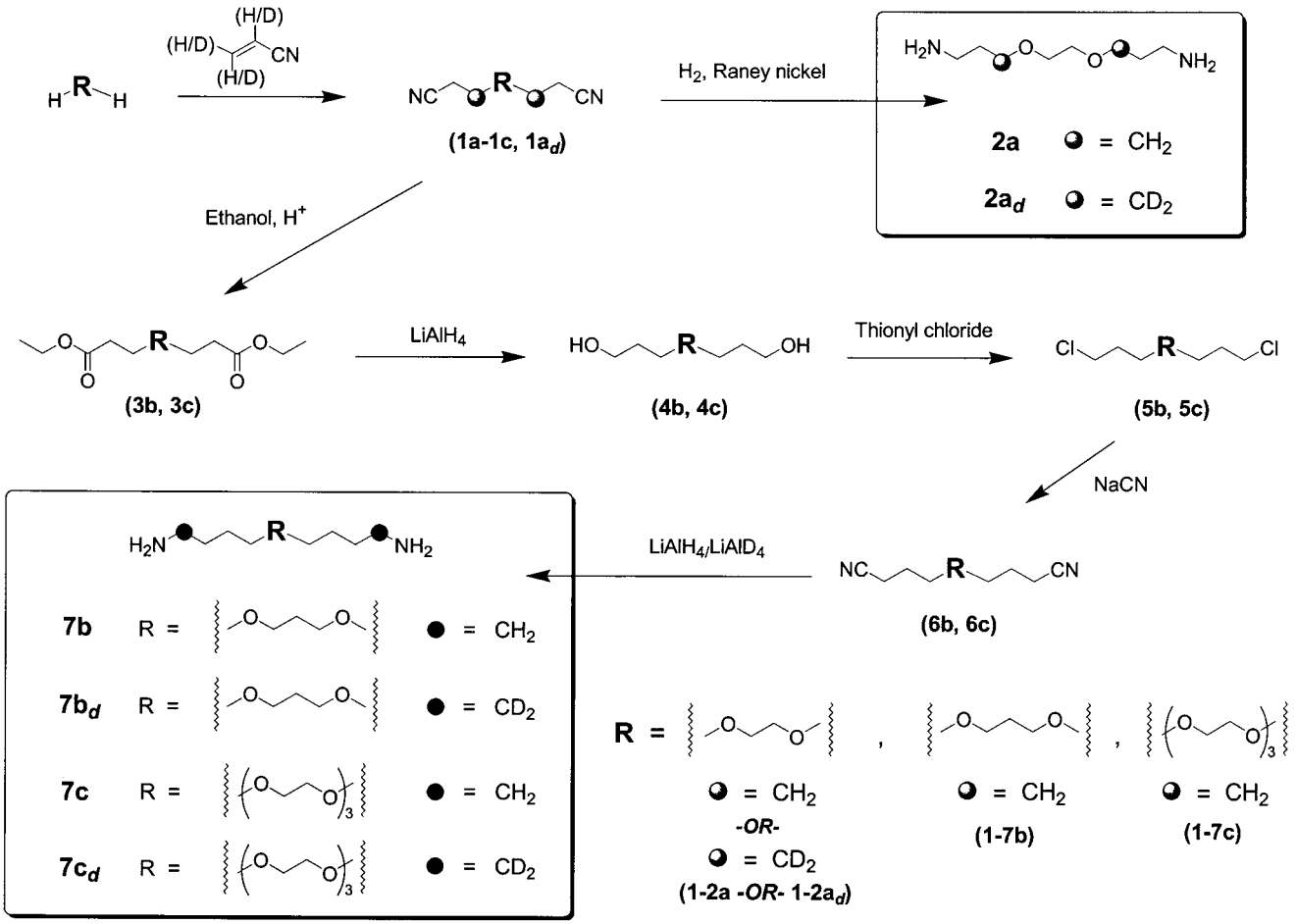
*Tetrafluorophenyl N-Methyl-N-biotinyl Glycinate (**15**).* In a modification of a procedure by Wilbur (*7*), 2.5 mg (7.4 μmol) of **14** (previously dried in vacuo in the presence of P_2O_5) was dissolved in 0.2 mL of anhydrous dimethylformamide containing three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C) by orbital shaking, under argon atmosphere. Then 5 μL of distilled [CaH_2] triethylamine was added, followed after 2 h of orbital shaking by 1.5 μL (8.8 μmol) of tetrafluorophenyl trifluoroacetate. The formation of active ester (15−30 min) was monitored by silica TLC (R_f 0.5, 5:1 dichloromethane/methanol, UV) with brief drying of the spotted TLC plate with a stream of air prior to development. The active ester was used within 1 h and without further purification or characterization.

*1-[N-(N-Methyl-N-biotinyl glycine)amido]-5,9-dioxa-13-tridecaneamine (**16**).* **7b** (42 mg, 0.2 mmol) was dried in vacuo in the presence of P_2O_5 overnight prior to use, followed by dissolution in 200 μL of anhydrous dimethylformamide by orbital shaking under argon. Three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C) were added, followed by 5 μL (0.03 mmol) of distilled [CaH_2] triethylamine. After 2 h of orbital shaking, **15** [freshly prepared from 10 mg (0.03 mmol) of **14**, in dimethylformamide] was added dropwise by dry syringe, and the reaction was allowed to proceed for 1 h at room temperature while mixing with orbital shaking. The molecular sieves were removed from the reaction vessel and rinsed with 0.5 mL of methanol, and the combined organic solvents were removed by vacuum centrifugation at room temperature. The residue was reconstituted in water, the pH was adjusted to 3−4 with trifluoroacetic acid, and the final product was purified by reversed-phase HPLC (preparative column, gradient 0−5 min, 0% B, 5−60 min, 0−26% B, t_R = 47.2 min) and dried by vacuum centrifugation to yield 13 mg (82%) of a thin, colorless film. ^{1}H NMR (D_2O) δ 4.49 (m, 1H), 4.32 (m, 1H), 4.08 (d, 2H), 3.55 (m, 8H), 3.41 (t, 2H), 3.22 (m, 1H), 3.04 (d, 3H), 2.93 (dd, 1H), 2.88 (t, 2H), 2.71 (d, 1H), 2.39 (dt, 2H), 1.81 (m, 2H), 1.69 (m, 4H), 1.60 (m, 8H), 1.49 (m, 2H). ESI-MS $(M+H^+)^+$: 516.33.

*Conjugate of **15** and **13b** (**17b**).* To 5.5 mg (6.8 μmol) of **13b** (previously dried in vacuo in the presence of P_2O_5) in a dry, 4 mL screw-cap vial was added 300 μL of anhydrous dimethylformamide under argon. The vial was agitated by orbital shaking to effect dissolution, followed by vacuum centrifugation to remove solvent. This was repeated, followed by additional drying in vacuo overnight in the presence of P_2O_5. The film was dissolved in 200 μL of anhydrous dimethylformamide, followed by 5 μL of dry, distilled [CaH_2] triethylamine and three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C) under argon. The mixture was agitated by orbital shaking for 2 h at room temperature, followed by addition of **15** [prepared from 2.2 mg (7 μmol) of **14**, in dimethylformamide]. After shaking for an additional 2 h, the molecular sieves were removed from the reaction vessel and rinsed with 0.5 mL of methanol, and the combined organic solvents were removed by vacuum centrifugation at room temperature. The resulting residue was reconstituted in water, the pH was adjusted to 3−4 with trifluoroacetic acid, and the product was purified by reversed-phase HPLC [preparative column, gradient (2), t_R = 52.4 min] and dried by vacuum centrifugation to yield 4.5 mg (62%) of a thin, colorless film. ^{1}H NMR (d_6-acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40−3.65 (broad m, 10H), 3.25 (m, 3H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS $(M+H^+)^+$: 1101.55, $(M+2H^+)^{2+}$: 551.62.

*Conjugate of **15** and **13b$_d$** (**17b$_d$**).* As prepared previously for **17b**, using **13b$_d$**. Yield 70%, preparative column, gradient (2), t_R = 52.5 min. ^{1}H NMR (d_6-acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40−3.65 (broad m, 8H), 3.24

Scheme 1



(m, 1H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS $(M+H^+)^+$: 1105.65, $(M+2H^+)^{2+}$: 553.58.

*Conjugate of **15** and **13c** (**17c**).* As prepared previously for **17b**, using **13c**. Yield 65%, preparative column, gradient (2), t_R = 52.7 min. ^{1}H NMR (d_6-acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40−3.65 (broad m, 18H), 3.25 (m, 3H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS $(M+H^+)^+$: 1175.79, $(M+2H^+)^{2+}$: 588.67.

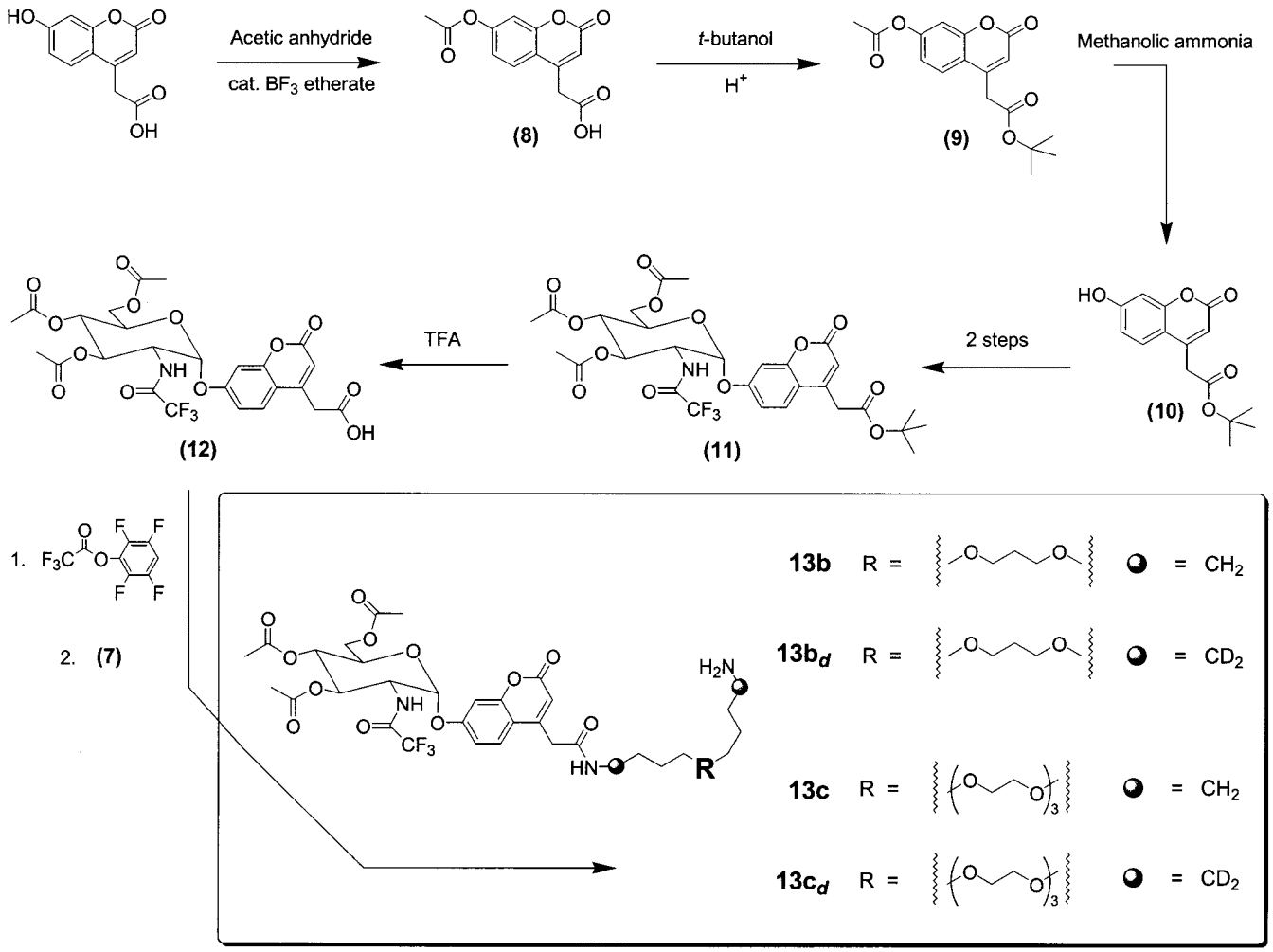
*Conjugate of **15** and **13c$_d$** (**17c$_d$**).* As prepared previously for **17b**, using **13c$_d$**. Yield 68%, preparative column, gradient (2), t_R = 53.0 min. ^{1}H NMR (d_6-acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40−3.65 (broad m, 16H), 3.24 (m, 1H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS $(M+H^+)^+$: 1179.59, $(M+2H^+)^{2+}$: 590.51.

*p-Aminophenyl-α-D-2-deoxy-2-acetamidoglucopyranoside (**20**).* *p*-Nitrophenyl-α-D-2-deoxy-2-acetamidoglucopyranoside (**18**, 20 mg, 0.07 mmol, Sigma) was added to 5 mg of palladium on activated carbon (previously washed with methanol by inversion and decantation) in 3 mL of methanol with stirring in a 5 mL septum-lined vial. The septum was pierced by a 16-gauge needle, through which the vial headspace was purged with H_2 gas. H_2 gas was allowed to slowly bubble through the solution for 2 h with stirring. After 45 min, all noncatalyst material was dissolved. The catalyst was removed by filtration over diatomaceous earth (Celite). The solvent was removed by rotary evaporation, and the product was further dried in vacuo to yield 18 mg of an off-white solid (90%). ^{1}H NMR (D_2O) δ 7.24 (d, 2H), 7.16 (d, 2H), 5.44 (d, 1H), 4.02 (dd, 1H), 3.88 (t, 1H), 3.68 (m, 3H), 3.49 (t, 1H), 1.97 (s, 3H). ESI-MS $(M+H^+)^+$: 313.22.

*p-Aminophenyl-α-D-(2-deoxy-2-N-trifluoroacetamido)-3,4,6-tri-O-acetylglucopyranoside (**21**).* As done previously for **20**, using **19** which was prepared by the method of Voznyi et al. (*13*). Yield 86%, R_f 0.55, 2:1 dichloromethane/ethyl acetate, UV, ninhydrin. ^{1}H NMR ($CDCl_3$) δ 7.35 (d, 2H), 6.92 (d, 2H), 6.77 (d, 1H), 5.69 (d, J = 3.1 Hz, 1H), 5.48 (t, 1H), 5.26 (t, 1H), 5.50 (td, 1H), 4.22 (dd, 1H), 4.01 (m, 2H), 2.07 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H). ESI-MS $(M+H^+)^+$: 493.29.

*p-Acrylamidophenyl-α-D-2-deoxy-2-acetamidoglucopyranoside (**23**).* **20** (10 mg, 0.03 mmol) was added to 15 mL of methanol and 100 μL of triethylamine with stirring. The solution was chilled in an ice bath. Acryloyl chloride (15 mg, 0.17 mmol) was dissolved in 2 mL of distilled [CaH_2] dichloromethane and added dropwise to the stirred solution over 5 min. The reaction was allowed to return to room temperature, followed by 2 h of stirring. The solution was treated with successive anion (hydroxide form) and cation (proton form) exchange resins until neutral pH was obtained with moist pH paper. Solvent was removed by rotary evaporation to yield an off-white

Scheme 2



solid (11 mg, 95%). ^{1}H NMR (D_2O) δ 7.36 (d, 2H), 7.03 (d, 2H), 6.32 (dd, 1H), 6.23 (d, 1H), 5.78 (d, 1H), 5.50 (d, 1H), 4.02 (dd, 1H), 3.88 (t, 1H), 3.68 (m, 3H), 3.49 (t, 1H), 1.94 (s, 3H). ESI-MS $(M+Na^+)^+$: 389.14.

*p-Acrylamidophenyl-α-D-(2-deoxy-2-N-trifluoroacetamido)-3,4,6-tri-O-acetylglucopyranoside (**24**).* As prepared previously for **23**, using **21**. Yield 87%, R_f 0.63, 9:1 dichloromethane/methanol. ^{1}H NMR ($CDCl_3$) δ 7.51 (d, 2H), 7.05 (d, 2H), 6.41 (d, 2H), 6.18 (dd, 1H), 5.75 (dd, 1H), 5.04 (d, 1H), 5.48 (t, 1H), 5.25 (t, 1H), 4.45 (td, 1H), 4.26 (dd, 1H), 4.08 (m, 2H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H).

*p-Acrylamidophenyl-β-D-galactopyranoside (**25**).* As prepared previously for **23**, using *p*-aminophenyl-β-D-galactopyranoside (**22**, Sigma). Yield 97%. ^{1}H NMR (d_7-dimethylformamide) δ 10.28 (s, 1H), 7.69 (d, 2H), 7.03 (d, 2H), 6.52 (dd, 1H), 6.27 (dd, 1H), 5.69 (dd, 1H), 5.18 (s, 1H), 4.83 (d, J = 10.3 Hz, 1H), 4.79 (s, 1H), 4.77 (s, 1H), 4.55 (s, 1H), 3.93 (t, 1H), 3.77 (m, 1H), 3.70 (m, 1H), 3.58 (m, 1H), 3.39 (s, 2H).

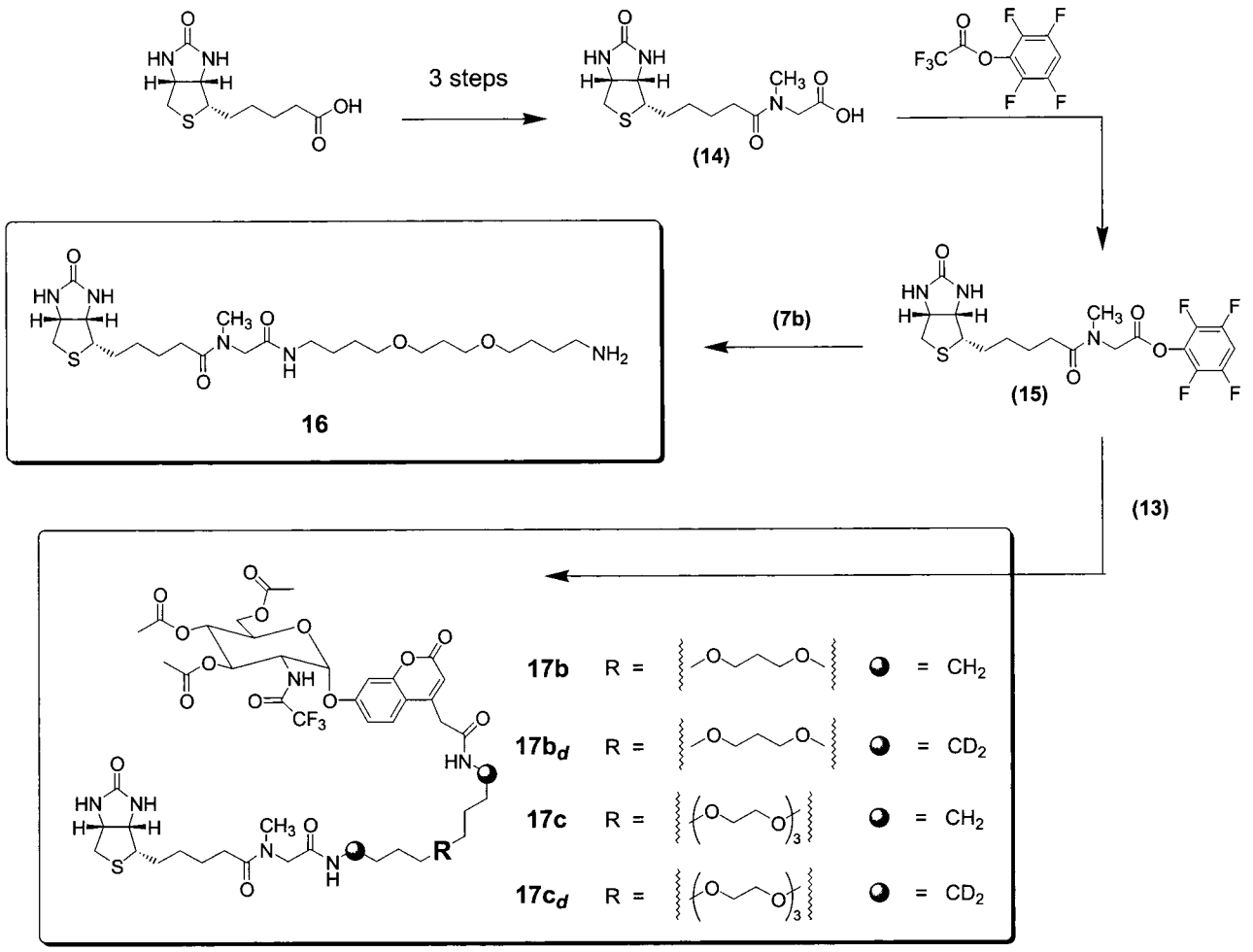
*Michael Addition Product of **2a** and **23** (**26**).* **23** (20 mg, 0.07 mmol) was added to a stirred solution of 80 mg (0.35 mmol) of **2a** in 5 mL of 0.2 M sodium carbonate, pH 10.5, warmed to 37 °C. The reaction was allowed to continue for 3 days at 37 °C, after which the solution was neutralized with dilute trifluoroacetic acid in water, concentrated by rotary evaporation, and purified by reversed-phase HPLC (preparative column, gradient 0−10 min, 0% B, 10−50 min, 0−19% B, t_R = 33.5 min). The purified product was dried by vacuum centrifugation to yield 14.4 mg (38%) as a film. ^{1}H NMR (D_2O) δ 7.33 (d, 2H), 7.22 (d, 2H), 5.88 (d, 1H), 3.76 (t, 1H), 3.70 (m, 3H), 3.58 (m, 8H), 3.47 (m, 1H), 3.32 (m, 3H), 3.05 (t, 2H), 2.94 (t, 2H), 2.80 (t, 2H), 1.95 (s, 3H), 1.72 (m, 2H), 1.60 (m, 6H). ESI-MS $(M+H^+)^+$: 543.19, $(M+2H^+)^{2+}$: 272.09.

*Michael Addition Product of **2a$_d$** and **25** (**27**).* As done previously for **26**, using **25** and **2a$_d$**. Yield 42%, preparative reversed-phase HPLC, gradient 0−10 min, 0% B, 10−50 min, 0−19% B, t_R = 32.9 min. ^{1}H NMR (d_7-dimethylformamide) δ 10.49 (s, 1H), 7.73 (d, 2H), 7.10 (d, 2H), 4.91 (d, 1H), 3.96 (d, 1H), 3.75 (m, 3H), 3.68 (m, 2H), 3.61 (m, 4H), 3.45 (m, 2H), 3.30 (s, 2H), 3.20 (s, 2H), 3.01 (t, 2H). ESI-MS $(M+H^+)^+$: 506.23, $(M+2H^+)^{2+}$: 253.60.

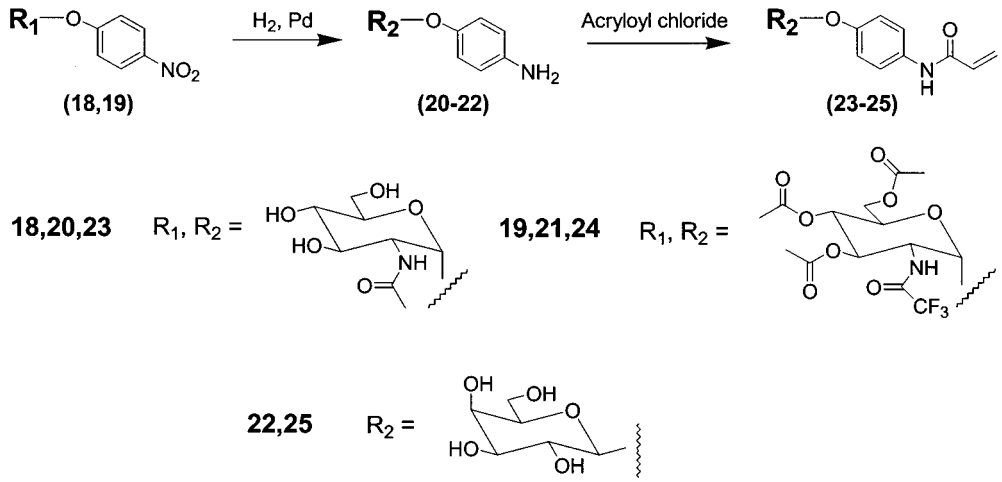
*Michael Addition Product of **7b$_d$** and **23** (**28**).* As done previously for **26**, using **23** and **7b$_d$**. Yield 36%, preparative reversed-phase HPLC, gradient 0−10 min, 0% B, 10−50 min, 0−19% B, t_R = 33.8 min. ^{1}H NMR (D_2O) δ 7.33 (d, 2H), 7.22 (d, 2H), 5.88 (d, 1H), 3.76 (t, 1H), 3.70 (m, 3H), 3.58 (m, 8H), 3.47 (m, 1H), 3.32 (m, 3H), 3.05 (t, 2H), 2.94 (t, 2H), 2.80 (t, 2H), 1.95 (s, 3H), 1.72 (m, 2H), 1.60 (m, 6H). ESI-MS $(M+H^+)^+$: 589.25, $(M+2H^+)^{2+}$: 295.22.

*Michael Addition Product of **16** and **24** (SFC Substrate) (**29**).* As done previously for **26**, using **24** and **16**, except 3 mL of 0.2 M sodium carbonate was used, and **16** was added as a solution in 2 mL of methanol. Yield 32%, preparative reversed-phase HPLC, gradient 0−5 min, 0% B, 5−60 min, 0−22% B, t_R = 40.2 min, broadly eluting.

Scheme 3



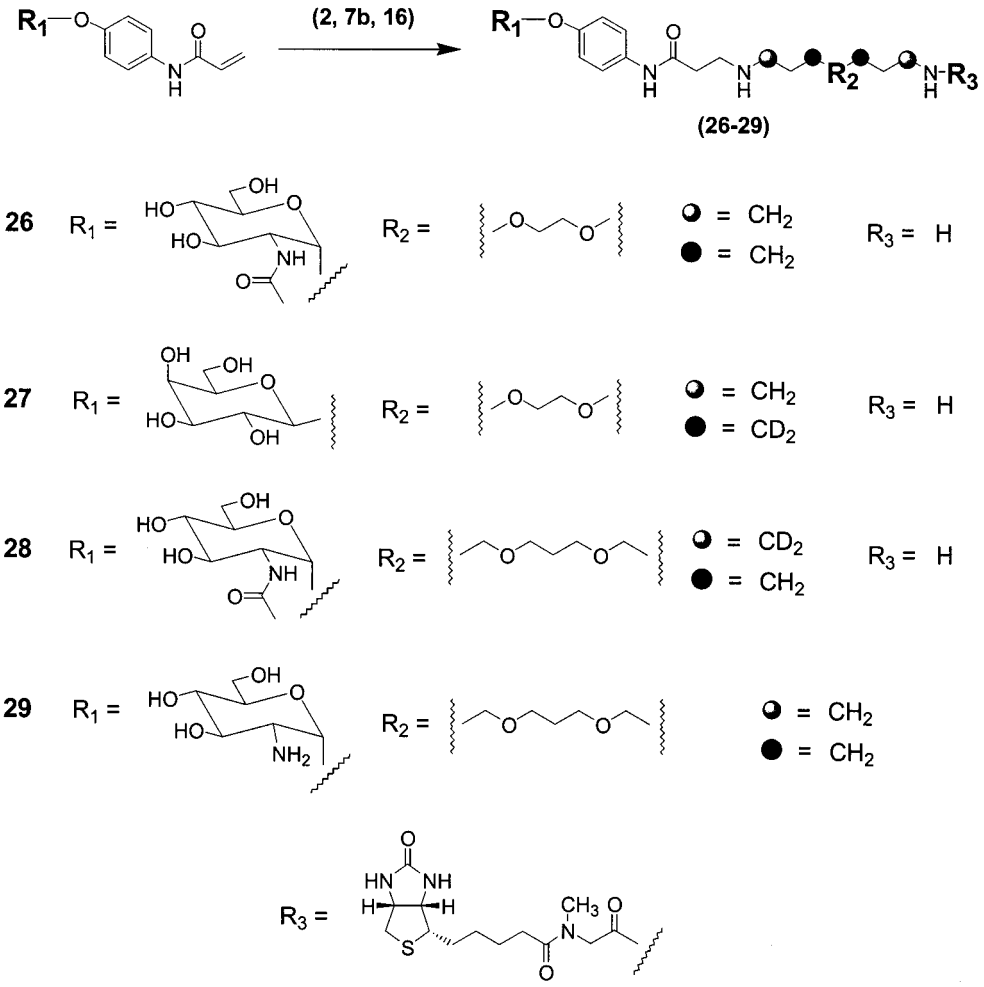
Scheme 4



^{1}H NMR (D_2O) δ 7.40 (d, 2H), 7.18 (d, 2H), 5.82 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.07 (t, 1H), 4.05 (d, 2H), 3.80 (m, 3H), 3.55 (m, 10H), 3.38 (t, 2H), 3.32 (m, 3H), 3.05 (t, 2H), 3.00 (d, 3H), 2.94 (t, 2H), 2.80 (t, 2H), 2.40 (dt, 2H), 1.72 (m, 2H), 1.60 (m, 12H). ESI-MS $(M+H^+)^+$: 840.57, $(M+2H^+)^{2+}$: 420.72.

*Conjugate of **15** and **27** (SFB Substrate) (**30**).* To 3 mg (5.5 μmol) of **26** previously dried in vacuo overnight in the presence of P_2O_5 was added 200 μL of anhydrous dimethylformamide and three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C) under argon to redissolve and further dry the film. Dry, distilled [CaH_2] triethylamine was added (2 μL), followed after 2 h of orbital shaking by **15** [prepared from 2 mg (5.5 μmol) of **14**]. After an additional 2 h of orbital shaking, the molecular sieves were removed and washed with 0.5 mL of methanol, and the combined solvents were removed by vacuum centrifugation. The final product was purified by reversed-phase HPLC [preparative column, gradient (1), t_R = 30.5 min] and dried by vacuum centrifugation to yield 4.6 mg of a clear film (62%). After quantitation by ^{1}H NMR, the conjugate was diluted in purified water to 2.0 mM final concentration and frozen in aliquots at −20 °C until use. ^{1}H NMR (D_2O) δ 7.45 (d, 2H), 7.18 (d,

Scheme 5



2H), 5.06 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.11 (m, 2H), 4.05 (d, 3H), 3.80 (m, 1H), 3.55 (m, 9H), 3.51 (t, 2H), 3.41 (t, 2H), 3.26 (m, 3H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 2.05 (s, 3H), 1.78 (m, 4H), 1.60 (m, 4H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 840.66, $(M+2H^+)^{2+}$: 420.78.

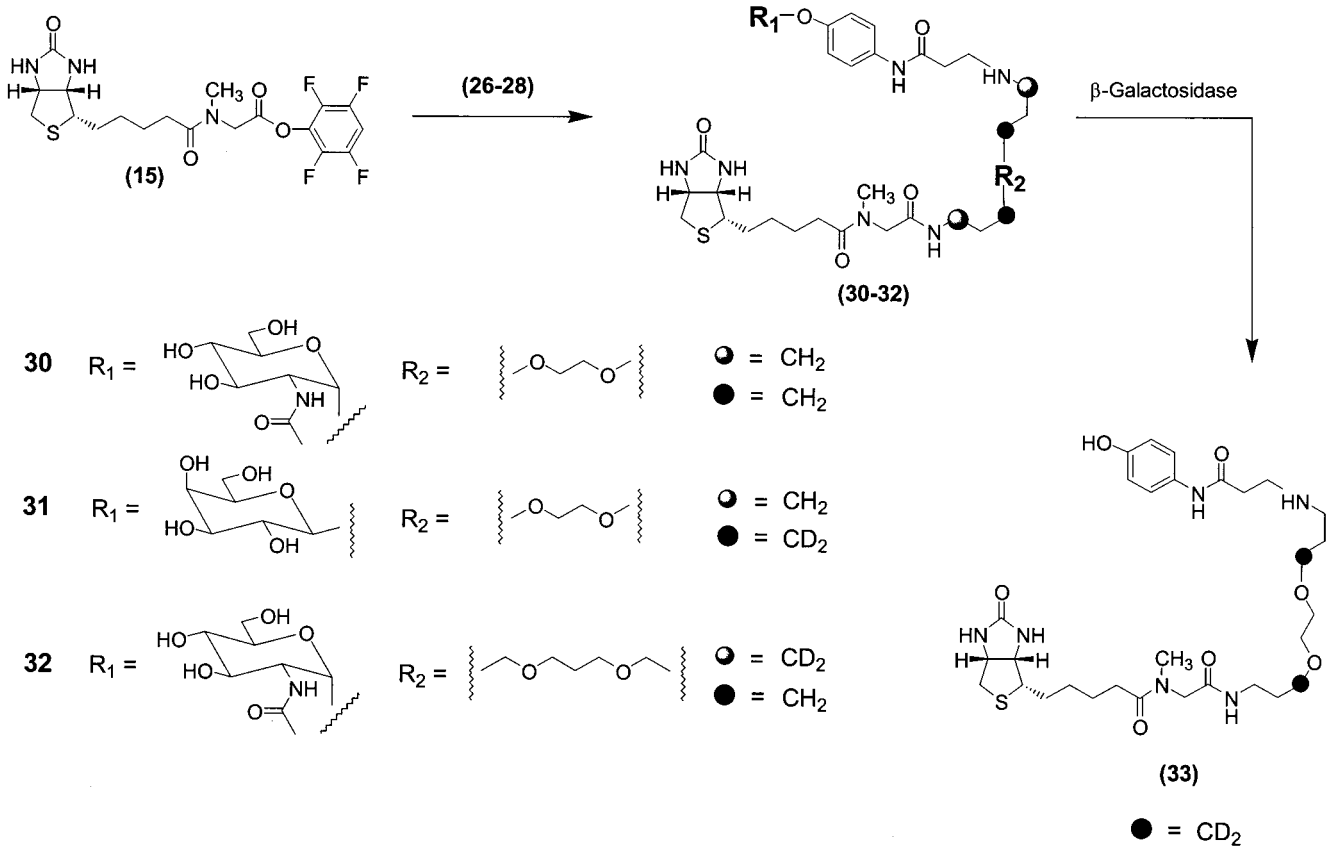
*Conjugate of **15** and **27** (for SFB Internal Standard) (**31**).* As done previously for **30** using **27**. Yield 58%, preparative reversed-phase HPLC, gradient (1), $t_R = 29.6$ min. ^{1}H NMR (D_2O) δ 7.42 (d, 2H), 7.18 (d, 2H), 5.05 (d, 1H), 4.62 (m, 1H), 4.42 (m, 1H), 4.11 (d, 2H), 3.80 (t, 1H), 3.75 (m, 2H), 3.65 (m, 11H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.78 (m, 4H), 1.60 (m, 4H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 803.55.

*Conjugate of **15** and **28** (SFC Internal Standard) (**32**).* As done previously for **30** using **28**. Yield 68%, preparative reversed-phase HPLC, gradient 0−5 min, 0% B, 5−60 min, 0−22% B, $t_R = 41.8$ min, broadly eluting. After quantitation by ^{1}H NMR, the conjugate was diluted in purified water to 1.0 mM final concentration and frozen in aliquots at −20 °C until use. ^{1}H NMR (D_2O) δ 7.42 (d, 2H), 7.18 (d, 2H), 5.58 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.11 (m, 2H), 4.05 (d, 3H), 3.80 (m, 1H), 3.55 (m, 9H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 2.05 (s, 3H), 1.78 (m, 6H), 1.60 (m, 8H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 886.77, $(M+2H^+)^{2+}$: 443.64.

*SFB Internal Standard Conjugate (**33**).* **31** (1.8 mg) was dissolved in 2 mL of 100 mM Tris-HCl, 10 mM $MgCl_2$ buffer, pH 7.3, with stirring at 37 °C. Recombinant β-D-galactosidase (10 units, Sigma) was added, and after 12 h of additional stirring at 37 °C, the mixture was acidified to pH 3−4 with trifluoroacetic acid and concentrated by rotary evaporation to 0.75 mL. The final product was purified by reversed-phase HPLC [semipreparative column, gradient (1), $t_R = 41.3$ min] and dried by vacuum centrifugation to yield 1.3 mg (89%) of a film. After quantitation by ^{1}H NMR, the conjugate was diluted in purified water to 1.0 mM final concentration and frozen in aliquots at −20 °C until use. ^{1}H NMR (D_2O) δ 7.18 (d, 2H), 6.79 (d, 2H), 5.58 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 3.97 (d, 2H), 3.55 (m, 8H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.68 (m, 2H), 1.60 (m, 6H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 641.51.

*Deprotection of Conjugate **17b** (**34b**).* To **17b** was added 1 mL of saturated ammonia in methanol and 0.35 mL of 2 M ammonium hydroxide with stirring. The solution was heated to 45 °C and allowed to stir overnight, followed by removal of solvent by vacuum centrifugation, redissolution in purified water, and pH adjustment to 3 with dilute trifluoroacetic acid and purification by reversed-phase HPLC [semipreparative column, gradient (2), $t_R = 32.0$ min]. The final product was dried by vacuum centrifugation to yield 3 mg (90%) as a thin, colorless film. ^{1}H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 10H), 3.41 (t, 2H), 3.26 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.69

Scheme 6



(t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 879.79, $(M+2H^+)^{2+}$: 440.41.

*Deprotection of Conjugate **17b$_d$** (**34b$_d$**).* As done previously for **17b**, using **17b$_d$**. Yield 82%, semipreparative column, gradient (2), t_R = 32.2 min. ^{1}H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 10H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.69 (t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 883.77, $(M+2H^+)^{2+}$: 442.42.

*Deprotection of Conjugate **17c** (**34c**).* As done previously for **17b**, using **17c**. Yield 92%, semipreparative column, gradient (2), t_R = 32.6 min. ^{1}H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 18H), 3.41 (t, 2H), 3.26 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 953.74, $(M+2H^+)^{2+}$: 477.47.

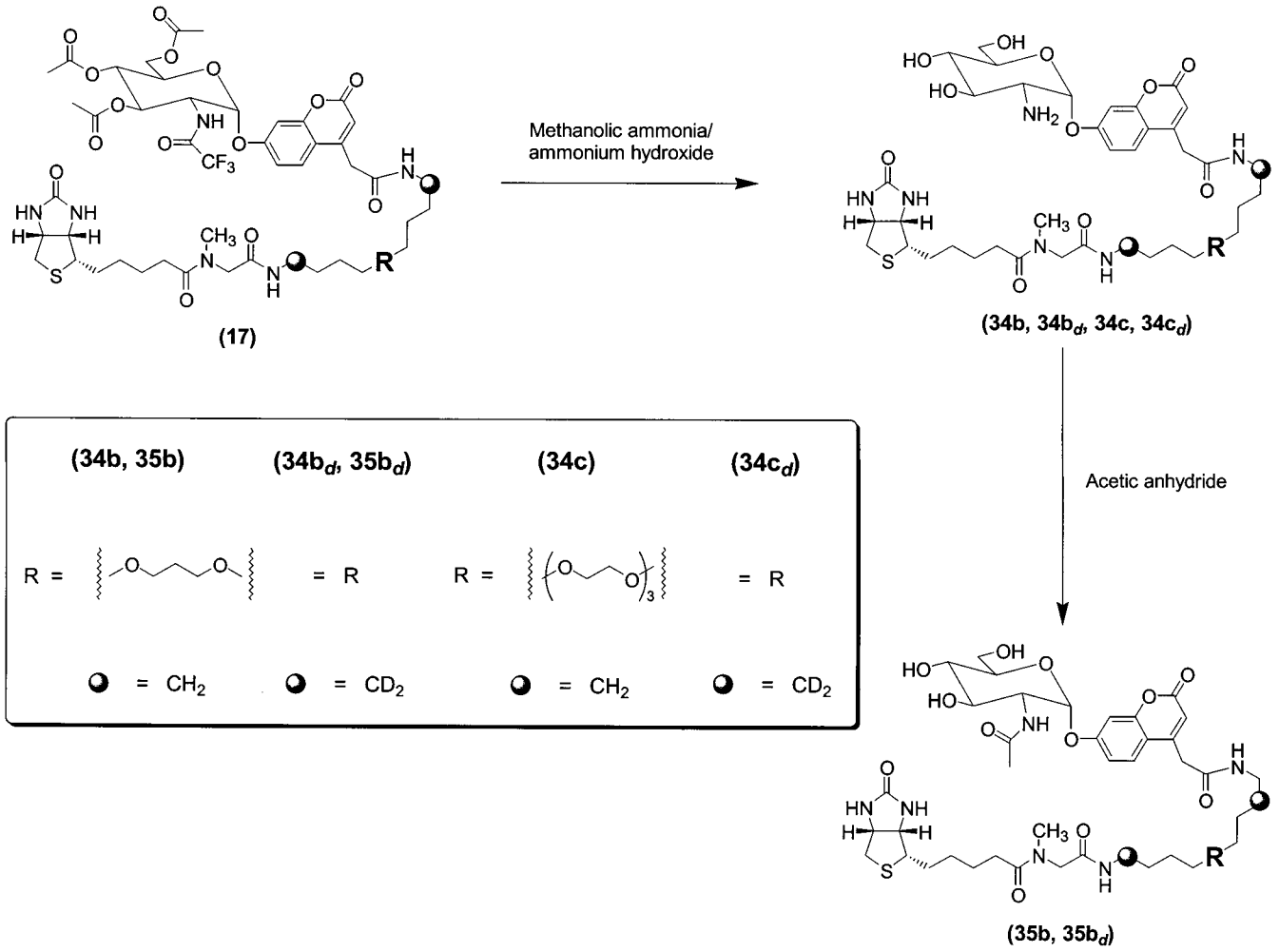
*Deprotection of Conjugate **17c$_d$** (SFA Internal Standard Conjugate) (**34c$_d$**).* As done previously for **17b**, using **17c$_d$**. Yield 88%, semipreparative column, gradient (2), t_R = 32.8 min. ^{1}H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 18H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 957.67, $(M+2H^+)^{2+}$: 479.43.

*N-Acetylation of **34b** (**35b**).* To 3 mg (3.7 μmol) of **34b** in 0.75 mL of purified water was added 4 mg (37 μmol) of sodium carbonate with stirring, followed after complete dissolution by 2 μL (18 μmol) of acetic anhydride. The reaction was allowed to stir for 4 h at room temperature, followed by adjustment of the pH to 3 with dilute trifluoroacetic acid and purification by reversed-phase HPLC [semipreparative column, gradient (2), t_R = 34.3 min]. The final product was dried by vacuum centrifugation to yield 3 mg (95%) as a thin, colorless film. ^{1}H NMR (D_2O) δ 7.67 (d, 1H), 7.12 (m, 2H), 7.19 (dd, 1H), 6.28 (s, 1H), 5.62 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.05 (dd, 1H), 4.00 (d, 2H), 3.88 (t, 1H), 3.75 (s, 2H), 3.68 (s, 2H), 3.59 (m, 1H), 3.52 (m, 1H), 3.45 (m, 8H), 3.23 (m, 5H), 3.00 (d, 3H), 2.88 (dd, 1H), 2.75 (dd, 1H), 2.40 (dt, 2H), 1.97 (s, 3H), 1.69 (t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 921.81, $(M+Na^+)^+$: 943.85, $(M+2Na^+)^{2+}$: 483.47.

*N-Acetylation of **34b$_d$** (SFD Internal Standard Conjugate) (**35b$_d$**).* As done previously for **34b**, using **34b$_d$**. Yield 94%, semipreparative column, gradient (2), t_R = 34.7 min. ^{1}H NMR (D_2O) δ 7.67 (d, 1H), 7.12 (m, 2H), 7.19 (dd, 1H), 6.28 (s, 1H), 5.62 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.05 (dd, 1H), 4.00 (d, 2H), 3.88 (t, 1H), 3.75 (s, 2H), 3.68 (s, 2H), 3.59 (m, 1H), 3.52 (m, 1H), 3.45 (m, 8H), 3.23 (m, 1H), 3.00 (d, 3H), 2.88 (dd, 1H), 2.75 (dd, 1H), 2.40 (dt, 2H), 1.97 (s, 3H), 1.69 (t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 925.87, $(M+Na^+)^+$: 947.79.

*N-Sulfation of **34c** (SFA Substrate Conjugate) (**36**).* To 2.5 mg (2.6 μmol) of **34c** in 0.5 mL of purified water was added 3 mg (29 μmol) of sodium carbonate with stirring at room temperature, followed after complete dissolution by 2 mg (13 μmol) of sulfur trioxide−pyridine complex (Lancaster, Lancaster, U.K.). The reaction was allowed to stir for 4 h at room temperature, followed by adjustment of the pH to 3 with dilute trifluoroacetic acid. The mixture was first purified by reversed-phase HPLC using TFA [semipreparative column, gradient (2), t_R = 42.6 min], and the correct product fractions were concentrated by vacuum centrifugation to 0.5−1 mL and repurified by reversed-phase HPLC without TFA [semipreparative column, gradient (2), t_R = 20.5 min, broadly eluting]. The

Scheme 7



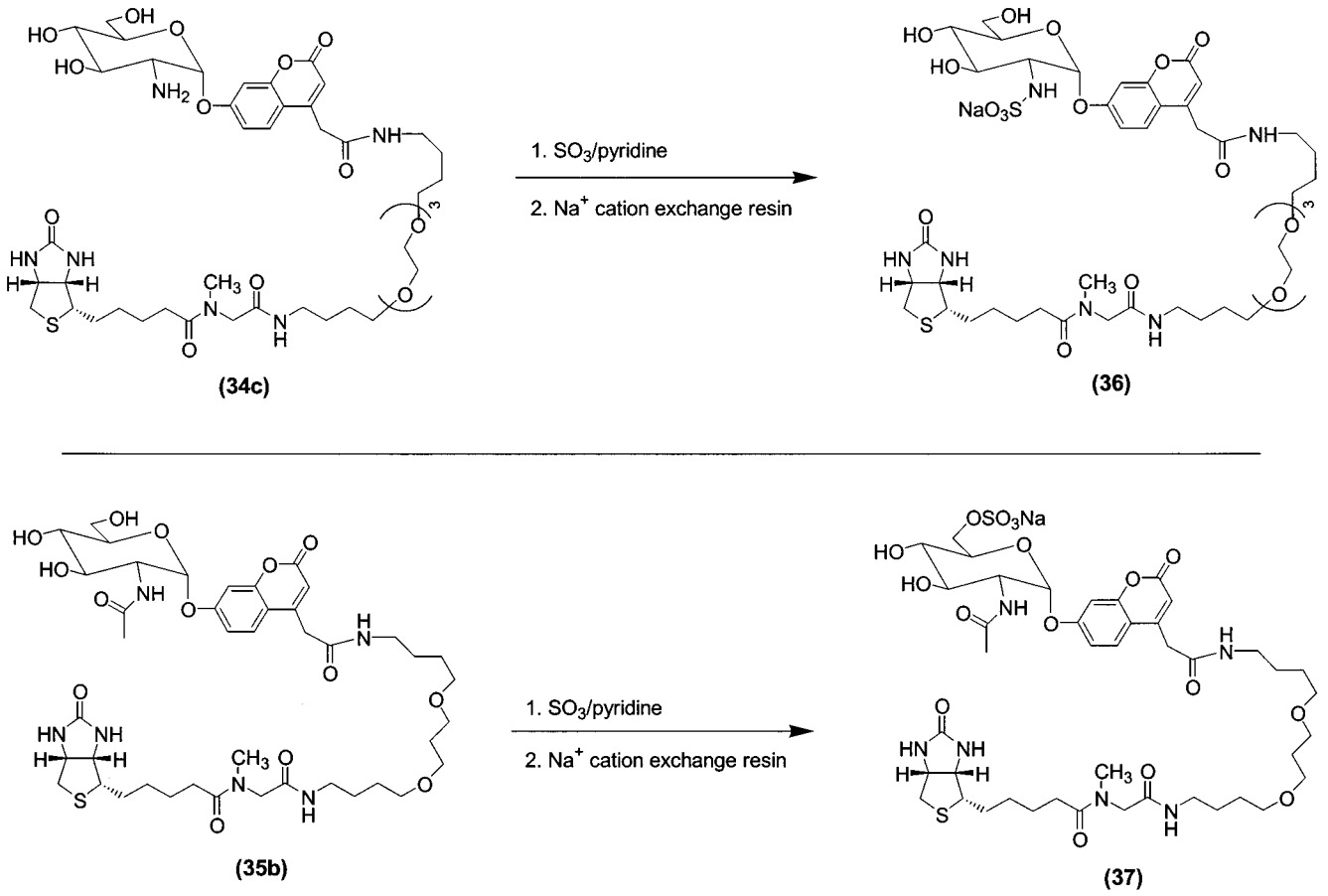
product fractions were again concentrated to 1.0 mL and applied to a short column (300 mg) of AG MP-1 cation-exchange resin, sodium form. The column was washed with 7 mL of purified water, and the combined eluants were concentrated to 1 mL and lyophilized to give 2.0 mg (72%) of an off-white powder. ^{1}H NMR (D_2O) δ 7.70 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.36 (s, 1H), 6.00 (d, 1H), 4.55 (m, 1H), 4.37 (m, 1H), 4.05 (d, 2H), 3.85 (m, 3H), 3.75 (m, 3H), 3.60 (m, 18H), 3.41 (t, 2H), 3.26 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 1056.81, $(M-Na^+)^-$: 1032.20.

N-Acetyl-glucosamine-6-O-sulfation of ***35b*** *(SFD Substrate Conjugate) (****37****).* 4.5 mg (4.9 μmol) of **35b** was dissolved in 300 μL of anhydrous dimethylformamide under argon. The solvent was then removed by vacuum centrifugation. This was repeated, followed by drying overnight in vacuo in the presence of P_2O_5. The film was redissolved in 400 μL of deuterio-dimethylformamide (d_7) in the presence of three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C) under argon. Then 10 μL of deuterio-pyridine (d_5) was added, and the mixture was allowed to agitate by orbital shaking for 30 min, followed by transfer to an NMR tube (previously dried for 48 h at 150 °C) containing a single activated molecular sieve. A stock solution of 1 μmol/μL sulfur trioxide−pyridine complex in deuterio-dimethylformamide was added in 3 μL aliquots followed by mixing by inversion for 30 min and ^{1}H NMR analysis to determine the extent of reaction.

The extent of reaction was evaluated by chemical shift change of the *N*-acetyl-glucosamine C-6 methylene protons upon sulfation. These protons shift from a broad singlet at 3.82 ppm to two *pro*-chiral multiplets at 4.06 and 4.17 ppm. Mono-sulfation of the unprotected substrate stops at 55−65% sulfation of the conjugate. Above this level, nonspecific di- and tri-*O*-sulfation occurred. A total of 27 μL (27 μmol) of sulfur trioxide−pyridine solution was used to obtain 60% sulfation.

The reaction solution was rinsed from the NMR tube with methanol. The molecular sieve was washed with methanol, and the combined solvents were removed by vacuum centrifugation. The resultant film was reconstituted with purified water, and the mixture was purified by reversed-phase HPLC without TFA [semipreparative column, gradient (2), t_R = 23.0 min, broadly eluting]. The product fractions were combined and concentrated by vacuum centrifugation to 1 mL and applied to a short-column (300 mg) AG MP-1 cation-exchange resin (sodium form). The column was washed with 7 mL of purified water, and the combined eluants were concentrated to 1 mL and lyophilized to yield 2 mg (45%) of an off-white powder. ^{1}H NMR (D_2O) δ 7.71 (d, 1H), 7.20 (m, 2H), 6.46 (s, 1H), 5.69 (d, 1H), 4.55 (m, 1H), 4.35 (m, 1H), 4.20 (s, 2H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.99 (m, 2H), 3.82 (d, 2H), 3.66 (t, 1H), 3.55 (m, 16H), 3.22 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 2.01 (s, 3H), 1.71 (m, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS $(M+H^+)^+$: 1023.79, $(M-Na^+)^-$: 999.75.

Scheme 8



RESULTS AND DISCUSSION

Preparation of Deuteratable, Polyether Diamine Linkers. As described in the Introduction, our enzyme analysis methodology makes use of a linker substructure, both deuterated and nondeuterated, to connect biotin to a particular substrate. Our initial attempts at linker synthesis were based on the use of 4,7,10-trioxa-1,13-tridecane diamine as a linker in a substrate conjugate made previously (*5*). The octa-deuterio form of that diamine was synthesized by Michael addition of d_2 (O−D) -ethylene glycol and d_3-acrylonitirile in D_2O. While this reaction produced the desired product in good yield and with little loss of deuterium, the subsequent reduction product was obtained in very low yield (due mostly to elimination of the Michael adduct), and with considerable exchange of the deuterium α to the cyano group. A procedure described previously (*15*) for the preparation of a polyether dichloride was modified to generate a diamine in good overall yield and with little or no loss of incorporated deuterium (Scheme 1). The diamine linkers used in the present study are prepared from 1,3-propanediol and triethylene glycol. However, identical methods were used to produce polyether diamines from ethylene, diethylene, and tetraethylene glycols. In addition, this procedure allows for a variety of levels of stable isotopic substitution (e.g., using commercially available d_4-ethylene glycol, d_3-acrylonitrile, or ^{13}C and/or ^{15}N sodium cyanide) to increase the linker mass by up to +24 amu, in 2 amu increments. Due to issues of scale, we resorted to preparative reversed-phase HPLC purification of the final diamine products, using UV detection at 210 nm. Vacuum distillation may be preferred when linkers are prepared in greater quantity.

Synthesis of Glucosamine−Coumarin Acid Glycoconjugates for Profiling Enzyme Activity in Sanfilippo Subtypes A and D. Our original chemistry to produce substrates for the enzymes relevant to Sanfilippo subtypes A and D was based on the attachment of the linker to the appropriate *p*-acrylamidophenyl glycosides (see below). These conjugates were found to be resistant to enzyme action. Due to the lack of information in the literature concerning the substrate specificity of these enzymes, we opted to conservatively modify the 4-methylumbelliferyl glycoconjugates previously reported as substrates for these enzymes (*16*, *17*). We used glycosides of umbelliferyl-4-acetic acid to further conjugate this substrate substructure to different linkers (Scheme 2), and, ultimately, to sarosinyl biotin (Scheme 3). Synthesis of the protected 7-hydroxy-coumarin-4-*tert*-butoxy acetate **10** and subsequent coupling of the trimethylsilyl ether of **10** with 1-fluoro-2-deoxy-2-(*N*-trifluoroacetyl)-3,4,6-tri-*O*-acetyl-glucosamine gave a mixture of **11** and **12** (partially deprotected by boron trifluoride−etherate in the coupling reaction to form **11**). The remaining **11** was easily deprotected without cleavage of the glycosidic linkage under anhydrous conditions using dry trifluoroacetic acid. The overall yield of the desired α-anomer was consistent with earlier syntheses of analogous 4-methylumbelliferyl glycoconjugates (*13*) (Scheme 2).

Activation and conjugation of **12** to diamines **7** yielded little or no product with a variety of coupling reagents, including DCC, EDC, and PYBOP under standard coupling conditions. When care was taken to azeotropically dry the starting material, and when activation was performed using 2,3,5,6-tetrafluorophenyl trifluoroacetate in the presence of an inert drying agent such as

molecular sieves, the active ester of **12** was consistently produced in very high yields. Several such successful reactions were performed in d_7-dimethylformamide in an NMR tube and monitored by ^{1}H NMR chemical shift change of the α-carboxylic acid methylene protons from 3.92 to 4.73 ppm upon activation. Although attempts to purify and isolate this activated intermediate were unsuccessful, in situ formation of the active ester and coupling to the diamines **7** consistently produced the desired amide products in at least 85% yield.

Further conjugation of the products **13** to sarcosinyl biotin was performed by a similar route involving careful preparation of tetrafluorophenyl *N*-methyl-*N*-biotinyl glycinate via a minor modification of a procedure by Wilbur (*7*) (Scheme 3). From this point, removal of protecting groups (Scheme 7) and functionalization of the carbohydrate (Schemes 7 and 8) ultimately afford the desired substrate and internal standard conjugates for profiling enzyme activity in Sanfilippo subtypes A and D (**34cd**, **35b**$_d$, **36**, and **37**). It should be noted that sulfation of the primary *N*-acetyl glucosamine hydroxyl of **35b** without additional steps to protect the secondary alcohols was performed by addition of sulfur trioxide−pyridine to the substrate in deuterated dimethylformamide in sub-stoichiometric amounts, and the reaction was monitored by ^{1}H NMR. Although the total amount of sulfating reagent required was in roughly 3-fold excess, addition of this amount in one portion yielded multiply sulfated products. Furthermore, mono-sulfation of the carbohydrate was uniform until roughly 40% of the starting material remained. At this point, di- and tri-sulfation occurred, despite a considerable quantity of unsulfated starting material remaining.

Synthesis of *p*-Amidophenyl Glycoconjugates for Profiling Enzyme Activity Involved in Sanfilippo Subtypes B and C. The commercial availability of some *p*-nitrophenyl glycoconjugates initially led our synthetic efforts. Synthesis of the substrate for Sanfilippo subtype C led us to the chemistry by Voznyi et al. (*13*) for the preparation of commercially unavailable 2-amino carbohydrate conjugates. Reduction and acrylation of such conjugates by the method of Romanowska (*18*) was achieved in good yield (Scheme 4). However, the following Michael addition to diamines **2** was much less successful (Scheme 5). Final coupling to sarcosinyl biotin was effected by active ester chemistry described above (Scheme 6).

Application of ACESIMS to Sanfilippo Syndrome. The use of the above substrate and internal standard conjugates for ACESIMS profiling of enzyme velocities involved in the Sanfilippo syndrome will be described elsewhere (ref 8). The ACESIMS methodology has been demonstrated to be rapid, sensitive, and accurate in confirming diagnoses of Sanfilippo subtypes. In addition, the ACESIMS approach has allowed all four Sanfilippo enzymes to be profiled in two duplex incubations, reducing the number of assays required. Owing to the sensitivity of ESI-MS, ACESIMS enzyme velocities have been calculated using 3.5 μg of patient total cell protein (obtained from ∼2500 fibroblasts), requiring only 400 ng of substrate conjugate per assay. Although multistep synthesis is required to prepare these ACESIMS conjugates, several thousand assays can be performed per milligram of substrate. In addition, the modular nature of ACESIMS substructures allows for combinations of previously prepared deuterated and nondeuterated linkers, substrates, and sarconsinyl biotin to develop conjugates for a host of clinical and biomedical applications.

ACKNOWLEDGMENT

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