Synthesis and characterization of biotin derivatives as multifunctional oligosaccharide tags

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Abstract
The synthesis of novel multifunctional oligosaccharide tags with amino, azido, and alkyne termini is described. Tags with an amino terminus can be introduced into the carbohydrate at the reducing end through reductive amination, while 1,3-dipolar cycloaddition ("click chemistry") can be used to label azido sugars with oligosaccharide tags having an alkyne terminus and vice versa.

Keywords: Oligosaccharide, sugar azide, reductive amination, Huisgen 1,3-dipolar cycloaddition, click chemistry

Introduction

Glycosylation of proteins represents the most structurally elaborate form of protein post-translational modification. Such a process can affect folding, stability, and biological activity of proteins; further it can also influence their interaction with other biomolecules. These glycans have been found to participate in molecular recognition, inter- and intracellular signaling, embryonic development, fertilization, immune defense, inflammation, cell adhesion and division processes, viral replication and parasitic infections.[1]

The analysis of carbohydrate oligomers is crucial for complete characterization of polysaccharides and glycoconjugates. The analysis of biologically important oligosaccharides is complicated by structural complexity (stereochemistry, linkage, and anomericity), by poor detectability in chromatography, and by limitation in sample amount.[2,3] Because carbohydrates lack chromophores, they have been labeled in the past with UV-active or fluorescent[4-8] tags to improve detectability during chromatography.[9-13] The label is most commonly introduced by reductive amination.[14,15]

Our group has previously synthesized a prototypical multifunctional small-molecule tag (Figure 1) for covalent introduction into oligosaccharides which helps to increase the UV

sensitivity, separation and isolation of the labeled oligosaccharides.[16] The structure includes a primary amine for reductive amination, a UV-active portion, and biotin functionality. The tag is different in that many other small-molecule tags described in the literature have an aromatic amine functionality with lower nucleophilicity.[2] The primary amine group of the new tag was successfully used in aminomethyl-*C*-glycoside formation through *in situ* reduction of the intermediate imine by sodium cyanoborohydride ($NaBH_3CN$). Low molecular weight carbohydrates and linear oligosaccharides could be labeled with high efficiency. However, subsequent experiments with higher molecular weight oligosaccharides resulted in greatly reduced labeling efficiency with concurrent aldehyde reduction (alditol formation) in the competing reaction channel. Here, we report modifications in the structure of **1**. The impact that length and polarity of the tether between the primary amino group and the chromophore had on the derivatization reaction was systematically investigated. We also introduced structural changes to the UV core.

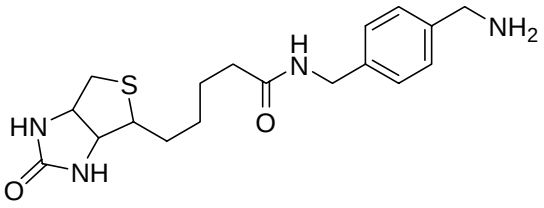


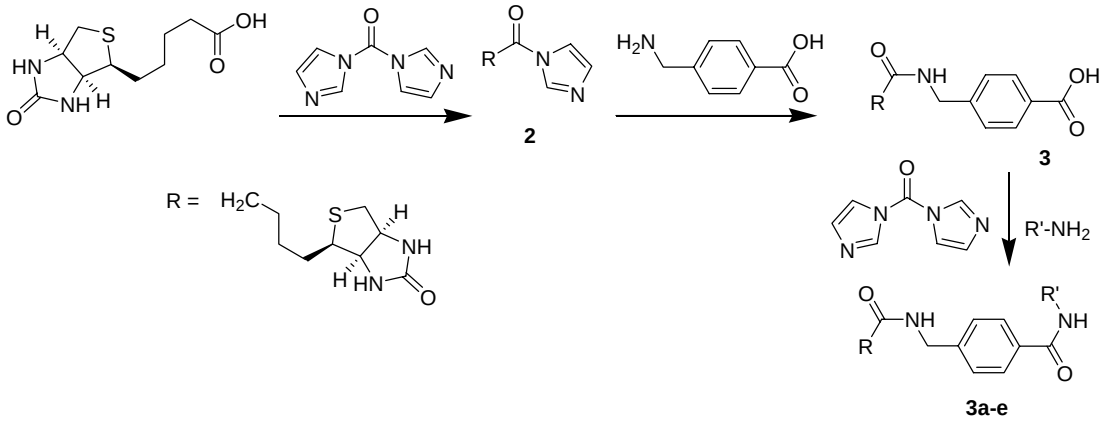
Figure 1. Oligosaccharide Tag[16], **(1)**

As an alternative to reductive amination, oligosaccharide azides or alkynes can also be labeled through Huisgen 1,3-dipolar cycloaddition with alkyne tags and azide tags, respectively, to afford triazoles. This very powerful approach has been popularized in the recent past as the "click" reaction.[17-23] The copper-catalyzed version of this reaction has proven very successful which was first investigated independently by Sharpless[24] and Meldal[25] groups. It has become a mild, efficient, and widely-used method to synthesize five-membered ring 1,2,3-triazoles and has high compatibility with functional groups (alcohols, carboxylic acids, amines) in different solvent systems, including water. In the field of carbohydrate chemistry, click chemistry has been used for the synthesis of glycoconjugates[26,27] and carbohydrate macrocycles[28,29] in which a sugar possessing an azido function is grafted onto a saccharide,[30] a peptide,[31] or a polymeric chain.[32] Here we report the synthesis of oligosaccharide tags bearing azido and alkyne termini which can be used to label oligosaccharides with alkyne or azido termini, respectively. We have applied this method to the covalent labeling of carbohydrate azides with new small-molecule alkyne-tags. Labeling of sugar azides with alkyne-tags or alternatively sugar alkynes with azide-tags under Huisgen cycloaddition conditions is possible with excellent yields and regioselectivity.

Results and Discussion

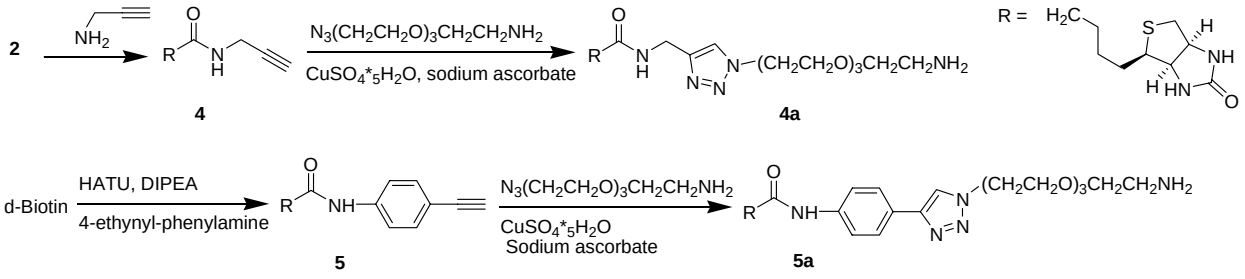
We had previously observed that the efficiency of labeling oligosaccharides with compound **1** decreased with increasing molecular mass and branching of the oligosaccharides; a fact that was attributed to insufficient length of the spacer between biotin and the amino group. The first modification we pursued was to increase the length of the tether by incorporating propane-1,3-diamine and hexane-1,6-diamine. D-Biotin was activated with 1,1'-carbonyldiimidazole in dry freshly-distilled dimethylformamide (DMF) to give compound **2** (2.05 g, 6.96 mmol, 85%) (Table 1). Moisture and amines in DMF are deleterious as they lead to hydrolysis and aminolysis, respectively, of the reactive intermediate **2**. Hence the use of dry, fresh DMF is crucial to the success of the reaction. The activated biotin **2** can be isolated and is stable under dry, inert (under nitrogen) conditions. Compound **2** was used for coupling with 4-aminomethylbenzoic acid to give **3** (2.78 g, 7.36 mmol, 90%) in excellent yield. In turn, compound **3** was activated with 1,1'-carbonyldiimidazole and was immediately added to a vigorously stirred solution of excess diamine (propane-1,3-diamine and hexane-1,6-diamine) to give **3a** (98 mg, 0.23 mmol, 85%) and **3b** (117 mg, 0.25 mmol, 64%) in good to very good yields. Use of excess solution of the diamine helps to avoid addition of activated **2** on both amino groups of the diamine. Surprisingly, the solubility of **3a** and **3b** was found to be better in polar solvents such as water and methanol in comparison to the original compound **1**. This led us to the additional hypothesis that the observed decrease in labeling efficiency may be connected to the solubility of the tag and the oligosaccharide in methanol or water. Consequently, to further improve the solubility of the tag, we incorporated 2,2'-[ethane-1,2-diylbis(oxy)]diethanamine as a spacer to obtain **3c** (105 mg, 0.21 mmol, 56%) which showed very good solubility in polar solvents and much improved labeling efficiency as discussed further below. For the investigation of 1,3-dipolar cycloaddition, we prepared compounds **3d** and **3e**. Compound **3d** requires a carbohydrate azide for coupling while compound **3e** requires a carbohydrate alkyne. Compounds **3d** (176 mg, 0.42 mmol, 80%) and **3e** (295 mg, 0.66 mmol, 50 %) were obtained from **3** after 1,1'-carbonyldiimidazole activation with an equimolar ratio of prop-2-yn-1-amine and 2-azidoethanamine (2.35 g, 27.30 mmol, 56%)[33], respectively.

Table 1. Synthesis of compound **3a-e**



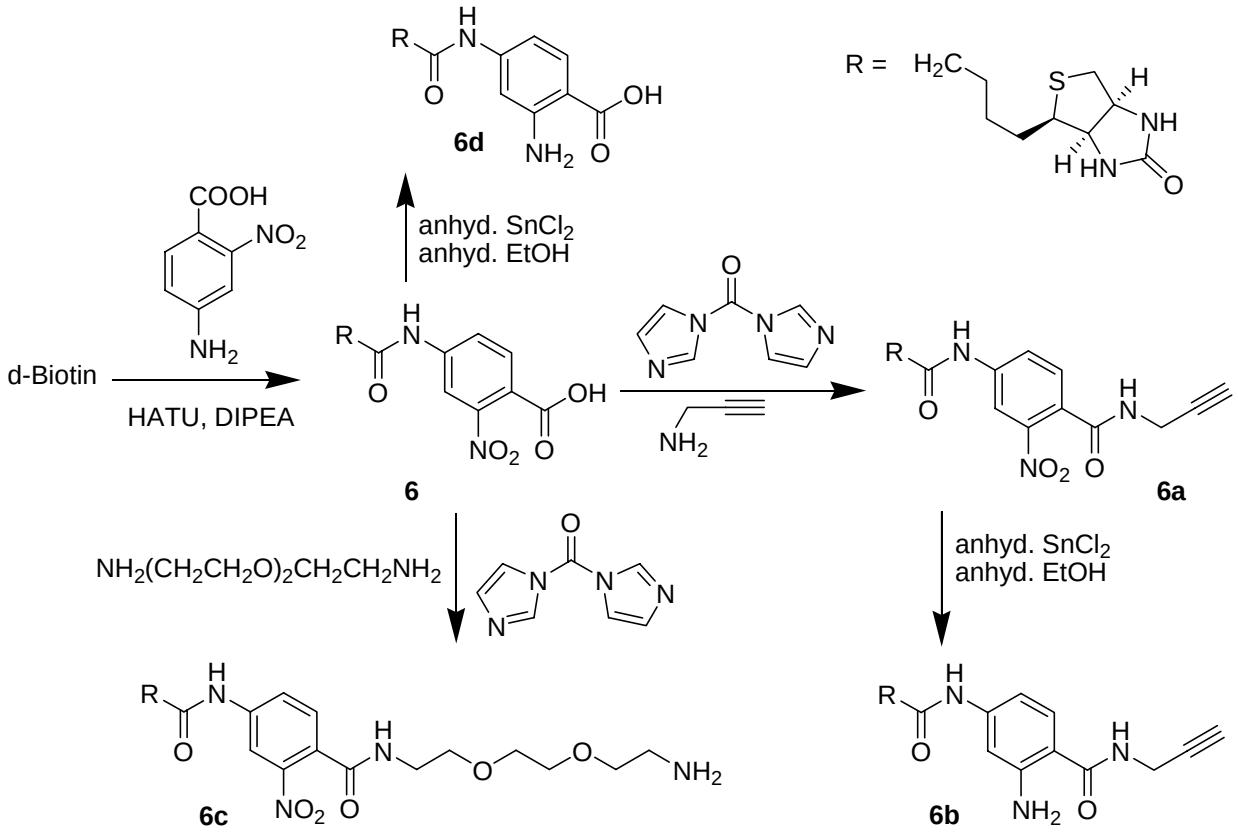
	Amines used	R′
3a	Propane-1,3-diamine	- $CH_2(CH_2)_2NH_2$
3b	Hexane-1,6-diamine	- $CH_2(CH_2)_5NH_2$
3c	2,2'-(Ethane-1,2-diylbis(oxy))diethanamine	-$CH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$
3d	Prop-2-yn-1-amine	-CH_2CCH
3e	2-Azidoethanamine	-$CH_2CH_2N_3$

The synthesis of **4** (0.71 g, 2.52 mmol, 78%) (Scheme 1) was accomplished through coupling of **2** with prop-2-yn-1-amine. Cycloaddition between **4** and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine in water gave **4a** (48 mg, 0.10 mmol, 54%) in moderate yield. In contrast to this result, the primary amine group of 4-ethynylbenzenamine failed to couple with **2** in DMF during the preparation of **5**. This was attributed to lower nucleophilicity of the amino group due to resonance with the aromatic ring. However, coupling promoted by *N*-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU)[34-37] gave **5** (202 mg, 0.59 mmol, 72%). Subsequent reaction of **5** with 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine gave **5a** (73 mg, 0.13 mmol, 56%) in moderate yield.



Scheme 1. Synthesis of **4a** and **5a**.

In an effort to increase the UV sensitivity of the label, we introduced an aromatic nitro moiety in the tags. Compound **6** (0.96 g, 2.35 mmol, 79%) (Scheme 2) was synthesized by coupling biotin with 4-amino-2-nitrobenzoic acid with the help of HATU. Compound **6** serves as intermediate for the synthesis of two tags, **6a** (85 mg, 0.19 mmol, 52%) and **6c** (105 mg, 0.21 mmol, 56%) downstream. The UV activity of **6a** and **6c** is not enhanced significantly as compared to original tag **1**. This was attributed to the electron withdrawing nature of all three functional groups on the aromatic ring in compound **6a** and **6c** including the nitro group. However, the nitro group on the aromatic ring can be selectively reduced $SnCl_2$[38] in anhydrous ethanol without affecting the other functional groups[39-42] to produce fluorescent compounds **6b** (20 mg, 0.05 mmol, 71%) and **6d** (quantitative yield).



Scheme 2. Synthesis of **6a-d**.

To evaluate the labeling efficiency of the tag (**3c**), we labeled standard oligosaccharides (*N*-acetylglucosamine, maltose, maltotriose, lacto-*N*-fucopentaose-II (LNFP-II) (human milk oligosaccharide) and lacto-*N*-difucohexaose-II (LNDFH-II) (human milk oligosaccharide)) with **3c** using a previously established procedure[16]. Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) analysis (Figure 2) of the labeling reaction showed that compound **3c** can be covalently attached to oligosaccharides without any side reaction such as

alditol formation to give quantitative conversion of oligosaccharides to their respective labeled derivatives.

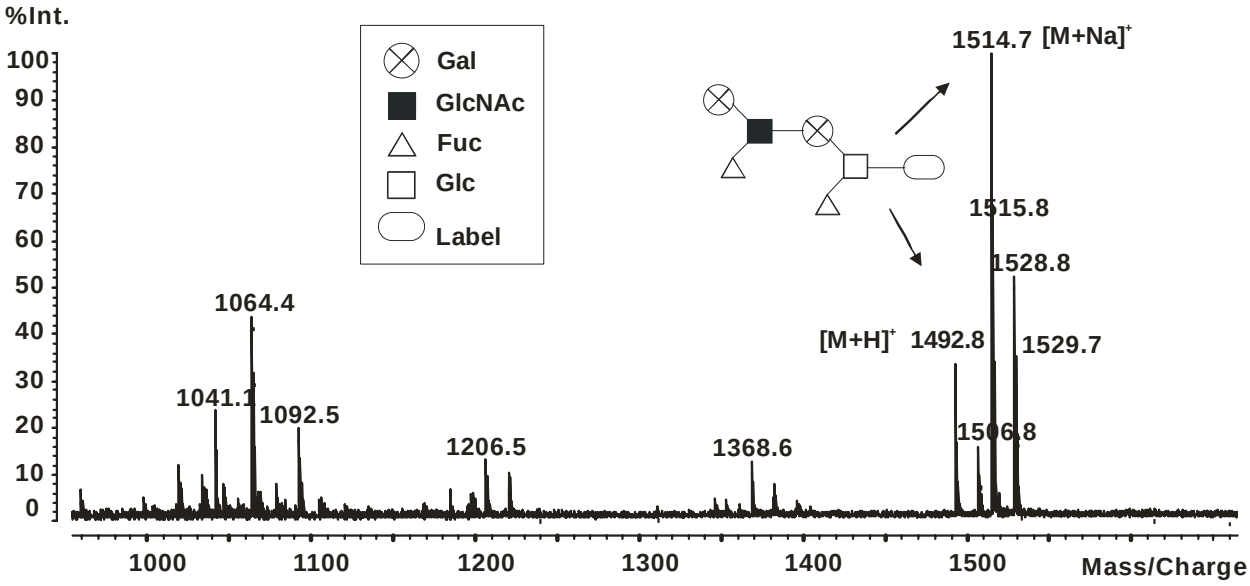


Figure 2. MALDI-TOF spectrum of LNDFH-II labeled with **3c**

Conclusions

Novel oligosaccharide tags were synthesized and characterized. During the synthesis, we observed that the intermediate, activated biotin can be isolated and is stable under dry and inert conditions. The compounds containing a primary amine group can be used to label oligosaccharides through reductive amination. In addition, compounds containing an azide functional group can be used to label oligosaccharide with an alkyne terminus and vice versa.

Experimental Section

General Procedures. Thin layer chromatography (TLC) was performed on plates coated with 0.25 mm of silica gel with UV-indicator (254 nm). Column chromatography was performed on silica gel (Premium Rf Grade, porosity 60Å, particle size 40-75μm, 200x400 mesh). ^{1}H-NMR, ^{13}C-NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer with specified deuterated solvents. Exact mass measurements were performed on the AccuTOF DART (Direct Analysis in Real Time, Time-of-Flight Mass Spectrometer) (JEOL Ltd.,Tokyo, Japan). Matrix-Assisted Laser Desorption/Ionization (MALDI) Time Of Flight (TOF) mass spectra were recorded on a Shimadzu/Kratos (Columbia, MD) AXIMA CFR mass spectrometer

in reflectron mode. The samples were coprecipitated with 2,5-dihydroxybenzoic acid (DHB, 5 mg/100 μL in acetonitrile:water (1:1) and were irradiated by a N_2-laser (λ = 335 nm) unless stated otherwise. Melting point ranges were determined using a Thomas Hoover Capillary Melting Point Apparatus and are uncorrected. During purification of labeled oligosaccharides, a heated CentriVap Concentrator (<?xml:namespace prefix = st1 ns = "urn:schemas-microsoft-com:office:smarttags" />Labconco, Kansas City, MO) was used to remove solvent and concentrate the samples. Aliquots of sample were desalted using the Porous Graphitized Carbon (PGC) cartridges (Supplier-Thermo Electron Hypersil Keystone, UK). The cartridges were washed with 60% acetonitrile and deionized water (H_2O) prior to use. Micro liter volumes of solvent were removed in a heated (40 °C) CentriVap Concentrator with spinning at reduced pressure (12 mTorr).

2-Azidoethanamine. The compound was prepared according to the method reported by Foster and Newman.[33] In brief, 2-bromoethylamine hydrochloride (10 g, 48.81 mmol) and sodium azide (6.35 g, 97.68 mmol) dissolved in water (20 mL) in a round bottom flask (50 mL) attached to a water condenser. The solution was heated to 70 °C for 5 hours. After cooling to room temperature, potassium hydroxide (20 g) was added, and 2-azidoethyl amine was separated by steam distillation. Addition of potassium hydroxide (20 g) to the distillate caused phase-separation of the free amine from the aqueous phase. The free amine was extracted from the aqueous phase with Et_2O. Solvent removal at 0 °C under reduced pressure gave the crude product (3.9 g). Distillation of the crude product from potassium hydroxide pellets gave the product as colorless oil (2.35 g, 27.30 mmol, 56%); ^{1}H-NMR (CD_3OD), δ 2.76 (t, *J* =6.0 Hz, 2H), 3.38 (t, *J* =6.0 Hz, 2H). ^{13}C-NMR (CD_3OD), δ 41.7, 55.0; HRMS calculated for $C_2H_6N_4$ $[M+H]^+$: 87.0671, found: 87.0660

Activated d-biotin (2). D-Biotin (2.0 g, 8.2 mmol) was dissolved in anhydrous DMF (15 mL) at 70°C in a round bottom flask with magnetic stirrer. The solution was cooled to room temperature, and 1,1'-carbonyldiimidazole (2.0 g, 12.33 mmol) was added with stirring at room temperature until a white precipitate formed (30 min). The crude precipitate of **2** was filtered and washed with dry chloroform (20 mL). The filter cake was resuspended in chloroform (40 mL) in a round bottom flask (100 mL) attached to a water condenser and was refluxed for 30 min. The suspension was cooled to room temperature and filtered to obtain pure **2** as a white solid (2.05 g, 6.96, mmol 85%); ^{1}H-NMR (DMSO-d_6), δ 1.34-1.74 (m, *J* = 6.9, 6 Hz), 2.59 (d, *J* = 12.4 Hz, 1H), 2.84 (dd, *J* = 12.4 Hz, *J* = 4.95 Hz, 1H), 3.03 (m, *J* = 6.9 Hz, 2H), 3.12 (m, *J* = 6.1 Hz, 1H), 4.15 (m, *J* = 6.1 Hz, *J* = 7.7 Hz, 1H), 4.31 (m, *J* = 7.7 Hz, *J* = 4.95 Hz, 1H), 6.38 (s, 1H), 6.46 (s, 1H), 7.07 (m, 1H), 7.71 (m, 1H), 8.43 (s, 1H). ^{13}C-NMR, (DMSO-d_6), δ 23.5, 27.7, 27.9, 34.0, 39.7, 55.2, 59.1, 60.9, 116.3, 130.1, 136.8, 162.5, 171.4

Biotinylated benzoic acid (3). D-Biotin (2.0 g, 8.2 mmol) was dissolved in anhydrous DMF (15 mL) at 70 °C in a round bottom flask (25 mL) with a magnetic stirrer. The solution was cooled to room temperature and 1,1'-carbonyldiimidazole (2.0 g, 12.33 mmol) was added followed by slow stirring at room temperature until a white precipitate formed. The compound to

be coupled, 4-(aminomethyl) benzoic acid (1.24 g, 8.2 mmol), was added, and the mixture was heated to 100 °C until clear solution obtained (15 min) and was stirred at 50 °C overnight. Cooling to room temperature followed by addition of dichloromethane resulted in a white precipitate which was filtered to yield **3** (2.78 g, 7.36 mmol, 90%); mp: > 250 ºC (decomp.); ^{1}H-NMR (DMSO-d_6), δ 1.32-1.76 (m, 6H), 2.15 (t, 2H), 2.59 (d, 1H), 2.83 (dd, 1H), 3.10 (m, 1H), 4.13 (m, 1H), 4.31 (m, 3H), 6.36 (s, 1H), 6.44 (s, 1H), 7.35 (m, 2H), 7.87 (m, 2H), 8.4 (t, 1H). ^{13}C-NMR (DMSO-d_6), δ 25.1, 27.9, 28.1, 35.0, 41.7, 42.7, 55.3, 59.1, 60.9, 126.8 , 127.0 (2C), 129.2 (2C), 144.8, 162.5, 167.0, 172.0; HRMS calculated for $C_{18}H_{24}N_3O_4S$ $[M+H]^+$: 378.1488, found: 378.1500.

Benzamide (3a). Compound **3** (100 mg, 0.26 mmol) was suspended in dry DMF (4 mL) in a round bottom flask (15 mL) with a magnetic stirrer, and the mixture was heated to 90 ºC to dissolve completely. The solution was cooled to room temperature and 1,1'-carbonyldiimidazole (90 mg, 0.55 mmol) was added to obtain a solution of activated compound **3**. The solution of activated **3** was added to propane-1,3-diamine (158 mg, 2.13 mmol) in DMF (5 mL) over the period of 3 hours with vigorous stirring. After addition, the reaction was stirred for additional 8 hours at room temperature. The product was separated by silica gel chromatography (methanol:ammonium hydroxide; 100:1) to obtain pure **3a** (98 mg, 0.23 mmol, 85%); mp: 140-142 ºC; ^{1}H-NMR (CD_3OD) δ 1.36-1.82 (m, *J* = 6.9 Hz, 8H), 2.27 (t, 2H), 2.7 (m, *J* = 6.9 Hz, 3H), 2.92 (dd, 1H), 3.17 (m, 1H), 3.44 (t, *J* = 6.9 Hz, 2H), 4.26 (dd, 1H), 4.41 (s, 2H), 4.47 (m, 1H), 7.37 (d, 2H), 7.77 (d, 2H). ^{13}C-NMR (CD_3OD) δ 26.9, 29.5, 29.8, 33.2, 36.8, 38.3, 39.8, 41.1, 43.8, 57.0, 61.7, 63.4, 128.6 (2C), 128.7 (2C), 134.6, 144.1, 166.1, 170.1, 176.1; HRMS calculated for $C_{21}H_{32}O_3N_5S$ $[M+H]^+$: 434.2226 found: 434.2220.

Benzamide (3b). Compound **3b** was synthesized analogously to **3a** except that propane-1,3-diamine was replaced with hexane-1,6-diamine. Compound **3** (145 mg, 0.38 mmol), anhydrous DMF (5 mL), 1,1'-carbonyldiimidazole (132 mg, 0.81 mmol), hexane-1,6-diamine (175 mg, 1.51 mmol) in DMF (5 mL). Chromatography: (SiO_2, methanol:ammonium hydroxide, 100:1), Yield (117 mg, 0.25 mmol, 64%); mp: 172-175 ºC; ^{1}H-NMR (CD_3OD) δ 1.43, (m, 14H), 2.27 (t, 2H), 2.68 (m, 3H), 2.92 (dd, 1H), 3.17 (m, 1H), 3.35 (t, 2H), 4.26 (dd, 1H), 4.41 (s, 2H), 4.48 (m, 1H), 7.37 (d, 2H), 7.77 (d, 2H). ^{13}C-NMR (CD_3OD) δ 26.9, 27.6, 27.9, 29.5, 29.8, 30.5, 33.1, 36.8, 40.9, 41.1, 42.3, 43.8, 57.0, 61.7, 63.4, 128.5 (2C), 128.6 (2C), 134.8, 144.0, 166.2, 170.0, 176.1; HRMS calculated for $C_{24}H_{38}O_3N_5S$ $[M+H]^+$: 476.2695, found: 476.2710.

Benzamide (3c). This reaction carried out analogously to synthesis of **3a** except propane-1, 3-diamine was replaced with 1,2-bis(2-aminoethoxy)ethane. **3** (140 mg, 0.37 mmol), anhydrous DMF (3 mL), 1,1'-carbonyldiimidazole (120 mg, 0.74 mmol), 1,2-bis(2-aminoethoxy)ethane (600 mg, 4.05 mmol in 5 mL DMF). Chromatography: (SiO_2, methanol:ammonium hydroxide, 100:1); yield (105 mg, 0.21 mmol, 56%); mp: 130-133 ºC; ^{1}H-NMR (CD_3OD), δ 1.43 (m, 2H), 1.65 (m, 4H), 2.27 (t, 2H), 2.69 (d, 1H), 2.84 (t, *J* = 5.1 Hz, 2H), 2.92 (dd, 1H), 3.17 (m, 1H), 3.56 (m, *J* = 5.1 Hz, 4H), 3.65 (m, 6H), 4.26 (m, 1H), 4.41 (s, 2H) 4.48 (m, 1H), 7.37 (d, 2H), 7.79 (m, 2H). ^{13}C-NMR (CD_3OD) δ 26.9, 29.5, 29.8, 36.8, 40.9, 41.1, 41.7, 43.7, 57.0, 61.7,

63.4, 70.6, 71.4 (2C), 71.9, 128.6 (4C), 134.5, 144.2, 166.2, 170.1, 176.1; HRMS calculated for $C_{24}H_{38}O_5N_5S$ $[M+H]^+$: 508.2594, found: 508.2580.

Benzamide (3d). Compound **3** (200 mg, 0.53 mmol) was dissolved in dry DMF (3 mL) in a round bottom flask (15 mL) with a magnetic stirring bar at 90 ºC and cooled down to room temperature. 1,1'-Carbonyldiimidazole (170 mg, 1.05 mmol) was added to obtain a solution of activated **3**. To the solution of activated **3** was added propargylamine (30 mg, 0.54 mmol) and the reaction was stirred for 8 hours at room temperature. Dichloromethane was added to the solution until the product precipitated. The precipitate was filtered and refluxed in 10 mL dichloromethane, cooled, filtered, and dried to yield 176 mg (0.42 mmol, 80%) of **3d**; mp: 164-166 ºC; ^{1}H-NMR (DMSO-d_6), δ 1.20-1.70 (m, 6H), 2.15 (t, 2H), 2.59 (d, 1H), 2.82 (dd, 1H), 3.08 (m, *J* = 2.4 Hz, 2H), 4.04 (dd, *J* = 2.4 Hz, 2H), 4.12 (m, 1H), 4.31 (m, 3H), 6.37 (s, 1H), 6.43 (s, 1H), 7.31 (m, 2H), 7.80 (m, 2H), 8.4 (t, 1H), 8.89 (t, 1H). ^{13}C-NMR (DMSO-d_6) δ 25.3, 28.0, 28.2, 35.1, 39.8, 41.7, 55.4, 59.2, 61.1, 72.8, 79.1, 81.4, 127.0 (2C), 127.3 (2C), 132.2, 143.4, 162.8, 165.8, 172.3; HRMS calculated for $C_{21}H_{27}N_4O_3S$ $[M+H]^+$: 415.1804, found: 415.1800.

Azide (3e). This reaction was carried out as analogously to the synthesis of **3a**, except propane-1,3-diamine was replaced with 2-azidoethanamine. Compound **3** (500 mg, 1.32 mmol), dry DMF (5 mL), 1,1'-carbonyldiimidazole (430 mg, 2.65 mmol), 2-azidoethanamine (150 mg, 1.74 mmol), dichloromethane (20 mL for reflux); Yield: 295 mg, 0.66 mmol, 50 %; mp: 147-150 ºC; ^{1}H-NMR (CD_3OD), δ 1.30-1.80 (m, 6H) 2.27 (t, 2H), 2.69 (d, 1H), 2.92 (dd, 1H), 3.17 (m, 1H), 3.47 (m, *J* = 5.4 Hz, 2H), 3.56 (m, *J* = 5.4 Hz, 2H), 4.26, (m, 1H), 4.41 (s, 2H), 4.48 (m, 1H), 7.38 (m, 2H), 7.79, (m, 2H). ^{13}C-NMR (CD_3OD) δ 26.9, 29.5, 29.8, 36.8, 40.7, 41.1, 43.8, 51.5, 57.0, 61.7, 63.4, 128.6 (2C), 128.7 (2C), 130.9, 144.3, 166.2, 170.3, 176.1; HRMS calculated for $C_{20}H_{28}N_7O_3S$ $[M+H]^+$: 446.1974, found: 446.1980.

Amide (4). Compound **2** (0.95 g, 3.23 mmol) and propargyl amine (0.55 g, 9.98 mmol) were dissolved in acetonitrile (30 mL) at 80 ºC. The reaction was cooled and stirred at room temperature overnight. White precipitate formed was filtered to yield **4** (0.71 g, 2.52 mmol, 78%); mp: 154-157 ºC; ^{1}H-NMR (CD_3OD), δ 1.36-1.80 (m, 6H), 2.20 (t, 2H), 2.57 (t, *J* = 2.47 Hz, 1H), 2.69 (d, 1H), 2.92 (dd, 1H), 3.20, (m, 1H), 3.93 (d, *J* = 2.47 Hz, 2H), 4.29 (dd, 1H), 4.48 (m, 1H). ^{13}C-NMR (CD_3OD), δ 26.7, 29.4, 29.5, 29.7, 36.5, 41.1, 57.0, 61.7, 63.4, 72.1, 80.7, 166.2, 175.7 ; HRMS calculated for $C_{13}H_{20}N_3O_2S$ $[M+H]^+$: 282.1276 found: 282.1270.

Amide (4a). To the suspension of **4** (50 mg, 0.18 mmol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine (41 mg, 0.19 mmol) in water (1.5 mL) were added sodium ascorbate (0.1 mol. equiv.) and $CuSO_4$*$5H_20$ (0.01 mol. equiv.). The reaction mixture was heated at 70 ºC for 2 hours. After completion of reaction (2 hours, TLC, solvent-chloroform:methanol, 2:1), water was removed *in vacuo* and the residue was dissolved in DMF and purified by silica gel chromatography (methanol:ammonium hydroxide, 100:1) to obtain pure **4a** (48 mg, 0.10 mmol, 54%). ^{1}H-NMR (CD_3OD), δ 1.32-1.8 (m, 6H), 2.23 (t, 2H), 2.70 (d, 1H), 2.77 (t, *J* = 5.23 Hz, 2H), 2.92 (dd, 1H), 3.20 (m, 1H), 3.50 (t, *J* = 5.23 Hz, 2H), 3.60, (d, 8H), 3.87 (t, *J* = 4.95 Hz, 2H), 4.29 (dd, 1H), 4.42 (s, 2H), 4.49 (m, 1H), 4.55 (t, *J* = 4.95 Hz,

2H), 7.90 (s, 1H). ^{13}C-NMR (CD_3OD) δ 26.7, 29.5, 29.7, 35.6, 36.6, 41.1, 42.2, 51.5, 57.0, 61.7, 63.4, 70.4, 71.3, 71.5 (2C), 71.6, 73.5, 125.0, 146.2, 166.1, 176.0; HRMS calculated for $C_{21}H_{38}N_7O_5S$ $[M+H]^+$: 500.2655, found: 500.2670.

Amide (5). D-Biotin (200mg, 0.82 mmol) and HATU (312 mg, 0.82 mmol) were suspended in anhydrous DMF (2.5 mL) followed by addition of DIPEA (210 mg, 1.63 mmol) and the reaction mixture and stirred at room temperature for 15 min. After adding 4-ethylaniline (96 mg 0.820 mmol), the reaction was heated to 70 ºC until completion (4 hours). The reaction was cooled to room temperature, concd and separated by silica gel chromatography (chloroform:methanol; 9:1) to obtain **5** as a white solid (202 mg, 0.59 mmol, 72%); mp: 228-230 ºC; ^{1}H-NMR (DMSO-d_6), δ 1.30-1.72 (m, 6H), 2.32 (t, 2H), 2.57 (d, 1H), 2.82 (dd, 1H), 3.12 (m, 1H), 4.06 (s, 1H), 4.13 (m, 1H), 4.30 (m, 1H), 6.36 (s, 1H), 6.44 (s, 1H), 7.39 (d, 2H), 7.61 (d, 2H), 10.05 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 24.9, 28.0, 28.1, 36.1, 38.4, 55.2, 59.1, 60.9, 79.6, 83.5, 115.7, 118.7 (2C), 132.2 (2C), 139.7, 162.6, 171.3; HRMS calculated for $C_{18}H_{22}N_3O_2S$ $[M+H]^+$: 344.1433 found: 344.1440

Amide (5a). Compound **5a** was prepared analogously to **4a**. Compound **5** (80 mg, 0.23 mmol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine (52 mg, 0.24 mmol), water (4 mL), sodium ascorbate (0.1 mol equiv), $CuSO_4$*$5H_20$ (0.01 mol. equiv.), chromatography (SiO_2, methanol:ammonium hydroxide, 100:1) to yield 73 mg (0.13 mmol, 56%) of **5a**; mp: 174-176 ºC; ^{1}H-NMR (CD_3OD), δ 1.44-1.84 (m, 6H), 2.41 (t, 2H), 2.71 (m, *J* = 5.22, Hz, 3H), 2.92 (dd, 1H), 3.22 (m, 1H), 3.43 (t, *J* = 5.22 Hz, 2H), 3.59 (m, 8H), 3.92 (t, *J* = 4.67 Hz, 2H), 4.31 (dd, 1H), 4.48 (m, 1H), 4.62 (t, *J* =4.67 Hz, 2H), 7.65 (d, 2H), 7.77 (d, 2H), 8.32 (s, 1H). ^{13}C-NMR (CD_3OD) δ 26.7, 29.6, 29.8, 37.7, 41.1, 42.0, 51.6, 57.0, 61.7, 63.4, 70.4, 71.3, 71.5, 71.6 (2C), 72.9, 121.5 (2C), 122.8, 127.2 (2C), 127.6, 140.1, 148.4, 166.2, 174.5; HRMS calculated for $C_{26}H_{40}N_7O_5S$ $[M+H]^+$: 562.2812, found: 562.2824.

Biotinylated benzoic acid (6). D-Biotin (725 mg, 2.97 mmol) was added to a round bottom flask (25 mL) containing dry DMF (10 mL) and a stirring rod. The mixture was heated to 90ºC to obtain a clear solution of biotin in DMF. The clear solution was cooled to room temperature followed by addition of HATU (1.13 g, 2.97 mmol) and stirred at room temperature for 15 min. DIPEA (775 mg, 6.0 mmol) and 4-amino-2-nitrobenzoic acid (600 mg, 3.29 mmol) were added and the reaction mixture was stirred at room temperature overnight. Dichloromethane (50 mL) was added until the product precipitated as a white solid. The product was isolated by vacuum filtration and washed with ethyl acetate (3 x 50 mL), dried to obtain **6** (0.96 g, 2.35 mmol, 79%) mp: 205-210ºC (decomp.); ^{1}H-NMR (CD_3OD), δ 1.45-1.83 (m, 6H), 2.42 (t, 2H), 2.69 (d, 1H), 2.92 (dd, 1H), 3.22 (m, 1H), 4.31 (dd, 1H), 4.48 (m, 1H), 7.54 (d, *J* = 8.7 Hz, 1H), 7.67 (dd, *J* = 2.1 Hz, 8.4 Hz, 1H), 8.26 (d, *J*=2.1 Hz, 1H). ^{13}C-NMR (CD_3OD) δ 26.5, 29.5, 29.8, 37.7, 41.1, 57.0, 61.7, 63.4, 115.1, 124.1, 130.4, 132.7, 140.5, 149.0 166.2, 173.4, 174.7; HRMS calculated for $C_{17}H_{21}N_4O_6S$ $[M+H]^+$: 409.1182, found: 409.1200.

Benzamide (6a). Compound **6** (150 mg, 0.37 mmol) was added to a round bottom flask (15 ml) containing dry DMF (1mL) and a stirring rod to obtain clear solution of **6** at room temperature, followed by addition 1,1'-carbonyldiimidazole (120 mg, 0.74 mmol). Solution was stirred

slowly for 10 min followed by addition of prop-2-yn-1-amine (20 mg, 0.36 mmol). Stirring continued for 6 hours. To the solution dichloromethane (~10 mL) was added to obtain a white precipitate which was isolated by filtration and washed with ethyl acetate (3 x 10 mL) and dried to obtain **6a** (85 mg, 0.19 mmol, 52%); mp: 228-230ºC; ^{1}H-NMR (CD_3OD), δ 1.45-1.83 (m, 6H), 2.44 (t, 2H), 2.65 (t, *J* = 2.7 Hz, 1H), 2.70 (d, 1H), 2.92 (dd, 1H), 3.22 (m, 1H), 4.12 (d, J = 2.4 Hz, 2H), 4.30 (dd, 1H), 4.49 (m, 1H), 7.50 (d, *J* = 8.1 Hz, 1H), 7.86 (dd, *J* = 2.1 Hz, 8.1 Hz, 1H), 8.44 (d, *J*=2.1 Hz, 1H). ^{13}C-NMR (CD_3OD) δ 26.4, 29.6, 29.8, 30.1, 37.7, 41.1, 57.0, 61.7, 63.4, 72.7, 80.0, 116.0, 124.5, 127.9, 130.7, 142.6, 148.8, 166.2, 168.7, 174.9; HRMS calculated for $C_{20}H_{24}N_5O_5S$ $[M+H]^+$: 446.1498, found: 446.1490.

Benzamide (6b). Compound **6a** (30 mg, 0.07 mmol) and $SnCl_2$ (64 mg, 0.34 mmol) was mixed with anhydrous ethanol (2 mL) in a round bottom flask (10mL) fitted with nitrogen supply. The mixture was stirred at 70 ºC. As the reaction proceeds, the color of the reaction mixture changes from white (starting material) to yellow (final product). Completion of the reaction (2 hours) was confirmed with TLC (dichloromethane:methanol; 4:1) and ESI-MS (electrospray ionization-mass spectrometry). The reaction mixture filtered through a small silica plug (solvent: methanol) and the collected fractions were concentrated on rotavap to obtain yellow colored **6b** (20 mg, 0.05 mmol, 71%); mp: 196-198 ºC; ^{1}H-NMR (DMSO-d_6), δ 1.30-1.70 (m, 6H), 2.30 (t, 2H), 2.58 (d, 1H), 2.82 (dd, 1H), 3.05 (t, J = 2.1 Hz, 1H), 3.12 (m, 1H), 3.95 (m, *J* = 2.4, *J* = 5.4, Hz, 2H) 4.14 (m, 1H), 4.30 (m, 1H), 6.37 (s, 1H), 6.44 (s, 1H), 6.60 (s, 2H), 6.67 (dd, *J* = 1.8 Hz, *J* = 8.4Hz, 1H), 7.12 (d, *J* = 2.1 Hz, 1H), 7.43 (dd, *J* = 8.7 Hz, 1H), 8.49 (t, *J* = 6.0 Hz, 1H). ^{13}C-NMR (DMSO-d_6) δ 25.0, 27.9, 28.0, 28.1, 35.7, 36.2, 55.3, 59.1, 61.0, 72.3, 81.7, 105.3, 106.2, 108.5, 128.6, 142.5, 150.9, 162.6, 168.1, 171.4; HRMS calculated for $C_{20}H_{26}N_5O_3S$ $[M+H]^+$: 416.1756, found: 416.1750.

Benzamide (6c). Compound **6** (100 mg, 0.24 mmol) was added to a round bottom flask (10 mL). Dry DMF (1 mL) was added to the flask at room temperature to obtain clear solution of **6** in DMF followed by addition 1,1'-carbonyldiimidazole (80 mg, 0.49 mmol) and stirred slowly for 15 min. 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (50 mg, 0.34 mmol) was added, and the mixture was stirred at room temperature until the reaction was completed (1 hour) which was confirmed by TLC (methanol:ammonium hydroxide, 100:1). The product was isolated by chromatography (SiO_2, methanol:ammonium hydroxide, 100:1) to obtain pure **6c** (86 mg, 0.16 mmol 65%); mp: 65-67 ºC; ^{1}H-NMR (CD_3OD), δ 1.45-1.83 (m, 6H), 2.44 (t, 2H), 2.70 (m, 1H), 2.79 (m, *J* = 5.4 Hz, 2H), 2.92 (dd, 1H), 3.22 (m, *J* = 4.5 Hz, 3H), 3.49-3.68 (m, *J* = 5.4 Hz, *J* = 4.5 Hz, 8H), 4.30 (dd, 1H), 4.49 (m, 1H), 7.52 (m, 1H), 7.85 (m, 1H), 8.44 (m, 1H). ^{13}C-NMR (CD_3OD) δ 26.4, 29.6, 29.8, 37.7, 41.0, 41.1, 42.0, 57.0, 61.7, 63.4, 70.3, 71.4, 71.6, 73.2, 115.9, 124.5, 128.5, 130.7, 142.4, 148.7, 166.2, 169.3, 174.9; HRMS calculated for $C_{23}H_{33}N_6O_7S$ $[M+H]^+$: 539.2288, found: 539.2280.

Biotinylated benzoic acid (6d). Compound **6** (200 mg, 0.49 mmol) and $SnCl_2$ (475 mg, 2.51 mmol) was mixed with anhydrous ethanol (5 mL) in a round bottom flask (10mL) fitted with nitrogen supply. The mixture was stirred at 70 ºC. Completion of the reaction (2 hours) was confirmed with TLC (methanol) and ESI-MS. The reaction mixture was cooled to room

temperature, was poured on crushed ice, and was stirred for 30 min. The mixture was filtered, and the white solid was collected and dried. To the dried solid a minimum amount of sodium hydroxide (1 mL, 2M) was added to get a clear solution. The solution was filtered and dilute sulphuric acid was added dropwise to the filtrate to obtain a white precipitate, which was collected by filtration and dried to obtain **6d** in quantitative yield. TLC (methanol) showed one spot; mp: > 250 ºC; ^{1}H-NMR (DMSO-d_6), δ 1.26-1.70 (m, 6H), 2.30 (t, 2H), 2.59 (d, 1H), 2.82 (dd, 1H), 3.11 (m, 1H), 4.13 (m, 1H), 4.30 (m, 1H), 6.37 (s, 1H), 6.45 (s, 1H), 6.60 (dd, *J* = 1.8 Hz, 9.0 Hz, 1H), 7.21 (d, *J* = 1.8 Hz, 1H), 7.58 (d, *J*= 9.0 Hz, 1H), 9.85 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 25.0, 28.0, 28.1, 36.2, 39.7, 55.3, 59.1, 61.0, 104.7, 104.7, 106.5, 131.9, 143.8, 152.4, 162.6, 169.1, 171.5; HRMS calculated for $C_{17}H_{23}N_4O_4S$ $[M+H]^+$: 379.1440, found: 379.1450.

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References

1. Varki, A. *Glycobiology* **1993**, *3*, 97.
2. Hase, S. J. *Chromat., A* **1996**, *720*, 173.
3. Harvey, D. J. *Int. J. Mass Spec.* **2003**, *226*, 1.
4. Anumula, K. R. *Anal. Biochem.* **1994**, *220*, 275.
5. Sato, K.; Sato, K.; Okubo, A.; Yamazaki, S. *Anal. Biochem.* **1998**, *262*, 195.
6. Neville, D. C. A.; Coquard, V.; Priestman, D. A.; te Vruchte, D. J. M.; Sillence, D. J.; Dwek, R. A.; Platt, F. M.; Butters, T. D. Anal. Biochem. **2004**, *331*, 275.
7. Bigge, J. C.; Patel, T. P.; Bruce, J. A.; Goulding, P. N.; Charles, S. M.; Parekh, R. B. *Anal. Biochem.* **1995**, *230*, 229.
8. Toomre, D. K.; Varki, A. *Glycobiology* **1994**, *4*, 653.
9. Kallin, E.; Loenn, H.; Norberg, T. *Glycoconj. J.* **1986**, *3*, 311.
10. Poulter, L.; Karrer, R.; Burlingame, A. L. *Anal. Biochem.* **1991**, *195*, 1.
11. Ohta, M.; Hamako, J.; Yamamoto, S.; Hatta, H.; Kim, M.; Yamamoto, T.; Oka, S.; Mizuochi, T.; Matsuura, F. *Glycoconj. J.* **1991**, *8*, 400.
12. Wang, W. T.; LeDonne, N. C., Jr.; Ackerman, B.; Sweeley, C. C. *Anal. Biochem.* **1984**, *141*, 366.
13. Higashi, H.; Ito, M.; Fukaya, N.; Yamagata, S.; Yamagata, T. *Anal. Biochem.* **1990**, *186*, 355.
14. Borch, R. F.; Bernstein, M. D.; Durst, H. D. *J. Am. Chem. Soc.* **1971**, *93*, 2897.

15. Anumula, K. R. *Anal. Biochem.* **2006**, *350*, 1.
16. Hsu, J.; Chang, S. J.; Franz, A. H. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 194.
17. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2001**, *40*, 2004.
18. Kolb, H. C.; Sharpless, K. B. *Drug Discovery Today* **2003**, *8*, 1128.
19. Brase, S.; Gil, C.; Knepper, K.; Zimmermann, V. *Angew. Chemie, Int. Ed.* **2005**, *44*, 5188.
20. St. Hilaire, P. M.; Lowary, T. L.; Meldal, M.; Bock, K. *J. Am. Chem. Soc.* **1998**, *120*, 13312.
21. Lin, C. H.; Shimazaki, M.; Wong, C. H.; Koketsu, M.; Juneja, L. R.; Kim, M. *Bioorg. Med. Chem.* **1995**, *3*, 1625.
22. Jiao, H.; Hindsgaul, O. J. *Carbohydr. Chem.* **1999**, *18*, 499.
23. Tornoe, C. W.; Meldal, M. Peptides: The Wave of the Future, Proceedings of the Second International and the Seventeenth American Peptide Symposium, San Diego, CA, United States, June 9-14, 2001; 263.
24. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chemie, Inter. Ed.* **2002**, *41*, 2596.
25. Tornoe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057.
26. Fazio, F.; Bryan, M. C.; Blixt, O.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124*, 14397.
27. Perez-Balderas, F.; Ortega-Munoz, M.; Morales-Sanfrutos, J.; Hernandez-Mateo, F.; Calvo-Flores, F. G.; Calvo-Asin, J. A.; Isac-Garcia, J.; Santoyo-Gonzalez, F. *Org. Lett.* **2003**, *5*, 1951.
28. Bodine, K. D.; Gin, D. Y.; Gin, M. S. *J. Am. Chem. Soc.* **2004**, *126*, 1638.
29. Bodine, K. D.; Gin, D. Y.; Gin, M. S. *Org. Lett.* **2005**, *7*, 4479.
30. Chen, Q.; Yang, F.; Du, Y. *Carbohydr. Res.* **2005**, *340*, 2476.
31. Hotha, S.; Kashyap, S. *J. Org. Chem.* **2006**, *71*, 364.
32. Ladmiral, V.; Mantovani, G.; Clarkson, G. J.; Cauet, S.; Irwin, J. L.; Haddleton, D. M. *J. Am. Chem. Soc.* **2006**, *128*, 4823.
33. Forster, M. O.; Newman, S. H. *J. Chem. Soc., Trans.* **1911**, *99*, 1277.
34. Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397.
35. Carpino, L. A.; El-Faham, A. *J. Org. Chem.* **1994**, *59*, 695.
36. Carpino, L. A.; El-Faham, A.; Albericio, F. *J. Org. Chem.* **1995**, *60*, 3561.
37. Carpino, L. A.; Ionescu, D.; El-Faham, A. *J. Org. Chem.* **1996**, *61*, 2460.
38. Bellamy, F. D.; Ou, K. *Tetrahedron Lett.* **1984**, *25*, 839.
39. George, J.; Chandrasekaran, S. *Synth. Comm.* **1983**, *13*, 495.
40. Gamble, A. B.; Garner, J.; Gordon, C. P.; O'Conner, S. M. J.; Keller, P. A. *Synth. Comm.* **2007**, *37*, 2777.
41. Pogorelic, I.; Filipan-Litvic, M.; Merkas, S.; Ljubic, G.; Cepanec, I.; Litvic, M. *J. Mol. Cat. A* **2007**, *274*, 202.
42. Gowda, D. C.; Gowda, S. *Indian J. Chem., Sect. B: Org. Chem.* **2000**, *39B*, 709.