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SYNTHESIS OF LABELED GALACTOSYLHYDROXYLYSINE

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Summary

A concise synthesis of isotopically labeled galactosylhydroxylysine (**2**) was achieved via glycosylation of labeled hydroxylysine derivative (**3**) with (+)-acetobromo-α-D-galactose (**4**) and subsequent hydrolysis in good overall yield. The hydroxylysine derivative (**3**) was prepared from (*S*)-(–)-methyl-2-[bis-(*tert*-butoxycarbonyl)amino]-5-oxopentanoate (**4**) with the introduction of label ($^{13}CD_2$) via a Wittig reaction using $^{13}CD_3PPh_3I$.

Key words: collagen, hydroxylysine, galactosylhydroxylysine, osteoporosis

Introduction

Galactosylhydroxylysine (GHL, **1**) (Figure 1) is a structural component of bone collagen (1, 2), which is formed by post-translational glycosylation of hydroxylysine (3, 4). During the process of bone resorption, GHL (**1**) is released into the serum and excreted in urine (5). It has been found that this degradation product of bone collagen, [GHL (**1**)], is clinically useful as a marker for diagnosis of osteoporosis (6,

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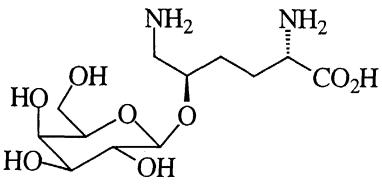
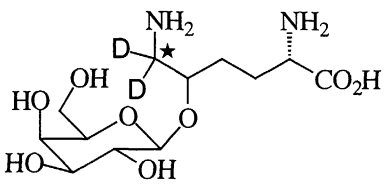
Figure 1



(−)-Galactosylhydroxylysine (1)



Labeled Galactosylhydroxylysine (2)

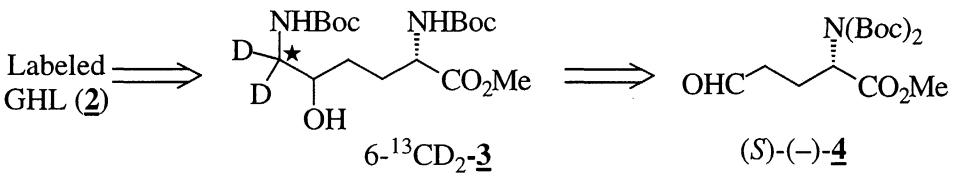
7) and other metabolic bone diseases (8, 9, 10). Additionally, it was also reported that the collagenous glycopeptides containing GHL (1), were recognized by T-cells and may have implications in rheumatoid arthritis (11) and cancer (12, 13). We have been interested in the measurement of GHL (1) in urine by immunoassays for the diagnosis of osteoporosis. Thus, recently we reported a chiral synthesis of GHL (1) (14) as well as the immunogens and tracers (15) required for the development of immunoassays. In this paper, we describe the synthesis of labeled galactosylhydroxylysine (2), which is necessary as an internal standard (IS) for the quantification of GHL (1) by mass spectrometry.

Results and Discussion

Any newly developed clinical immunoassay needs to be correlated to a known method in order to ensure its accuracy. In recent years application of electrospray ionization mass spectrometry (ESI-MS) (16) in combination with liquid chromatography has evolved into a widely applied and routinely used technique for quantification of a variety analytes. The success of ESI-MS in quantification can be attributed to the ease of operation coupled with the robustness of LC-MS interface based on low temperature ionization. Application of an internal standard (IS) is critical for achieving high precision and accuracy in the quantification of analytes by mass spectrometry (16). There are number of important issues which need to be considered when choosing the IS, such as molecular weight, physicochemical properties, and the stability under the assay conditions. Typically, isotopically

labeled analyte is an ideal compound for use as an internal standard, because of its similar properties to the analyte except the molecular weight. In preparing the isotopically labeled IS, it is important to incorporate the isotope in the analyte (ex., **1**) at a position which is stable and non exchangeable. We envisioned (Figure 1) that an analog such as **2**, in which the (6)-CH_2 is replaced by (6)-$^{13}CD_2$ in the hydroxylysine skeleton, would meet this criteria. Thus, the strategy for the synthesis of labeled GHL (**2**) (Figure 2) first involved the synthesis of labeled hydroxylysine derivative (**3**) from (*S*)-(–)-methyl-2-[bis-(*tert*-butoxycarbonyl)amino]-5-oxopentanoate (**4**) (16) followed by the introduction of the galactosyl unit and subsequent hydrolysis.

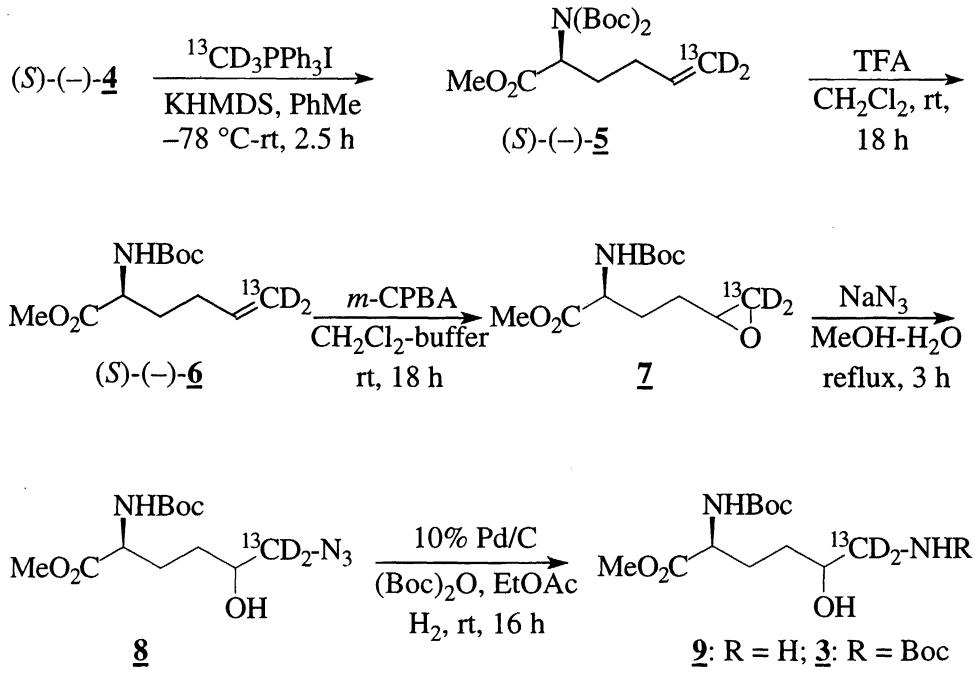
Figure 2



Accordingly, the aldehyde (*S*)-(–)-**4**, which was prepared from a commercially available *L*-glutamic acid dimethyl ester (17, 18), was subjected to the Wittig reaction (Scheme 1) with the ylide generated from the labeled ($^{13}CD_3$)-methyl triphenyl phosphonium iodide (19, 20) using KHMDS in toluene. Purification of the crude product by silica gel column chromatography afforded the olefin (*S*)-(–)-**5** in 85% yield. It was interesting to note that the Wittig reaction of (*S*)-(–)-**4** using *n*-BuLi in THF, under the conditions developed previously (20) for olefin (*S*)-(–)-**5** (which had *t*-butyl ester vs methyl ester), gave (*S*)-(–)-**5** in very poor yield (5-10%) with no unreacted aldehyde (*S*)-(–)-**4**. Selective hydrolysis of the Boc group in (*S*)-(–)-**5** using TFA in CH_2Cl_2 gave (*S*)-(–)-**6** in excellent yield (18). Oxidation of the olefin (*S*)-(–)-**6** using *m*-chloroperoxybenzoic acid in CH_2Cl_2 and buffer (pH = 8.0) gave the diastereomeric mixture of epoxide (**7**) in 1:1 ratio and 75% yield. The diastereomeric mixture of **7** was treated with sodium azide in MeOH-water at reflux

temperature to afford the azide (**8**) in 70% yield. Hydrogenation of the azide (**8**) using 10% Pd/C in ethyl acetate in the presence of $(Boc)_2O$ formed the amino compound (**9**) which was isolated as the corresponding *tert*-butoxycarbonyl (Boc) to afford the labeled hydroxylysine derivative (**3**) in excellent yield (67%).

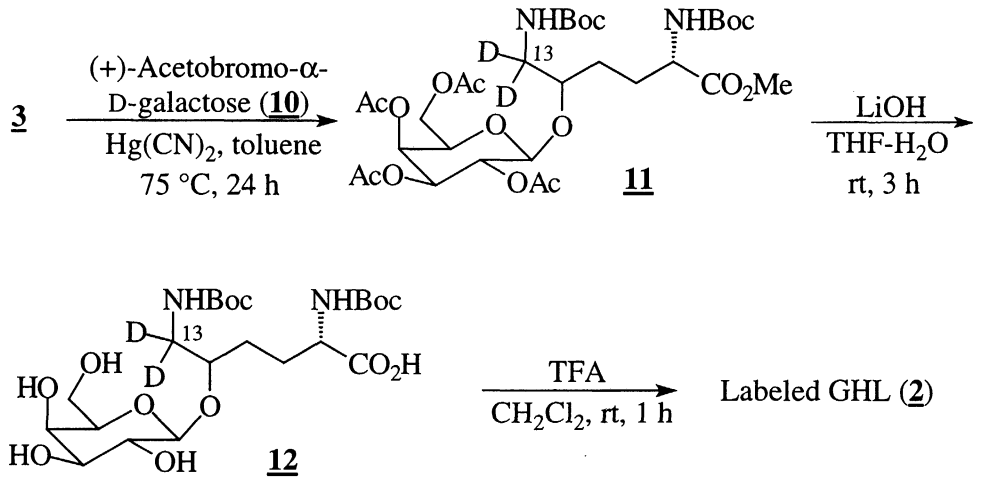
Scheme 1



The glycosylation of hydroxylysine derivation (**3**) (Scheme 2) was carried out using 2.0 equiv. of (+)-acetobromo-α-D-galactose (**10**) and 2.2 equiv. of mercury (II) cyanide in toluene (14, 15) at 75 °C temperature for 24 h. The crude product was purified by preparative reversed phase HPLC and lyophilized to afford the glycosylated product **11** in >99% purity but in low yield (20%) as a white powder. Treatment of compound **11** with LiOH in THF-H_2O to hydrolyze both the methyl ester and the acetate protective groups followed by purification of the crude product by reverse phase HPLC afforded acid **12** in 76% yield. Finally, the Boc protective groups in **12** were cleaved by treatment with TFA in CH_2Cl_2 and the crude product

was purified by reversed phase HPLC to afford the labeled GHL (**2**) in 98% yield as its TFA salt.

Scheme 2



In summary, a concise synthesis of a labeled galactosylhydroxylysine (**2**), which is needed as an internal standard for the quantification of GHL (**1**) by mass spectrometry, was described.

Experimental

General methods and materials: ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini spectrometer (300 MHz) and the chemical shifts (δ) were reported in ppm relative to TMS and coupling constants (*J*) were reported in Hz. Electrospray ionization mass spectrometry (ESI-MS) were carried on a Perkin-Elmer (Norwalk, CT) Sciex API 100 Benchtop system employing Turbo Ionspray ion source and HRMS were obtained on a Nermang 3010 MS-50, JEOL SX102-A mass spectrometers. The solvents were freshly distilled (THF from sodium and benzophenone; CH_2Cl_2 from CaH_2; toluene from CaH_2) under nitrogen. All reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and all solvents employed were of HPLC grade from EM Science (Gibbstown, NJ). Analytical reversed phase (RP) HPLC was performed

using a Waters, RCM, C18, Symmetry, 7.0 μm (8 × 100 mm). Preparative reversed phase (RP) HPLC was performed using Waters RCM, C18, Symmetry, 7.0 μm (40 × 100 mm). Optical rotations were measured on Autopol III polarimeter, Rudolph Research, Flanders, NJ.

(*S*)-(–)-Methyl-2-[bis-(*tert*-butoxycarbonyl)amino]-5-oxopentanoate (**4**) was prepared on a 25 g scale from commercially available *L*-glutamic acid dimethyl ester (17, 18) in three steps.

(*S*)-(–)-[(6)-$^{13}CD_2$]-1-Methyl-2-[bis-(*tert*-butoxycarbonyl)amino]-5-hexenoate (5): KHMDS (0.5 M soln in toluene, 36.52 mL, 18.26 mmol, 1.0 equiv.) was added dropwise to a 0 °C cooled suspension of ($^{13}CD_3$)-methyltriphenylphosphonium iodide (7.45 g, 18.26 mmol, 1.0 equiv.) in toluene (70 mL) under nitrogen. The resulting pale yellow mixture was stirred for 30 min and cooled to –78 °C. A solution of (*S*)-(–)-**4** (6.3 g, 18.26 mmol, 1.0 equiv.) in toluene (70 mL) was added via double ended needle and stirred the mixture for 1.5 h. Cooling bath was removed and the mixture was allowed to warm to room temperature with stirring. After 1 h, it was cooled to –78 °C and quenched with saturated aq NH_4Cl solution (36 mL). The mixture was allowed to warm and diluted with EtOAc (600 mL) and water (100 mL). The aqueous layer was separated and the organic layer was washed with brine (100 mL), dried ($MgSO_4$) and concentrated on a rotary evaporator. The crude compound was purified by silica gel column chromatography (20% EtOAc in hexanes) to afford 5.37 g of (*S*)-(–)-**5** in 85% yield as a colorless viscous oil. R_f: 0.65 (20% EtOAc in hexanes); $[\alpha]^{23}_D$ –35.7 (c 1.15, MeOH); Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/70:30; 2.0 mL/min at 225 nm, R_t: 7.86 min, 96.3%; ^1H NMR ($CDCl_3$): δ 5.84–5.76 (m, 1H), 4.90–4.85 (m, 1H), 3.71 (s, 3H), 2.29–1.94 (m, 4H), 1.50 (s, 18H); ^{13}C NMR ($CDCl_3$): δ 171.4, 152.1, 137.4 (qd), 115.4 (quin), 83.0, 57.5, 52.1, 30.3 (t), 29.3 (d), 28.0; ESI-MS (*m/z*): 347 $(M + H)^+$; HRMS (FAB, *m/z*): calcd for $C_{16}H_{28}C[_{13}]D_2NO_6$, 347.2232 $(M + H)^+$; observed, 347.2228.

(*S*)-(–)-[(6)-$^{13}CD_2$]-1-Methyl-2-[(*tert*-butoxycarbonyl)amino]-5-hexenoate (6): Trifluoroacetic acid (1.78 mL, 23.2 mmol, 1.5 equiv.) was added to a solution of (*S*)-(–)-**5** (5.35 g, 15.4 mmol) in CH_2Cl_2 (150 mL) at room temperature under nitrogen. The mixture was stirred for 18 h and concentrated on a rotary evaporator. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ (100 mL), dried (Na_2SO_4) and concentrated on a rotary evaporator. Purification the crude compound by silica gel column chromatography (20% EtOAc in hexanes) afforded 3.21 g of **(*S*)-(–)-6** in 85% yield as a colorless viscous oil. R_f: 0.43 (25% EtOAc in hexanes); $[\alpha]^{23}_D$ –12.2 (c 0.74, MeOH); Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/50:50, 2.0 mL/min at 225 nm, R_t: 8.32 min, 99%; ^1H NMR ($CDCl_3$): δ 5.81–5.71 (m, 1H), 5.11–5.04 (m 1H), 4.36–4.26 (m, 1H), 3.73 (s, 3H), 2.00–2.10 (m, 2H), 1.95–1.82 (m, 1H), 1.77–1.64 (m, 1H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 173.2, 155.2, 137.1(qd), 115.0 (quin), 79.7, 52.8, 52.1, 31.84 (q), 29.2, 28.2; ESI-MS (*m/z*): 246 $(M)^+$; HRMS (FAB, *m/z*): calcd $C_{11}H_{19}C[_{13}]D_2NO_4$, 247.1596 $(M + H)^+$; observed: 247.1588.

Methyl-(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-{[(1)-$^{13}CD_2$]-2-oxiranyl} butanoate (7): *m*-Chloroperoxybenzoic acid (50-70% grade, 8.76 g, 50.76 mmol) was added to a solution of (*S*)-(–)-**6** (6.27 g, 25.48 mmol) in CH_2Cl_2 (100 mL) and phosphate buffer (pH: 8.0, 125 mL) at room temperature and the mixture stirred vigorously for 17 h. The organic layer was separated, diluted with CH_2Cl_2 (200 mL), washed with saturated $NaHCO_3$ (3 × 100 mL), brine (100 mL), dried (Na_2SO_4) and concentrated on a rotary evaporator. Purification the crude compound by silica gel column chromatography (30% EtOAc in hexanes) afforded 5.01 g of epoxide (**7**) in 75% yield as a mixture of diastereomers (1:1 ratio, colorless viscous oil). R_f: 0.36 (40% EtOAc in hexanes); Analytical RP HPLC: MeCN:water/50:50, 2.0 mL/min at 225 nm, R_t: 7.0 min, 98%; ^1H NMR ($CDCl_3$): δ 5.20–5.05 (m, 1H), 4.40–4.30 (m, 1H), 3.75 (s, 3H), 3.12–2.90 (m, 1H), 2.12–1.91 (m, 1H), 1.93–1.50 (m, 3H), 1.45 (s,

9H); ESI-MS (*m/z*): 263 (M + H)$^+$; HRMS (FAB, *m/z*): calcd for $C_{11}H_{19}C[13]D_2NO_5$, 263.1545 (M + H)$^+$; observed: 263.1533.

[(6)-$^{13}CD_2$]-Methyl-(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-5-hydroxy-6-azidohexanoate (8): Sodium azide (0.260 g, 4.0 mmol, 2.0 equiv.), ammonium chloride (0.107 g, 2.0 mmol, 1.0 equiv.) were added to the mixture of epoxide (7, 0.524 g, 2.0 mmol) in MeOH (20 mL) and water (2.0 mL) and the mixture was gently refluxed for 3 h. The solvent was removed on a rotary evaporator and the residue was dissolved in EtOAc (100 mL). It was washed with water (2 × 50 mL), brine (50 mL), dried (Na_2SO_4) and concentrated on a rotary evaporator. Purification of the crude compound by silica gel column chromatography (30% EtOAc in hexanes) afforded 0.417 g of azide (8) in 70% yield as a mixture of diastereomers (1:1 ratio, colorless viscous oil). R_f: 0.36 (40% EtOAc in hexanes); Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/50:50, 2.0 mL/min at 225 nm, R_t: 3.6 min, 95%; ^{1}H NMR ($CDCl_3$): δ 5.20–5.10 (m, 1H), 4.42–4.30 (m, 1H), 3.86–3.78 (m, 1H), 3.74 (s, 3H), 2.00–1.50(m, 4H), 1.44 (s, 9H) ; ESI-MS (*m/z*): 306 (M + H)$^+$; HRMS (FAB, *m/z*): calcd for $C_{11}H_{20}C[13]D_2N_4O_5$, 306.1715 (M + H)$^+$; observed: 306.1708.

[(6)-$^{13}CD_2$]-Labeled hydroxylysine derivative (3): 10% Pd/C (0.350 g) in EtOAc (40 mL) was stirred at room temperature under hydrogen atmosphere (55 psi) for 20 min. To this mixture, a solutions of $(Boc)_2O$ (3.0 g, 13.77 mmol, 1.2 equiv.) in EtOAc (20 mL) and azide (8, 3.5 g, 11.47 mmol) in EtOAc (20 mL) were added and the mixture was stirred at room temperature under hydrogen atmosphere (55 psi) for 16 h. An additional amount of 10% Pd/C (0.200 g) and $(Boc)_2O$ (0.500 g, 2.23 mmol, 0.2 equiv.) were added and continued stirring under hydrogen atmosphere (55 psi) for 24 h. The mixture was filtered through celite powder (5.0 mm thickness) and washed with EtOAc (50 mL). The filtrate was concentrated and the crude compound was purified by silica gel column chromatography (60% EtOAc in hexanes) to afford 2.90 g of hydroxylysine derivative (3) in 67% yield as a mixture of diastereomers

(1:1 ratio, colorless viscous oil). R_f: 0.37 (60% EtOAc in hexanes); Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/50:50; 2.0 mL/min at 225 nm, R_t: 5.9 min, >99%; ^{1}H NMR ($CDCl_3$): δ 5.36–5.20 (m, 1H), 5.04 (bs, 1H), 4.38–4.26 (m, 1H), 3.75 (s, 3H), 3.72–3.66 (m, 1H), 3.20–3.10 (bs, 1H), 2.20–1.60 (m, 4H), 1.44 (s, 18H); ESI-MS (*m/z*): 380 $(M + H)^+$; HRMS (FAB, *m/z*): calcd for $C_{16}H_{30}C[_{13}]D_2N_2O_7$, 380.2335 $(M + H)^+$; observed: 380.2327.

[$^{13}CD_2$]-Glycosylated compound (10): (+)-Acetobromo-α-D-galactose (**9**, 3.06 g, 7.44 mmol, 2.0 equiv.) and $Hg(CN)_2$ (2.35 g, 9.3 mmol, 2.5 equiv.) were added to a solution of hydroxylysine derivative (**3**, 1.41 g, 3.72 mmol) in toluene (214 mL) and the mixture was gently heated in a oil bath at 75 °C (bath temperature) for 24 h. The solvent was removed to dryness on a rotary evaporator and the residue was purified by silica gel column chromatography (50% EtOAc in hexanes) to afford 0.750 g of product (**10**). The product (**10**) was further purified by preparative reversed phase HPLC (MeCN:water/50:50, 45 mL/min at 225 nm). Lyophilization of the product afforded analytically pure 0.520 g of (**10**) in 20% yield as a mixture of diastereomers (1:1 ratio and colorless viscous oil). R_f: 0.6 (50% EtOAc in hexanes); Analytical RP HPLC: MeCN:water/50:50; 2.0 mL/min at 225 nm, R_t: 13.6 min, 97% ; ^{1}H NMR ($CDCl_3$): δ 5.39 (d, 1H, *J*=1.9 Hz), 5.20–5.10 (m, 1H), 5.00 (dd, 1H, *J*=32.0, 18.0 Hz), 4.77 (bs, 1H), 4.50 (d, 1H, *J*=7.9 Hz), 4.30 (bs, 1H), 4.13 (dd, 2H, *J*=15.0, 10.1 Hz), 3.90 (q, 1H, *J*=7.2, 14.1 Hz), 3.70 (bs, 4H), 2.17 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H), 1.80–1.50 (m, 4H), 1.43 (s, 18H); ESI-MS (*m/z*): 710 $(M + H)^+$; HRMS (FAB, *m/z*): calcd for $C_{30}H_{48}C[_{13}]D_2N_2O_{16}$, 732.3212 $(M + Na)^+$; observed: 732.3215.

[$^{13}CD_2$]-Acid (11): Lithium hydroxide (monohydrate, 0.148 g, 3.52 mmol, 10.0 equiv.) was added to a mixture of glycosylated compound (**10**, 0.250g, 0.352 mmol) in THF (10 mL) and water (4 mL) at room temperature. The mixture was stirred for 1 h and concentrated to dryness on rotary evaporator. The residue was dissolved in

MeCN-water (10 mL, 70:30 ratio) and the pH was adjusted to 3.0 using 1N HCl and a pH meter. The mixture was purified by preparative HPLC (MeCN:0.1 aq trifluoroacetic acid/23:77/45 mL/min at 225 and lyophilyzed to afford 0.141 g of acid (**11**) in 76% yield as a white powder. Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/25:75; 2.0 mL/min at 225 nm, R_t: 8.8 min, 99%; ^1H NMR ($CDCl_3$): δ 4.80 (bs, 2H), 4.18 (d, 1H, *J*=7.1 Hz), 3.84–3.98 (m, 1H), 3.58–3.75 (m, 4H), 3.35–3.45 (m, 4H), 3.21 (quin, 1H, *J*=1.6 Hz), 1.86–2.02 (m, 1H), 1.54–1.70 (m, 3H), 1.35 (s, 18H); ESI-MS (*m/z*): 528 $(M + H)^+$; HRMS (FAB, *m/z*): calcd for $C_{21}C[_{13}]H_{38}D_2N_2O_{12}$, 528.2819$(M + H)^+$; observed: 528.2825.

Labeled Galactosylhydroxylysine (2): Dichloromethane (3.0 mL) and trifluoroacetic acid (2.0 mL) were added sequentially to the acid (**11**, 0.088 g, 0.166 mmol) at room temperature. After stirring the mixture for 0.5 h, it was concentrated on a rotary evaporator (below 40 °C bath temperature) to dryness. The crude compound was dissolved in 0.1% aq trifluroacetic acid (2.0 mL) and purified by preparative HPLC (0.1% aq trifluoroacetic acid/25.0 mL/min at 225 nm). Lyophilization of the product gave 0.087 g of labelled GHL-TFA salt (**2**) in 98% yield as colorless glassy material (mixture of diastereomers). Analytical RP HPLC: 0.1% aq trifluoroacetic acid/1.0 mL/min at 225 nm; R_t: 2.2 min, >99%; ^1H NMR (CD_3OD): δ 4.40 (d, 1H, *J*=7.8 Hz), 4.06–4.10 (m, 2H), 3.82 (d, 2H, *J*=2.0 Hz), 3.76–3.99 (m, 2H), 3.63–3.57 (m, 2H), 3.48–5.52 (dd, 1H, *J*=10 3 Hz), 3.33–3.31 (m, 1H), 2.06–2.25 (m, 2H), 1.85–2.04 (m, 1H), 1.81–1.79 (m, 1H); ESI-MS (*m/z*): 328 $(M + H)^+$, HRMS (FAB, *m/z*): calcd for $C_{11}C[_{13}]H_{22}D_2N_2O_8$, 328.1770 $(M + H)^+$; observed, 328.1757.

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