Supplemental Data

Synthesis of GLA-S, GLA-IS, GAA-S and GAA-IS

The synthetic scheme is shown in the figure below.

General Methods. Thin layer chromatography (TLC) was carried out on silica plates (Merck, 60F<sub>254</sub>), and flash column chromatography was carried out with silica gel (Merck, 230-400 Mesh). Preparative HPLC was carried out on a C18 reverse phase column (Vydac 218TP1022, 2.2 cm x 25 cm). Elution of compounds was monitored with a UV detector (λ = 254 nm). Dry CH<sub>2</sub>Cl<sub>2</sub> was obtained by distillation from CaH<sub>2</sub> under Ar, and other dry solvents were obtained from Aldrich (Sure-Seal). As noted below, reactions were carried out in a round bottom flask (RBF) or in a vial with a Teflon septum-lined screw cap. <sup>1</sup>H-NMR spectra were obtained on a Bruker DPX200 spectrometer (200 MHz) unless otherwise noted.

Acetic acid 4-nitro-phenyl ester (1): Acetic anhydride (50 ml) was added to a solution of 4-nitrophenol (5.56 g, 40 mmol) in dry pyridine (50 ml). The solution was stirred at ambient temperature for 2 hr and then at 70 °C overnight with a reflux condenser under Ar. The mixture was poured onto ice, and a white precipitate formed after standing for several hours. Water (400 ml) was added, and the white solid was collected by vacuum filtration and dried in vacuo to yield a white solid (5.1 g, 70%). ESI-MS (M+H)<sup>+</sup>: 182.2. <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ 8.30 (2H, d, J = 9.0 Hz, NO<sub>2</sub>CC<sub>6</sub>H<sub>4</sub>), 7.31 (2H, d, J = 9.0 Hz, OCC<sub>6</sub>H<sub>4</sub>), 2.25 (3H, s, CH<sub>3</sub>).

Acetic acid 4-acryloylamino-phenyl ester (2): H<sub>2</sub> was bubbled through a solution of 1 (280 mg, 1.54 mmol) and 10 mg of 10% Pd on carbon in 20 ml of MeOH for 1 hr. The catalyst was removed by filtration. Triethylamine (410 µl, 3.08 mmol) was added to the
filtrate which was chilled on ice, then acryloyl chloride (250 µl, 3.08 mmol, Aldrich) in 10 ml of dry CH₂Cl₂ was added dropwise with stirring over 0.5 hr under Ar. The reaction was allowed to return to ambient temperature, followed by 2 hr of stirring. Anion exchange resin (Bio-Rad, AG-MP1, OH⁻) (4 equivalents based on acryloyl chloride) was added, the mixture was filtered, and the filtrate was treated with sufficient cation exchange resin (Dowex, 50W X 8, H⁺) to bring the mixture to neutrality (moist pH paper). The resin was removed by filtration, and the solvent was removed by rotary evaporation to yield an off-white solid (268 mg, 85%). ESI-MS (M+H)⁺: 206.1. ¹H-NMR (acetone-d₆) δ 9.15 (1H, br, NH), 7.78 (2H, d, J = 9.0 Hz, NHCCH), 7.08 (2H, d, J = 9.0 Hz, NHCCCH), 6.55~6.37 (2H, m, COCHCH (anti to each other)), 5.75 (1H, dd, J = 9.8 and 2.2 Hz, COCHCHH (syn to COCH)).

N-(4-Hydroxy-phenyl)-acrylamide (3). To 2 (200 mg, 0.98 mmol) in 1.5 ml of MeOH in a 5 ml screw-capped vial was added 1.0 ml of 0.5 M of sodium methoxide in MeOH. The mixture was stirred at ambient temperature, and the reaction was complete in 10 min. The mixture was neutralized by addition of cation exchange resin (Dowex, 50W X 8, H⁺) (moist pH paper). The resin was removed by filtration and washed with MeOH. The combined filtrate and wash was concentrated by rotary evaporation to yield an off-white solid (152 mg, 95%), ESI-MS (M+H)⁺: 164.2. ¹H-NMR (acetone-d₆), δ 9.15 (1H, br, NH), 7.59 (2H, d, J = 9.0 Hz, NHCCH), 6.82 (2H, d, J = 9.0 Hz, NHCCCH), 6.52~6.35 (2H, m, COCHCHH (anti to each other)), 5.70 (1H, dd, J = 9.8 and 2.2 Hz, COCHCHH (syn to COCH)).

4-Acrylaminophenyl α-D-galactopyranoside (4): The compound was prepared as described for 2 using 1 g of 4-nitrophenyl α-D-galactopyranoside (Sigma) to obtain 0.94
g (87%) of 4; ESI-MS (M+H): 326.3. \(^1\)H-NMR (D\(_2\)O) \(\delta\) 7.43 (2H, d, \(J = 9.0\) Hz, NHCH\(_2\)), 7.16 (2H, d, \(J = 9.0\) Hz, NHCHCH\(_2\)), 6.47–6.24 (2H, m, COCHCH\(_2\)) (anti to each other)), 5.81 (1H, dd, \(J = 9.8\) and 2.2 Hz, COCHCH\(_2\)) (syn to COCH)), 5.52 (1H, d, \(J = 3.4\) Hz, H-1), 4.01–3.86 (4H, m, H-2,3,4,5), 3.63–3.60 (2H, d, \(J = 6.2\) Hz, H-6, 6’).

4-Acrylaminophenyl \(\alpha\)-D-glucopyranoside (5): The compound was prepared as described for 4, using 1 g of 4-nitrophenyl \(\alpha\)-D-glucopyranoside (Sigma) to obtain 0.97 g (90%) of 5; ESI-MS (M+H): 326.3. \(^1\)H-NMR (D\(_2\)O) \(\delta\) 7.43 (2H, d, \(J = 9.0\) Hz, NHCH\(_2\)), 7.16 (2H, d, \(J = 9.0\) Hz, NHCHCH\(_2\)), 6.47–6.24 (2H, m, COCHCH\(_2\)) (anti to each other)), 5.81 (1H, dd, \(J = 9.8\) and 2.2 Hz, COCHCH\(_2\)) (syn to COCH)), 5.60 (1H, d, \(J = 3.6\) Hz, 1-H), 3.94–3.66 (5H, m, H-2,3,5,6,6’), 3.48 (1H, t, \(J = 9.2\) Hz, H-4).

N-(6-Amino-hexyl)-benzamide (6): To a stirred solution of 1,6-diaminohexane (10.0 g, 86.3 mmol, Aldrich) in 30 ml dry CH\(_2\)Cl\(_2\) was added benzoyl chloride (1 ml, 8.6 mmol) in 300 ml dry CH\(_2\)Cl\(_2\) dropwise at ambient temperature under Ar. A white precipitate formed as the reaction proceeded, and the mixture was stirred at ambient temperature for 5 hr after the addition was completed. Aqueous NaOH (3 ml of 4 N) was added to dissolve the precipitate. The reaction mixture was washed with water (3 x 60 ml), dried over Na\(_2\)SO\(_4\) and solvent was removed by rotary evaporation. The oil was purified by flash chromatograph on silica eluting (Merck 230-400 Mesh) with 30:1 acetone/concentrated ammonium hydroxide to yield product as a yellowish oil (0.75 g, 32%). \(R_f = 0.43\) (TLC, same solvent). ESI-MS (M+H): 221.3. \(^1\)H-NMR (acetone-\(d_6\)) \(\delta\) 7.82–7.75 (2H, m, COCCH\(_2\)), 7.55–7.35 (3H, m, COCHCHCH\(_2\)), 6.35 (1H, br, NH), 3.42 (2H, dt, \(J = 5.8\) and 6.8 Hz, CONHCH\(_2\)), 3.20 (1H, t, \(J = 6.8\) Hz, NH\(_2\)CH\(_2\)), 1.90-1.32 (8H, m, NHCH\(_2\)(CH\(_2\))\(_4\)).
N-(6-Amino-hexyl)-d5-benzamide (7): The compound was prepared as for 6 using d5-benzoyl chloride (Cambridge Isotope Inc.). ESI-MS (M+H): 226.3. 1H-NMR (acetone-d6) δ 6.35 (1H, br, NH), 3.42 (2H, dt, J = 5.8 and 6.8 Hz, CONHCH2), 3.20 (1H, t, J = 6.8 Hz, NH2CH2), 1.90-1.32 (8H, m, NHCH2(CH2)4).

N-(7-Amino-heptyl)-benzamide (8): The compound was prepared as for 6 using 1,7-diaminohexane (Aldrich). ESI-MS (M+H): 235.3. 1H-NMR (acetone-d6) δ 7.82~7.75 (2H, m, COCCH), 7.55~7.35 (3H, m, COCHCHCH), 6.35 (1H, br, NH), 3.42 (2H, dt, J = 5.8 and 6.8 Hz, CONHCH2), 3.20 (1H, t, J = 6.8 Hz, NH2CH2), 1.90-1.32 (10H, m, NHCH2(CH2)5).

N-(7-Amino-heptyl)-d5-benzamide (9): The compound was prepared as for 6 using d5-benzoyl chloride. ESI-MS (M+H): 240.3. 1H-NMR (acetone-d6) δ 6.35 (1H, br, NH), 3.42 (2H, dt, J = 5.8 and 6.8 Hz, CONHCH2), 3.20 (1H, t, J = 6.8 Hz, NH2CH2), 1.90-1.32 (10H, m, NHCH2(CH2)5).

(6-Benzoylamino-hexyl)-2-[4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenylcarbamoyl]-ethyl]-carbamic acid tert-butyl ester (GLA-S): Compound 4 (0.88 g, 2.7 mmol) and 6 (0.71 g, 3.2 mmol) in a solution of isopropanol (30 ml) and H2O (4 ml) was stirred at 65 °C (oil bath) in a capped 100 ml RBF for 48 hrs. TLC on silica showed that at least 85% of 4 was converted to the Michael addition product (Rf = 0, 30:1 acetone-concentrated ammonium hydroxide). The reaction was allowed to cool to ambient temperature, followed by the addition of powdered K2CO3 (0.44 g, 3.2 mmol) and di-tert-butylcarbonate (0.84 mg, 3.8 mmol, Aldrich). The mixture was stirred at ambient temperature for 3 hr. TLC showed at least 80% of Michael addition product was converted to the desired product (Rf = 0.17, 10:1 acetone-concentrated ammonium
hydroxide). The solid was collected by vacuum filtration and was washed with 30 ml of MeOH. The filtrates were combined, and solvent was removed by rotary evaporation to give an oily residue. MeOH (6.5 ml) was added to dissolve the residue, and the pH was adjusted to ~3-4 (moist pH paper) by addition of trifluoroacetic acid with chilling on ice. The desired product was purified by 10 runs of preparative HPLC: 50% MeOH in H₂O, at a flow rate of 6 ml/min; tᵣ = 27 min. Product fractions were pooled, and most of the solvent was removed by rotary evaporation at ambient temperature. The remaining solvent was removed by lyophilization, and the resulting residue was dissolved in 20 ml of MeOH. Solvent was removed by rotary evaporation, and the oily residue was dried in vacuum to give a white solid (1.1 g, 63%). ESI-MS (M+H)⁺: 646.6; ¹H-NMR (1:2.5 D₂O/acetone-d₆) δ 7.80~7.75 (2H, m, COC₆H), 7.55~7.35 (5H, m, COCHCH₃CH₃ and NHCC₆H), 7.05 (2H, d, J = 9.0 Hz, NHCCCH₃), 5.39 (1H, d, J = 3.4 Hz, H-1), 4.00~3.57 (6H, m, H-2,3,4,5,6,6’), 3.51 (2H, t, J = 6.8 Hz, COCH₂CH₂), 3.30 (2H, t, J = 7.0 Hz, CONHCH₂), 3.16 (2H, t, J = 7.0 Hz, CONH(CH₂)₃CH₂), 2.55 (2H, t, J = 6.4 Hz, COCH₂), 1.70~1.20 (17H, m, O-tert-C₄H₉ and NHCH₂(CH₂)₄.

(7-Benzoylamino-heptyl)-{2-[4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenylcarbamoyl]-ethyl}-carbamic acid tert-butyl ester (GAA-S): The compound was prepared as for GLA-S starting from 0.63 g of 5 and 0.55 g of 8. HPLC tᵣ = 40 min. Yield 60%. ESI-MS (M+H)⁺: 660.6. ¹H-NMR (1:2.5 D₂O/acetone-d₆) δ 7.80~7.75 (2H, m, COC₆H), 7.55~7.35 (5H, m, COCHCH₃CH₃ and NHCC₆H), 7.05 (2H, d, J = 9.0 Hz, NHCCCH₃), 5.39 (1H, d, J = 3.6 Hz, H-1), 3.90~3.57 (5H, m, H-2,3,5,6,6’), 3.51 (2H, t, J = 6.8 Hz, COCH₂CH₂), 3.45 (1H, t, j = 9.6 Hz, H-4), 3.30 (2H, t, J = 7.0 Hz,
CONHCH$_2$), 3.20 (2H, t, J = 7.0 Hz, CONH(CH$_3$)$_2$CH$_2$), 2.65 (2H, t, J = 6.4 Hz, COCH$_2$), 1.70-1.20 (19H, m, O-tert-$C_4H_9$ and NHCH$_2$(CH$_2$)$_3$).

(6-$d_5$-Benzoylamino-hexyl)-[2-(4-hydroxy-phenylcarbamoyl)-ethyl]-carbamic acid tert-butyl ester (GLA-IS): Compound 3, 10 mg, 0.06 mmol) and 7 (21 mg, 0.09 mmol) were dissolved in 1.5 ml of isopropanol in a screw capped vial. The mixture was stirred at 65 °C overnight. TLC showed that more than 85% of 3 had been converted into the Michael addition product (R$_f$ = 0.22, 30:1 acetone/concentrated ammonium hydroxide solution). After the reaction was cooled to ambient temperature, K$_2$CO$_3$ (10 mg, 0.07 mmol) and di-tert-butylcarbonate (16 mg, 0.07 mmol) were added, and the mixture stirred for 2 hr at the same temperature. TLC showed that all the Michael addition product had been converted into the desired product (R$_f$ = 0.93, 30:1 acetone/concentrated ammonium hydroxide solution). The final product was purified by HPLC (solvent A, H$_2$O; solvent B, MeOH; Gradient 0-30 min, 30-60% B; 30-70 min, 60-85%; flow rate 6 ml/min; t$_r$ = 45.4 min) to yield 22 mg of desired product (yield 75%). ESI-MS (M+H)$^+$: 489.5. $^1$H-NMR(CDCl$_3$) δ 8.78 and 8.48 (2H, br, NH), 7.35 (2H, d, J = 9.0 Hz, NHCCCH), 6.91 (1H, br, OH), 6.77 (2H, d, J = 9.0 Hz, HOCCCH), 3.47 (2H, t, J = 6.2 Hz, COCH$_2$CH$_2$), 3.34 (2H, dt, J = 5.8, 6.8 Hz, CONHCH$_2$), 3.09 (2H, t, J = 6.8 Hz, CONH(CH$_2$)$_3$CH$_2$), 2.55 (2H, t, J = 6.2 Hz, COCH$_2$), 1.70-1.10 (17H, m, O-tert-$C_4H_9$ and NHCH$_2$(CH$_2$)$_3$).

(6-Benzoylamino-hexyl)-[2-(4-hydroxy-phenylcarbamoyl)-ethyl]-carbamic acid tert-butyl ester (GLA-P): The compound was prepared as for GLA-IS using 10.4 mg of 6. HPLC t$_r$ = 45.3 min. Yield 72.1%. ESI-MS (M+H)$^+$: 484.5. $^1$H-NMR(CDCl$_3$) δ 8.78 and 8.48 (2H, br, NH), 7.84~7.79 (2H, m, COCCCH), 7.55~7.35 (5H, m, NHCCCH, COCHCHCHCH), 6.91 (1H, br, OH), 6.82 (2H, d, J = 9.0 Hz, HOCCCH), 3.57 (2H, t, J = 6.2
Hz, COCH$_2$CH$_2$), 3.42 (2H, dt, J = 5.8, 6.8 Hz, CONHCH$_2$), 3.20 (2H, t, J = 6.8 Hz, CONH(CH$_2$)$_2$CH$_2$), 2.64 (2H, t, J = 6.2 Hz, COCH$_2$), 1.70-1.10 (17H, m, O-tert-C$_4$H$_9$ and NHCH$_2$(CH$_2$)$_4$).

(7-$d_5$-Benzoylamino-heptyl)-[2-(4-hydroxy-phenylcarbamoyl)-ethyl]-carbamic acid tert-butyl ester (GAA-IS): The compound was prepared as for GLA-IS using 22 mg of 9. HPLC $t_R$ = 47.0 min. Yield 70.5%. ESI-MS (M+H)$^+$: 503.5. $^1$H-NMR(CDCl$_3$) $\delta$ 8.78 and 8.48 (2H, br, NH), 7.35 (2H, d, J = 9.0 Hz, NHCCCH), 6.91 (1H, br, OH), 6.77 (2H, d, J = 9.0 Hz, HOCCCH), 3.47 (2H, t, J = 6.2 Hz, COCH$_2$CH$_2$), 3.34 (2H, dt, J = 5.8, 6.8 Hz, CONHCH$_2$), 3.09 (2H, t, J = 6.8 Hz, CONH(CH$_2$)$_2$CH$_2$), 2.55 (2H, t, J = 6.2 Hz, COCH$_2$), 1.70-1.20 (19H, m, O-tert-C$_4$H$_9$ and NHCH$_2$(CH$_2$)$_3$).

(7-Benzoylamino-heptyl)-[2-(4-hydroxy-phenylcarbamoyl)-ethyl]-carbamic acid tert-butyl ester (GAA-P): The compound was prepared as for GAA-IS using 11 mg of 8. HPLC $t_R$ = 46.8 min. Yield 75.5%. ESI-MS (M+H)$^+$: 498.5. $^1$H-NMR(CDCl$_3$) $\delta$ 8.78 and 8.48 (2H, br, NH), 7.84–7.79 (2H, m, COCCH), 7.55–7.35 (5H, m, NHCCCH, COCHCHCH), 6.91 (1H, br, OH), 6.82 (2H, d, J = 9.0 Hz, HOCCCH), 3.57 (2H, t, J = 6.2 Hz, COCH$_2$CH$_2$), 3.42 (2H, dt, J = 5.8, 6.8 Hz, CONHCH$_2$), 3.20 (2H, t, J = 6.8 Hz, CONH(CH$_2$)$_2$CH$_2$), 2.64 (2H, t, J = 6.2 Hz, COCH$_2$), 1.70-1.10 (19H, m, O-tert-C$_4$H$_9$ and NHCH$_2$(CH$_2$)$_3$).
Synthesis of GLA-S, GLA-P, GLA-IS, GAA-S, GAA-P and GAA-IS using the following reagents: a, pyridine, reflux; b, H₂, Pd-C, MeOH, rt; c, CH₂=CH-COCl, Et₃N, MeOH, rt; d, NaOMe, MeOH, rt; e, CH₂Cl₂, rt; f, isopropanol, H₂O, 65 °C; g, K₂CO₃, (t-BuOCO)₂O, rt.
Loading the Filter Plate with Silica Gel.

This is facilitated by use of a plate loader similar to those available from Millipore (Cat. MACL 096). A homemade plate loader was used. The loader was prepared by boring wells into an aluminum plate such that each well holds 100 mg of silica gel when filled. The loader was used to fill the multiwell filter plate as described by Millipore.

Analysis of Additional alpha-Glucosidase Inhibitors.

We analyzed additional compounds for inhibition of acid alpha-glucosidases using the fluorimetric assay with 1.4 mM 4-methylumbelliferyl-alpha-glucopyranoside in citrate-phosphate buffer, pH 4.0 (Umapathysivam, K., Hopwood, J. J. and Meikle, P. J. (2001) Clin. Chem. 47, 1378-1383). The alpha-glucosidase inhibitors tested were: 1) Blintol (Johnston, B. D., Ghavami, A., Jensen, M. T., Svensson, B. and Pinto, B. M. (2002) J. Am. Chem. Soc., 124, 8245-8250); 2) Castanospermine (Sigma); 3) Miglitol (purified on a cation exchange resin from the drug Glyset); 4) Salacinol (Ghavami, A., Johnston, B. D. and Pinto, B. M. (2001) J. Org. Chem., 66, 2312 -2317). The following IC$_{50}$ values were obtained using recombinant GAA and PMN lysate as the source of acid alpha-glucosidase, respectively: Blintol (0.6 µM, 6 µM), Castanospermine (0.6 µM, 0.2 µM), Miglitol (26 µM, 53 µM) and Salacinol (0.9 µM, 8 µM). These data show that these additional inhibitors are not sufficiently selective to block RAAG for the selective detection of GAA.
Regression Analysis of the Callibration Curves Shown in Supporting Data Figure 1.

<table>
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<th>Assay</th>
<th>$n$</th>
<th>$m$</th>
<th>$b$</th>
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<th>$s_m$</th>
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$n$ = number of data points in calibration  
$m$ = slope  
$b$ = intercept  
$r^2$ = correlation coefficient (squared)  
$s_y$ = standard deviation of measured product/internal standard ratio  
$s_m$ = standard deviation of slope  
$s_b$ = standard deviation of intercept  
$s_x$ = standard deviation of product/internal standard ratio, as read from the least-squares calibration line.