Supporting Information

Structure-Activity Studies on Antiproliferative Factor (APF) Glycooctapeptide Derivatives

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Materials and Methods

Amino acids and resins were purchased from AnaSpec, Inc. (San Jose, CA) or EMD Chemicals (San Diego, CA); Ac_2O from Sigma Aldrich (St. Louis, MO); HOAt and HATU from AK Scientific, Inc. (Mountain View, CA); and solvents from American Bioanalytical (Natick, MA) or Fisher Scientific (Pittsburgh, PA). Peptide synthesis was performed either manually or on a Nautilus 2400 Parallel synthesizer (Argonaut Technologies, Foster City, CA). Preparative HPLC was performed on a Waters Prep LC 4000 System equipped with PDA detector (Waters 2996) on C_{18} column (mobile phase: Solvent A, 0.1% trifluoroacetic acid in H_2O, Solvent B, 0.1% trifluoroacetic acid in CH_3CN).

Glycopeptide synthesis. The synthesis of the peptide segments of the glycopeptides were carried out on a 0.1 mM scale by solid-phase methods by using standard Fmoc chemistry on 2ClTrt or Rink Amide resin. Protected amino acids (10 eq) were coupled using HATU (10 eq) and HOAt (10 eq) reagents in the presence of DIPEA (20 eq). The Fmoc group was removed with 20% piperidine in NMP. To estimate the obtained substitution level, an aliquot of dry resin (3 mg) was shaken with 20% piperidine in DMF for 20 min and the solution was analyzed for absorbance at 290 nm. The loading was calculated using the equation: Loading (mmol/g) = (Abs_{sample}) / (mg of sample x 1.75).[1] Typically, the obtained substitution level was 0.35-0.40 mmol/g. Each glycopeptide was cleaved from the resin with TFA:TIS:H_2O (95:2.5:2.5).

A mixture of DIPCDI/HOAt (5 eq each) in dry DMF was used for coupling of Fmoc-Thr(Ac_4Galβ1-3Ac_2GalNAcα-*O*-)-OH to the peptide chain (1 eq). The reaction was performed without any added base to prevent epimerization. This cycle was repeated two additional times to affect complete coupling. Even after this treatment, traces of uncoupled heptapeptide remained;

thus an HPLC purification step was performed after resin cleavage and ether precipitation. This pure material was treated with NaOMe/MeOH (pH ~ 9, 1.5 h) to remove the sugar acetyl groups, followed by a second HPLC purification. All intermediates and the final products were verified by HPLC-MS (Agilent 1200, Agilent Technologies, Inc., Santa Clara, CA); purity of > 95% was confirmed for all compounds by HPLC trace analysis at 227 nm). L-amino acids were used for the synthesis of all derivatives unless stated otherwise.

Circular dichroism. CD measurements were performed on an AVIV 202 spectrometer (Lakewood, NJ), over the range of 190-260 nm, using either 1, 0.1 or 0.01 cm quartz cuvettes at 25° C. The parameter settings of 0.5 nm resolution and 5 s averaging time were used for all CD measurements. The concentration of each glycopeptide was 50 μM in H_2O:TFE (1:1). Additionally, a spectrum of a 50 μM solution of *as*-APF8 in water (pH ~ 6) was recorded. A blank run of solvent alone was subtracted from the spectrum of each glycopeptide. All spectra were smoothed. All compounds were lyophilized immediately before measurements.

Patients. Normal controls, asymptomatic for urinary tract disease and undergoing cystoscopy following abdominal or pelvic surgery as standard of care, were consented to provide biopsy for the generation of normal bladder epithelial cell explants. These participants were all at least 18 years old and enrolled in accordance with guidelines of the Institutional Review Board of the University of Maryland, School of Medicine.

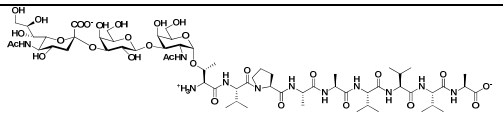
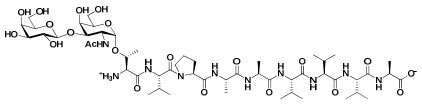
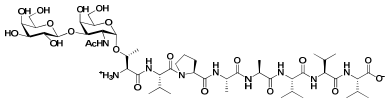
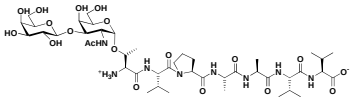
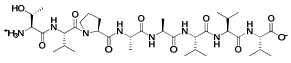
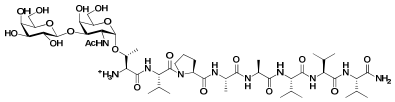
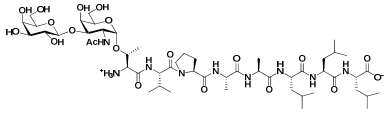
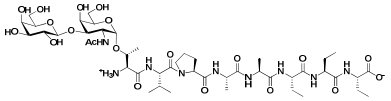
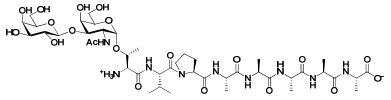
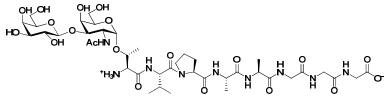
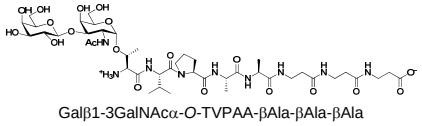
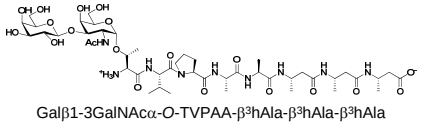
Cell Culture. Cystoscopy was performed under general anesthesia, and 4-mm^2 pieces of transitional epithelium with submucosal bladder tissue were obtained for the growth of primary bladder epithelial cells, as previously described.[2, 3]

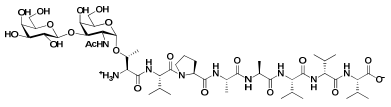
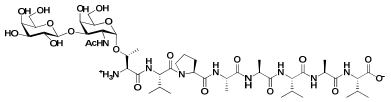
Normal bladder epithelial cells were propagated in DMEM-F12 (Media-Tech, Herndon VA) with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic solution, 1% glutamine, 0.25 units/mL insulin (all from Sigma, St. Louis, MO), and 5 ng/mL hEGF (R&D Systems, Minneapolis, MN) at 37°C in a 5% CO_2 atmosphere and characterized by binding of AE-1/AE-3 pancytokeratin antibodies (Signet, Dedham, MA). Culture medium was changed to MEM (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic solution, and 1% glutamine (all from Sigma) when cells were set up in flasks or plates for experiments; the cells were then further incubated in serum-free MEM medium overnight prior to treatment with *as*-APF derivatives.

^{3}H-Thymidine Incorporation. Cell proliferation was measured by ^{3}H-thymidine incorporation into explanted normal human bladder epithelial cells. Each purified lyophilized synthetic APF congener was resuspended in acetonitrile:distilled water (1:1), diluted in serum-free MEM (containing only glutamine and antibiotics/antimycotics), and applied to the cells; cell controls received acetonitrile:distilled water diluted in serum-free MEM alone. Cells were then incubated at 37°C in a 5% CO_2 atmosphere for 48 hours. The cell contents were harvested, methanol-fixed onto glass fiber filter paper and the amount of radioactivity incorporated determined. Significant inhibition of ^{3}H-thymidine incorporation was defined as a mean decrease in counts per minute of greater than 2 standard deviations from the mean of control cells for each plate.

Nuclear Magnetic Resonance Spectroscopy. NMR samples used for 1D and 2D ^{1}H NMR experiments were prepared by dissolving ~3 mg glycopeptide in 700 μL of 90% H_2O/10% D_2O CD_3COONa buffer (5 mM). The final pH of each sample was adjusted to ~4.6 using dilute solutions of DCl and NaOD as needed. Glycopeptides were characterized by 1D ^{1}H, TOCSY, and ROESY NMR spectroscopy. Spectroscopic data was collected on a Varian Unity INOVA 500 MHz spectrometer equipped with a variable temperature (VT) controller and a triple resonance (HCN) probe. The WATERGATE 3-9-19 pulse sequence with gradients was used for water suppression in all experiments.[4] TOCSY spectra were obtained using a DIPSI spinlock with a spinlock power of 7.5 kHz and a mixing time of 80 ms. Typically 2k data points in F2 and 256 experiments in F1 were acquired, with 4 transients per FID. ROESY experiments were performed using a spinlock field of 2.0 kHz with a mixing time of 300 ms. Typically 2k data points in F2 and 512 experiments in F1 were acquired, with 32 transients per FID. Data was processed using the NMRPipe[5] and NMRViewJ[6] software packages respectively. TOCSY and ROESY data were processed using a 90°–shifted sine-squared window function in both dimensions. Experiments were conducted at 298 K unless otherwise indicated, and all spectra were referenced to internal DSS.

Table S1. Structure, logP, and activity of APF analogues.

No	Derivative	logP [a]	% of activity [b]	P value [c]
1	 Neu5Acα2-3Galβ1-3GalNAcα-*O*-TVPAAVVVA	-7.14	100%	NS
2	 Galβ1-3GalNAcα-*O*-TVPAAVVVA	-4.67	100%	NS
3	 Galβ1-3GalNAcα-*O*-TVPAAVVV	-4.42	100%	NS
4	 Galβ1-3GalNAcα-*O*-TVPAAVV	-5.13	inactive	
5	 TVPAAVVV	-2.93	inactive	
6	 Galβ1-3GalNAcα-*O*-TVPAAVVV-NH_2	-2.79	inactive	
7	 Galβ1-3GalNAcα-*O*-TVPAALLL	-3.35	10%	< 0.001
8	 Galβ1-3GalNAcα-*O*-TVPAA-Abu-Abu-Abu	-5.73	inactive	
9	 Galβ1-3GalNAcα-*O*-TVPAAAAA	-7.31	100%	NS
10	 Galβ1-3GalNAcα-*O*-TVPAAGGG	-8.51	inactive	
11	 Galβ1-3GalNAcα-*O*-TVPAA-βAla-βAla-βAla	-8.81	0.1%	< 0.001
12	 Galβ1-3GalNAcα-*O*-TVPAA-β^{3}hAla-β^{3}hAla-β^{3}hAla	-7.51	inactive	

13	 Galβ1-3GalNAcα-*O*-TVPAAV-D-Val-V	-4.42	inactive
14	 Galβ1-3GalNAcα-*O*-TVPAAVAV	-5.38	inactive

[a]Calculated from the XLOGP3 program, http://www.sioc-ccbg.ac.cn/software/xlogp3/[7] [b]Due to the variability of cell response in the biological assay, the activity of each congener was normalized to the activity of **1** run simultaneously on the same plate according to the equation: $\% = \frac{\overline{IC_{50}(APF)}}{\overline{IC_{50}(derivative)}} \cdot 100\%$; the average IC_{50} value of **1** was ~ 1 nM. Percent of activity is expressed relative to **1** which served as a standard control on each plate. Derivatives with no significant activity at < 25 μM concentration (the cut-off limit for the biological assay) were considered to be inactive. [c]NS = not significant at $p > 0.05$.

Table S2. Analytical data for *as*-APF analogues.

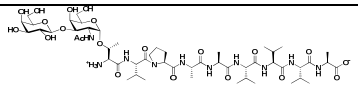
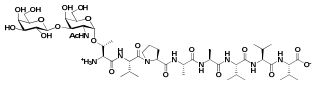
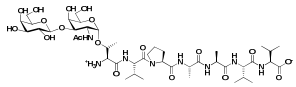
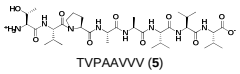
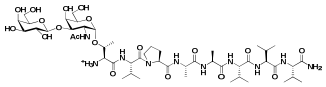
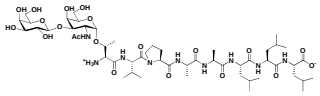
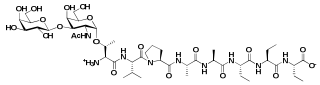
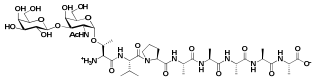
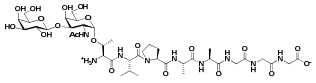
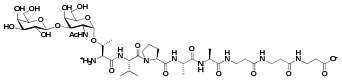
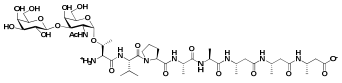
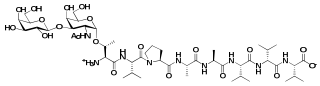
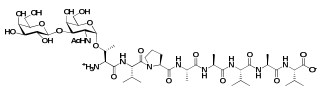
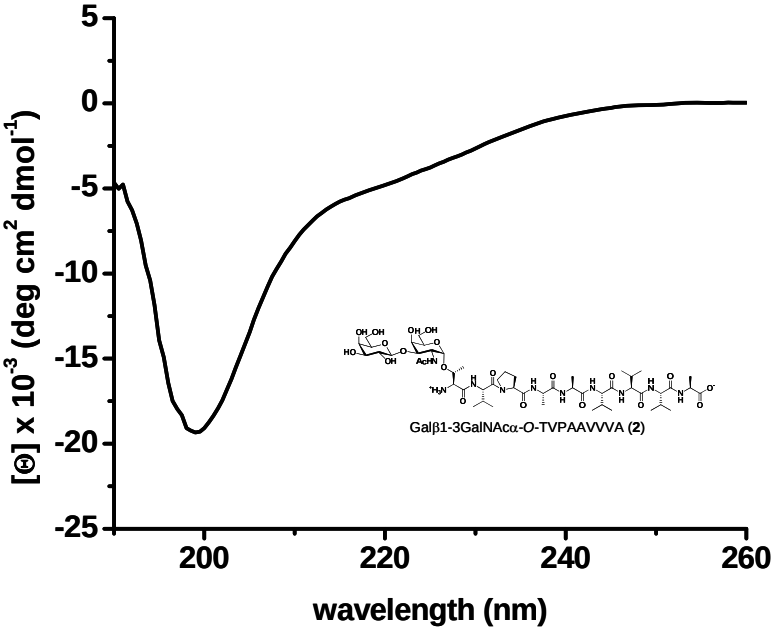
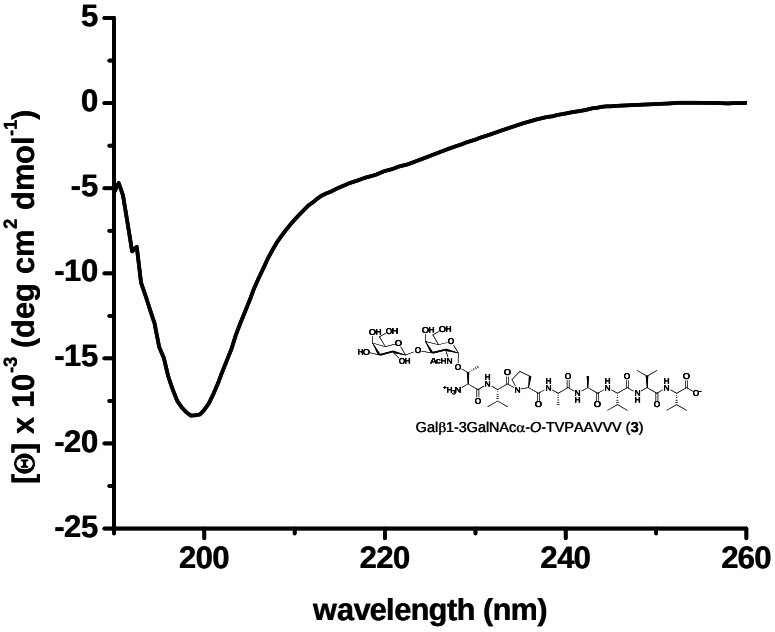
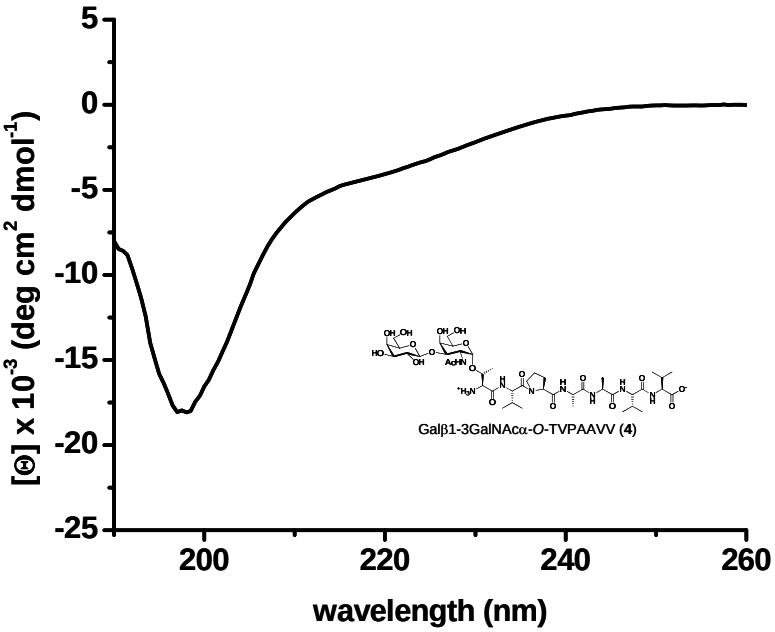
Compound	[M + H$^+$] calc	[M + H$^+$] obs	[M – H$^+$] calc	[M – H$^+$] obs	r_t[a] (min)	Purity
 Galβ1-3GalNAcα-*O*-TVPAAVVVA (**2**)	1191.64	1191.55	1189.62	1189.45	27.387	>99%
 Galβ1-3GalNAcα-*O*-TVPAAVVV (**3**)	1120.60	1120.45	1118.58	1118.35	28.359	>99%
 Galβ1-3GalNAcα-*O*-TVPAAVV (**4**)	1021.53	1021.45	1019.51	1019.50	26.473	>99%
 TVPAAVVV (**5**)	755.47	755.35	753.45	753.25	29.546	>99%
 Galβ1-3GalNAcα-*O*-TVPAAVVV-NH_2 (**6**)	1119.61	1119.55	1117.60	1117.45	26.935	>99%
 Galβ1-3GalNAcα-*O*-TVPAALLL (**7**)	1162.65	1162.45	1160.63	1160.35	34.028	>99%
 Galβ1-3GalNAcα-*O*-TVPAA-Abu-Abu-Abu (**8**)	1078.55	1078.45	1076.54	1076.35	25.212	>99%
 Galβ1-3GalNAcα-*O*-TVPAAAAA (**9**)	1036.51	1036.30	1034.49	1034.35	22.143	>99%
 Galβ1-3GalNAcα-*O*-TVPAAGGG (**10**)	994.46	994.30	992.44	992.20	20.035	>99%
 Galβ1-3GalNAcα-*O*-TVPAA-βAla-βAla-βAla (**11**)	1036.51	1036.30	1034.49	1034.35	20.996	>99%
 Galβ1-3GalNAcα-*O*-TVPAA-β^{3}hAla-β^{3}hAla-β^{3}hAla (**12**)	1078.55	1078.45	1076.54	1076.35	23.289	>99%
 Galβ1-3GalNAcα-*O*-TVPAAV-D-Val-V (**13**)	1120.60	1120.45	1118.58	1118.35	30.914	>99%
 Galβ1-3GalNAcα-*O*-TVPAAVAV (**14**)	1092.57	1092.55	1090.55	1090.45	26.642	>99%

Figure S1. CD spectra of *as*-APF analogues. Concentration: 50 μM. Solution: H_2O:TFE (1:1). Temperature: 25°C.






5
0
-5
-10
-15
-20
-25
200
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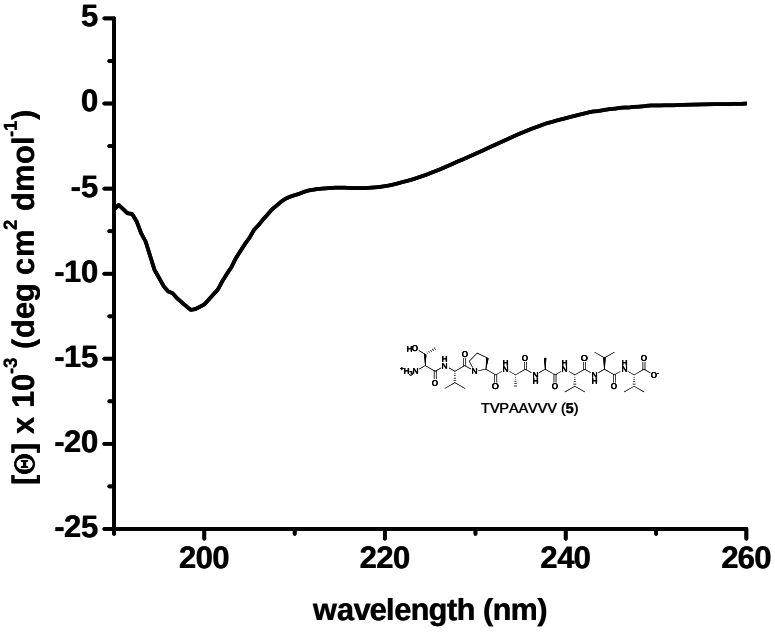
260
wavelength (nm)
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAAVV (4)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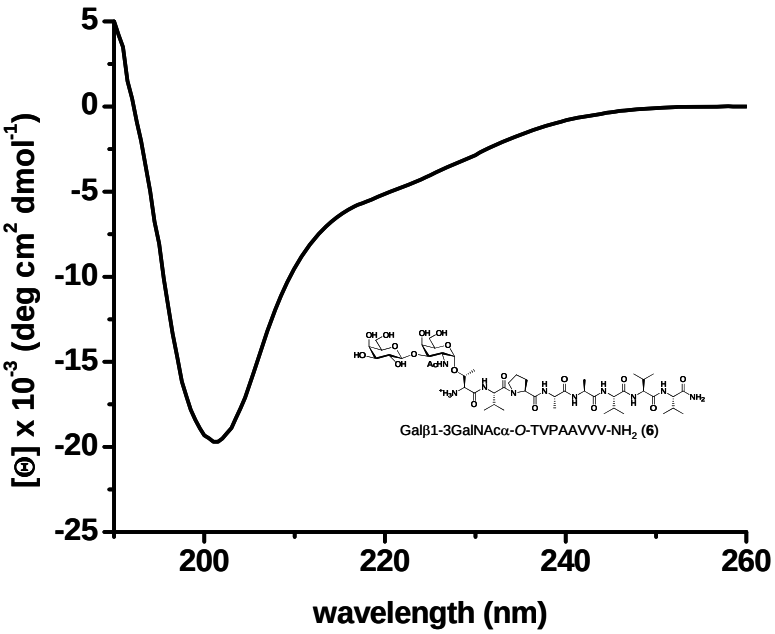
TVPAAVVV (5)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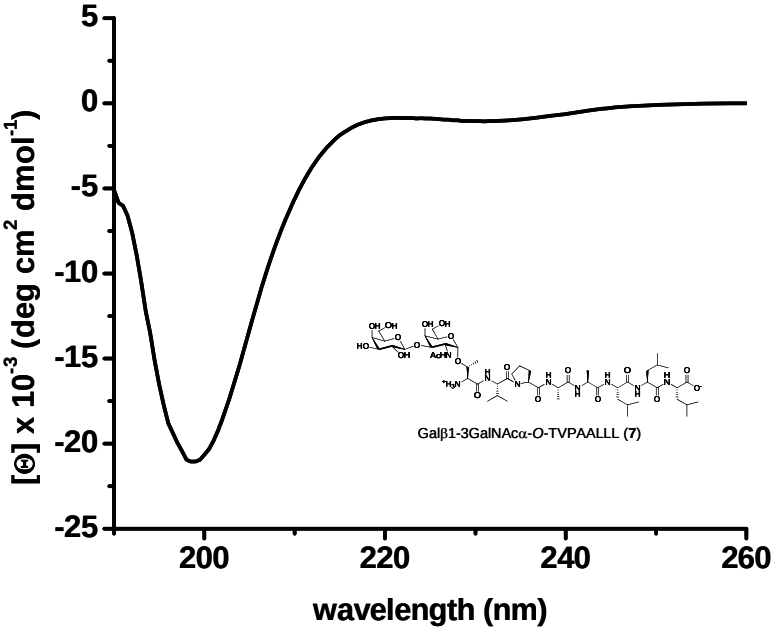
wavelength (nm)
Galβ1-3GalNAcα-O-TVPAAVVV-NH2 (6)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-15
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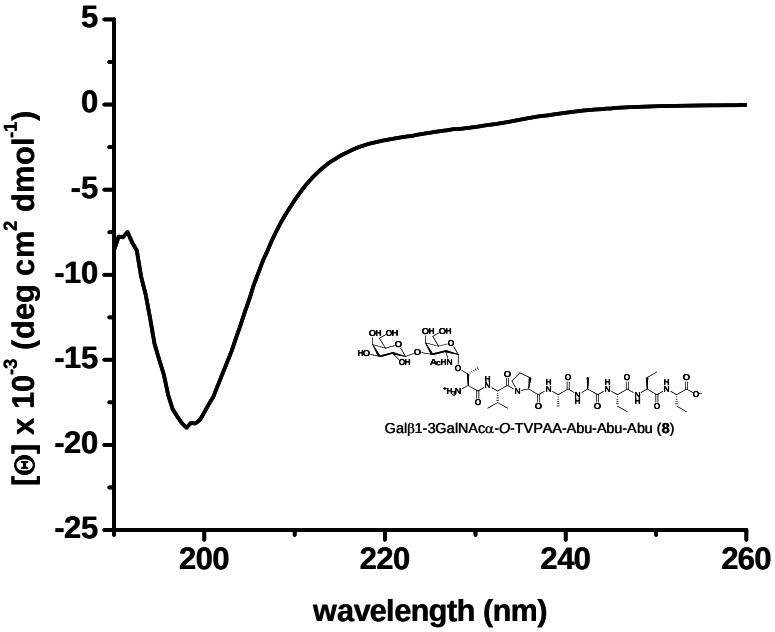
wavelength (nm)
Galβ1-3GalNAcα-O-TVPAALLL (7)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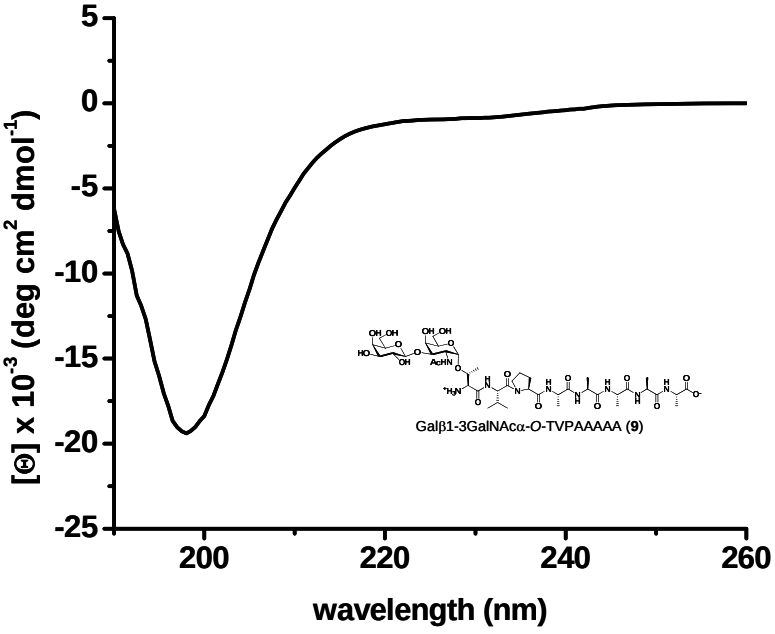
0
-5
-10
-15
-20
-25
200
220
240
260
wavelength (nm)
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAA-Abu-Abu-Abu (8)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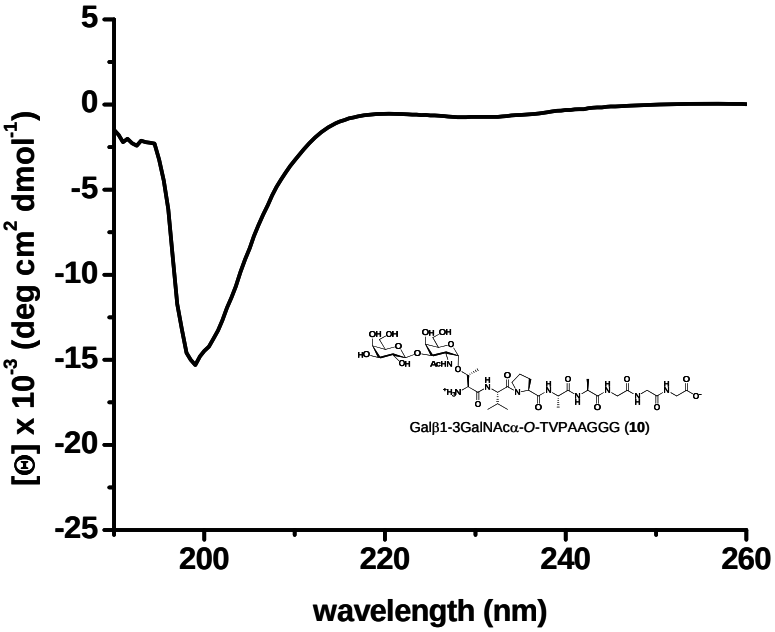
wavelength (nm)
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAAAAA (9)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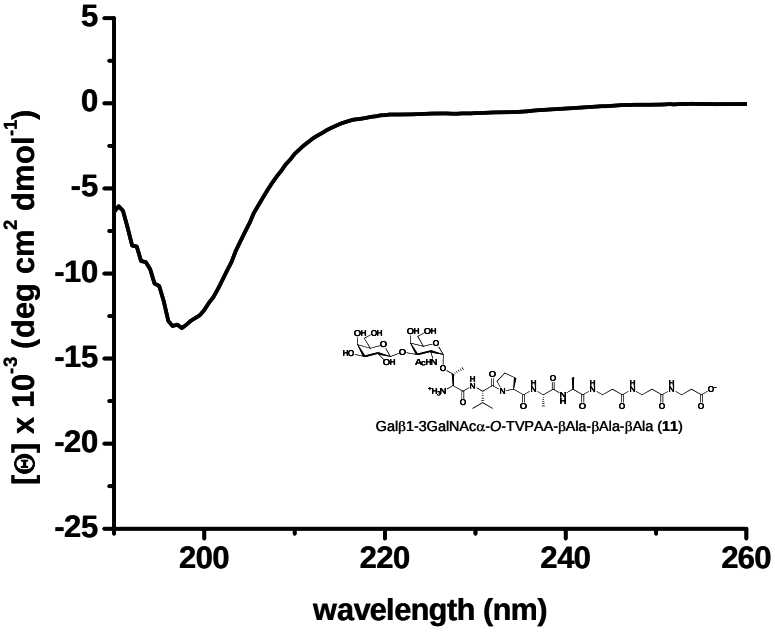
wavelength (nm)
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAAGGG (10)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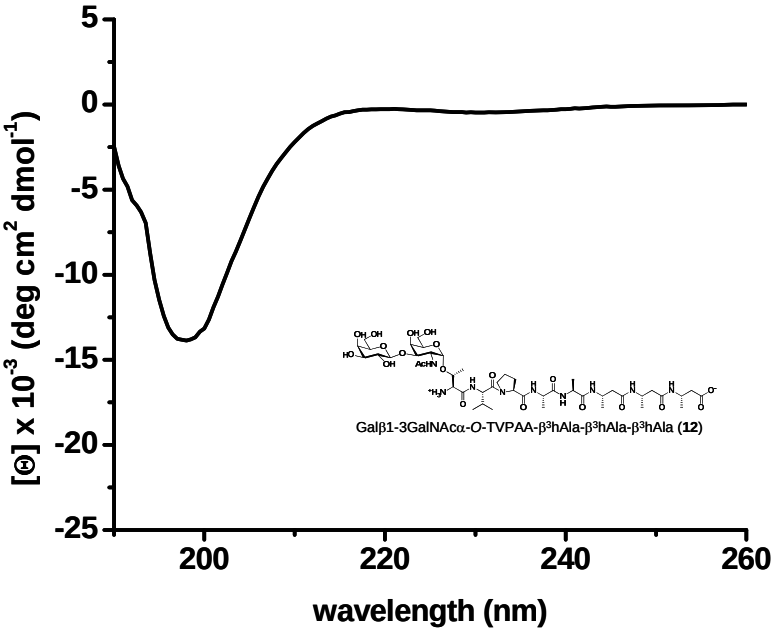
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAA-βAla-βAla-βAla (11)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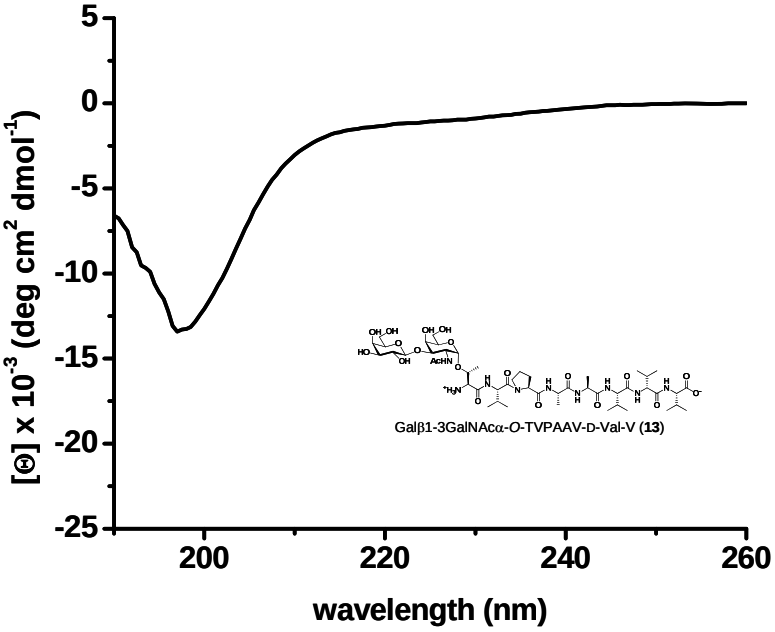
260
wavelength (nm)
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAA-β3hAla-β3hAla-β3hAla (12)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200
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wavelength (nm)
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAAV-D-Val-V (13)

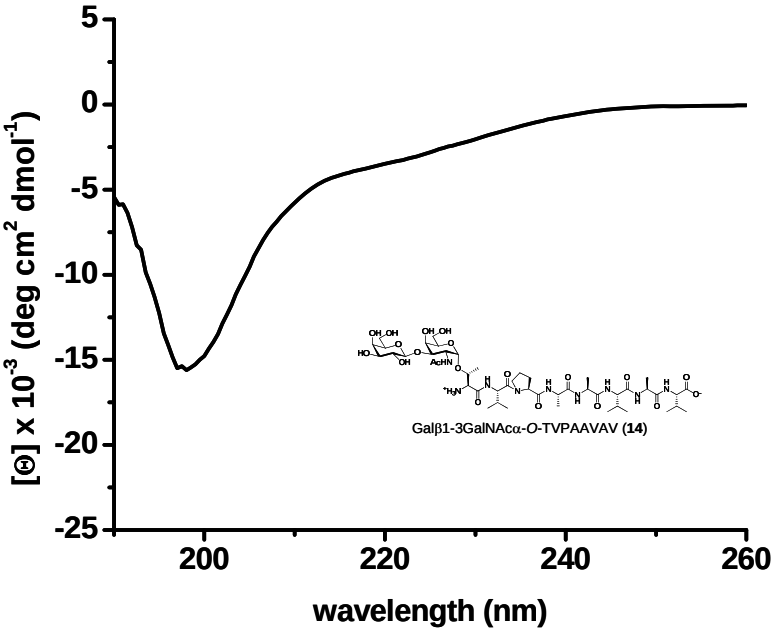
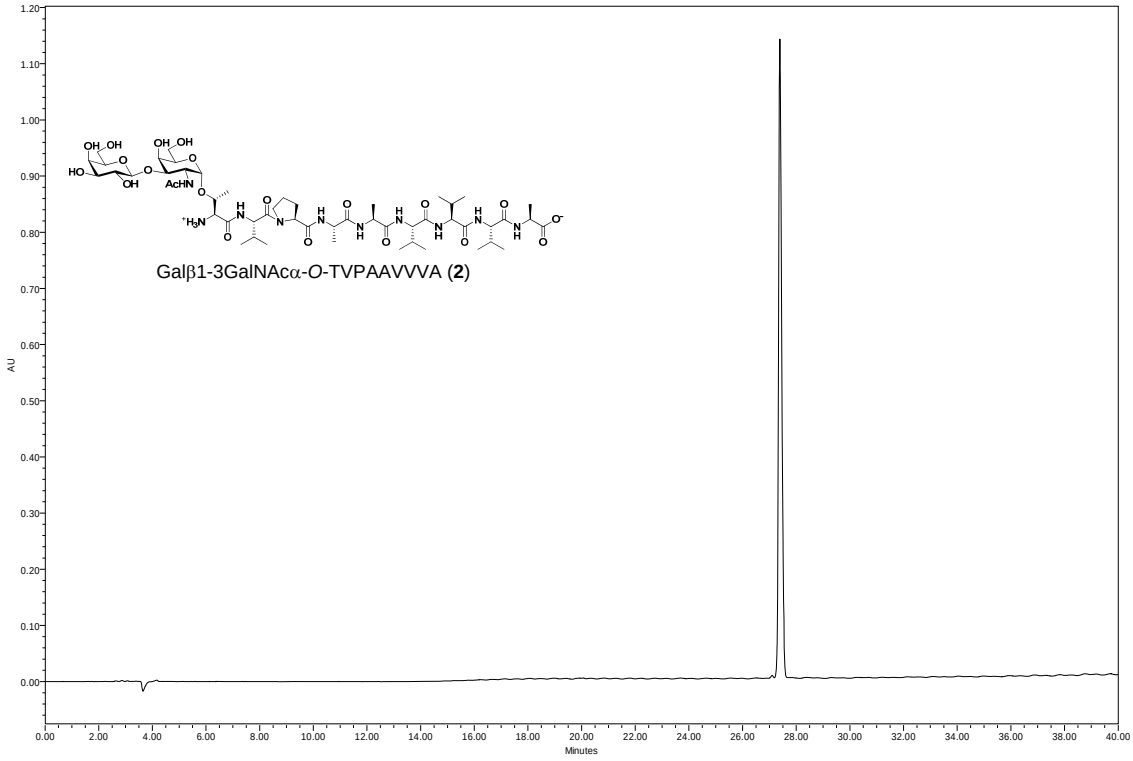
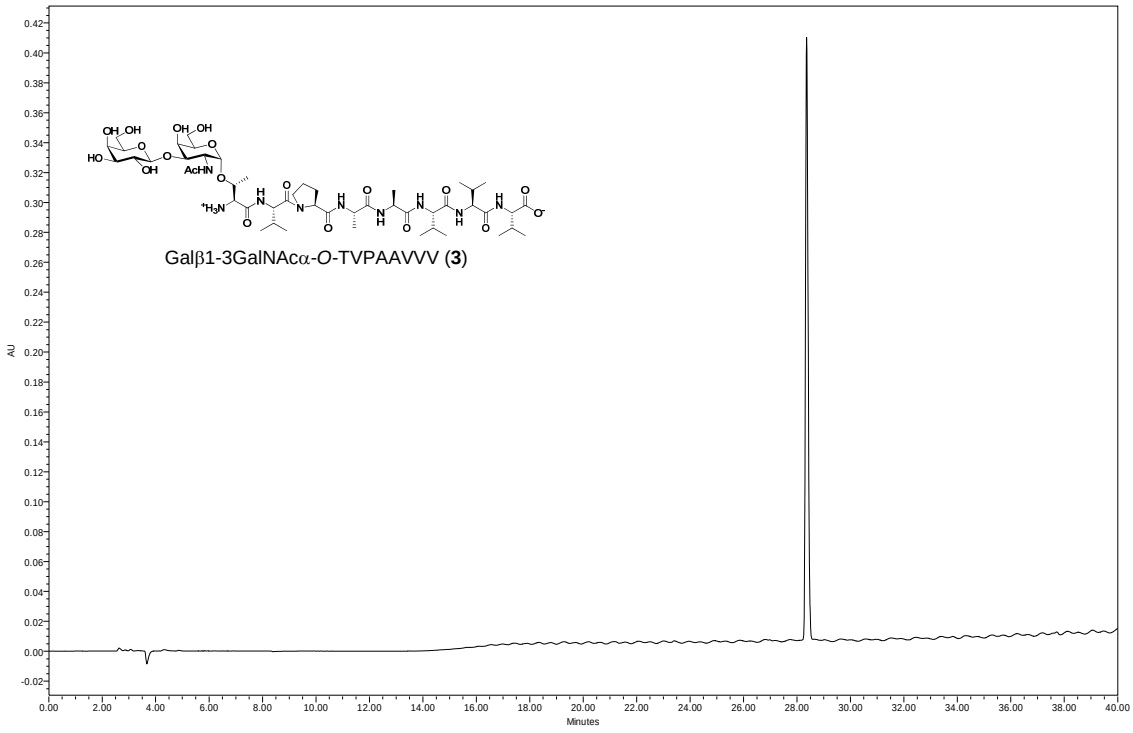
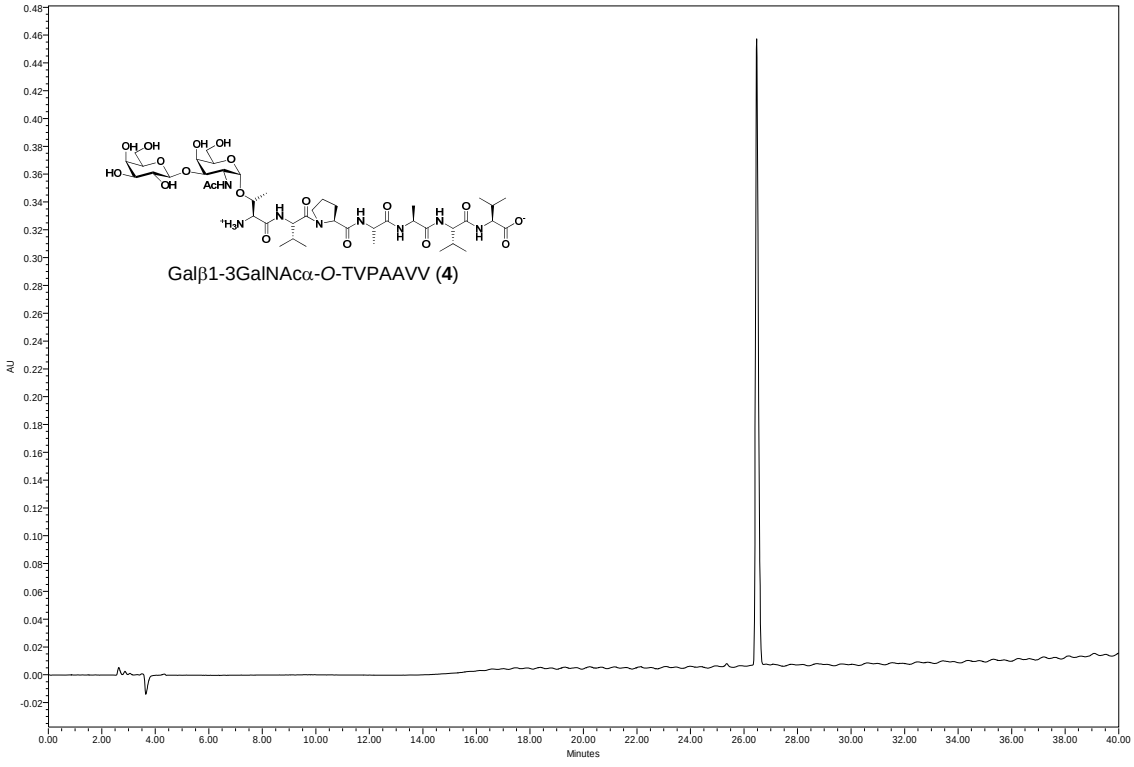

5
0
-5
-10
-15
-20
-25
200
220
240
260
wavelength (nm)
[Θ] x 10-3 (deg cm2 dmol-1)
Galβ1-3GalNAcα-O-TVPAAVAV (14)

Figure S2. HPLC traces. HPLC system: Waters Prep LC 4000 System equipped with PDA detector (Waters 2996) with UV detection (227 nm). Column: Cosmosil C_{18} 250 x 4.6 (Nacalai Tesque, Inc. Kyoto, Japan). Gradient: 0 → 5 min 1% B, 5 → 40 min 1-50% B; A – water (0.1% TFA); B – acetonitrile (0.1% TFA). Flow rate: 1 mL / min.

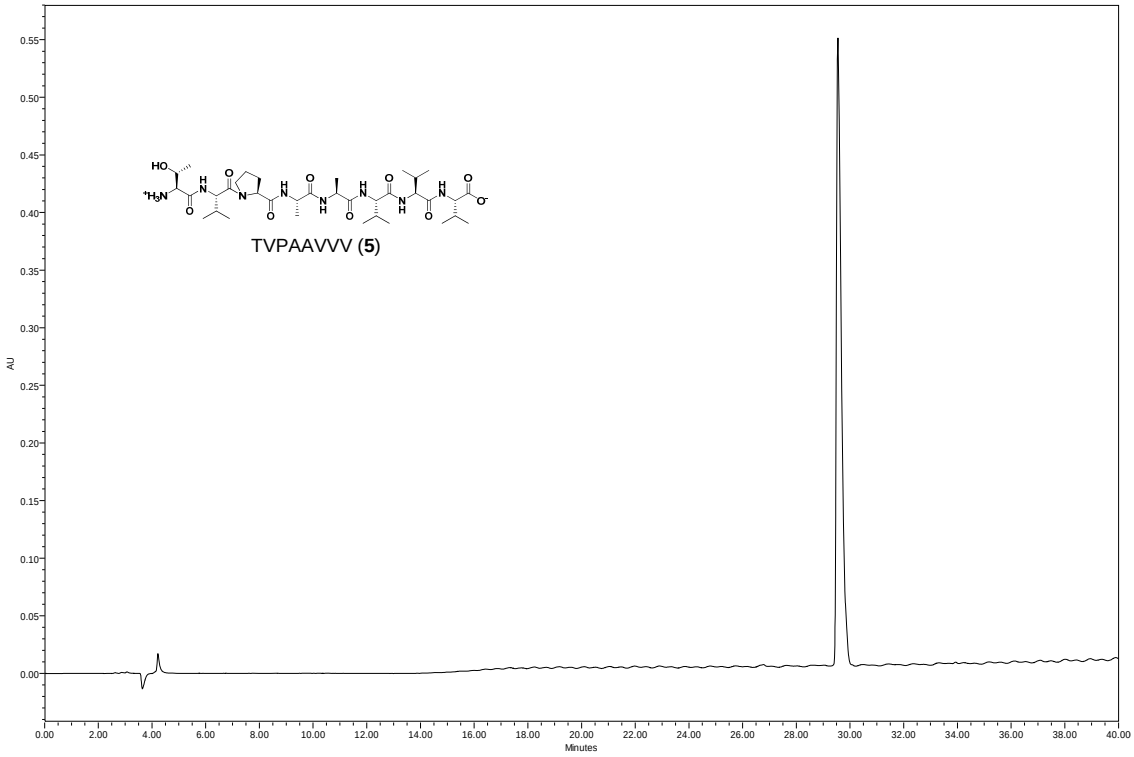


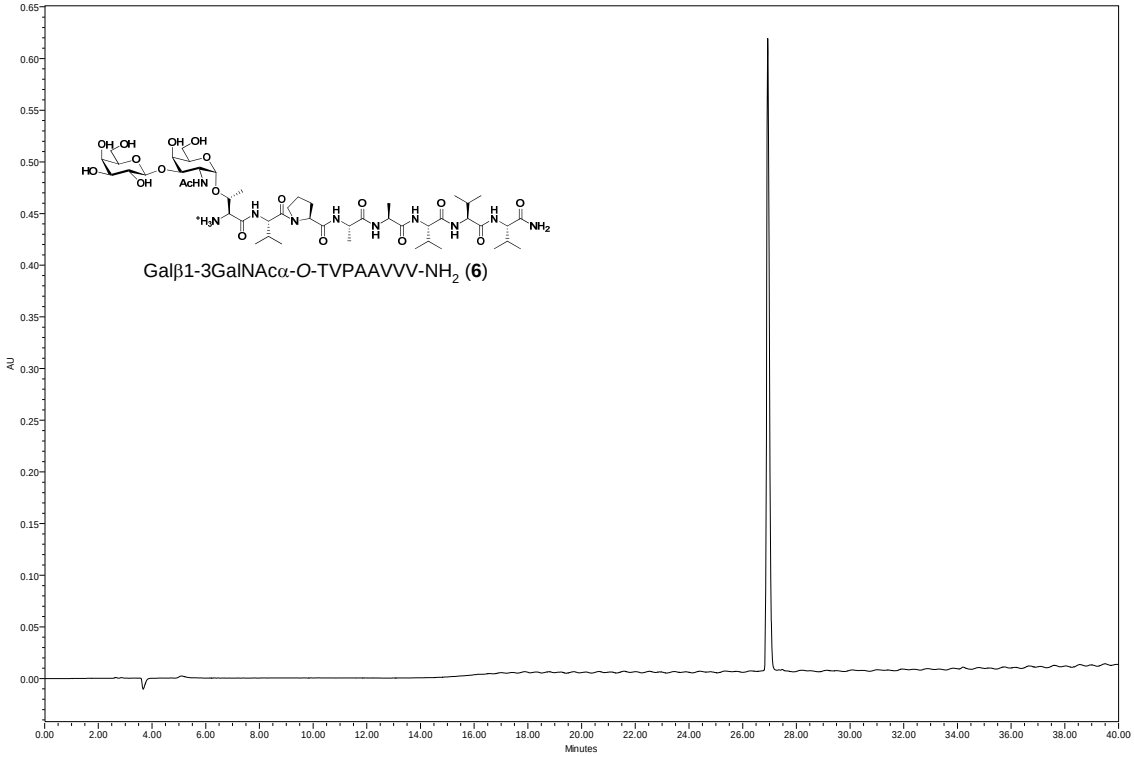


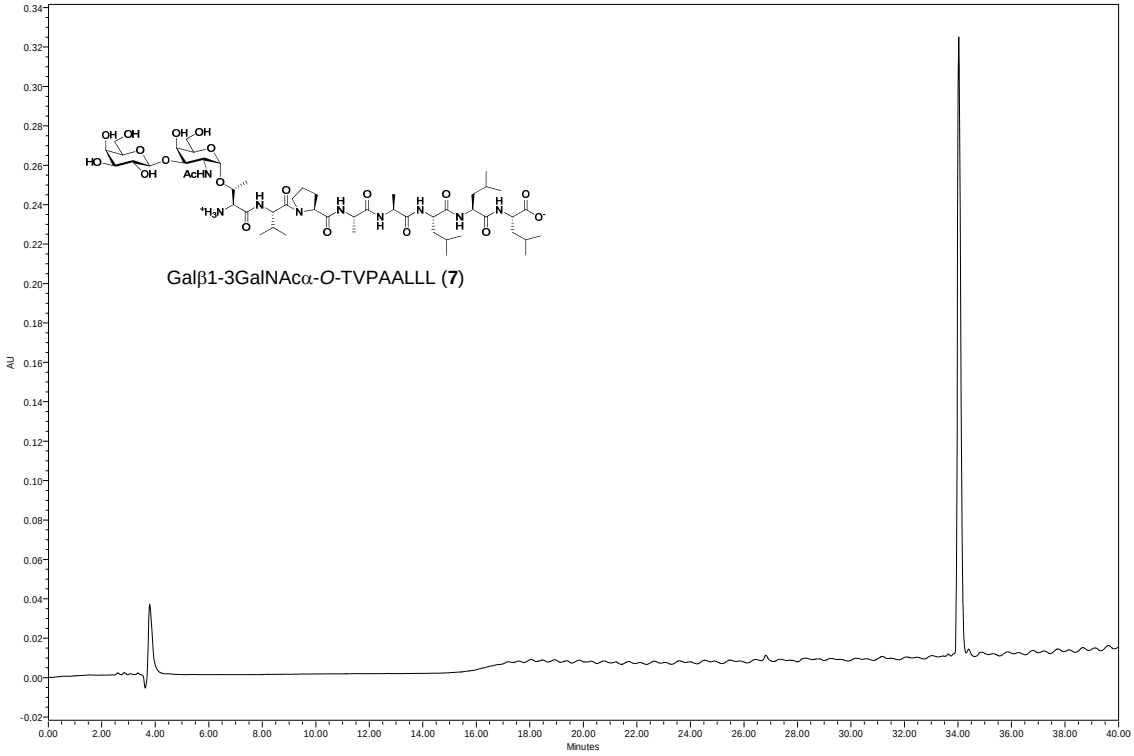
Galβ1-3GalNAcα-*O*-TVPAAVVV (**3**)



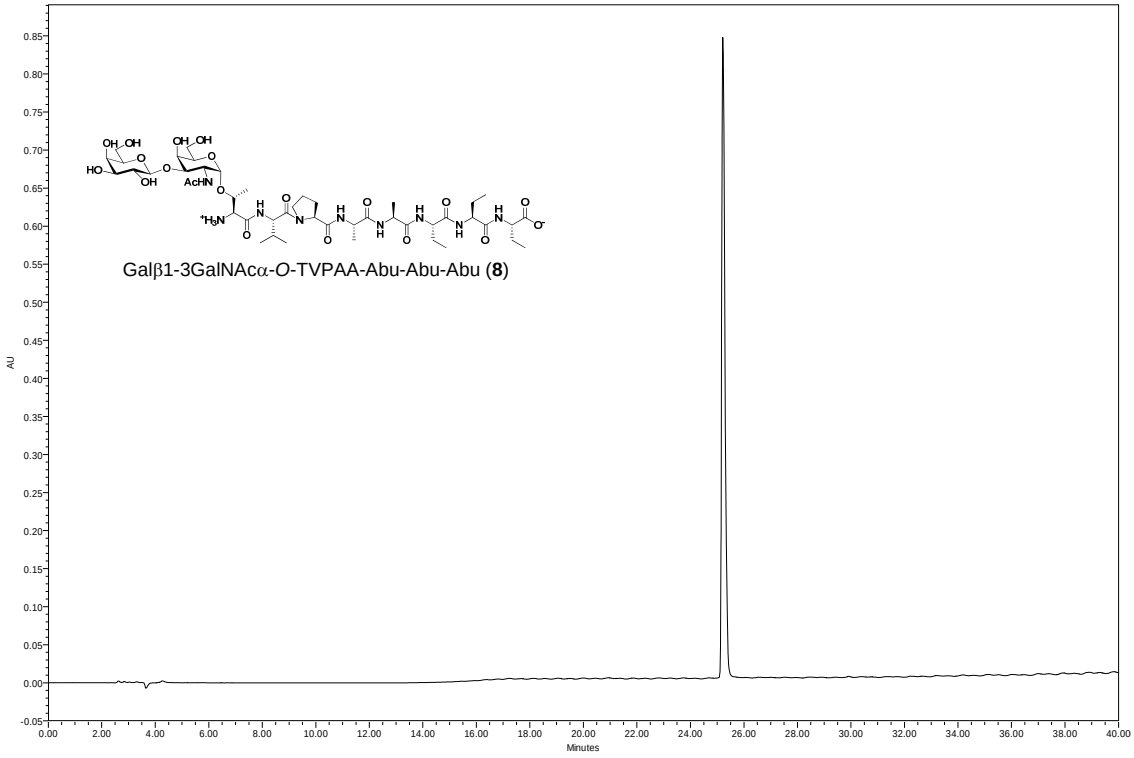
Galβ1-3GalNAcα-*O*-TVPAAVV (**4**)


TVPAAVVV (5)
AU
Minutes

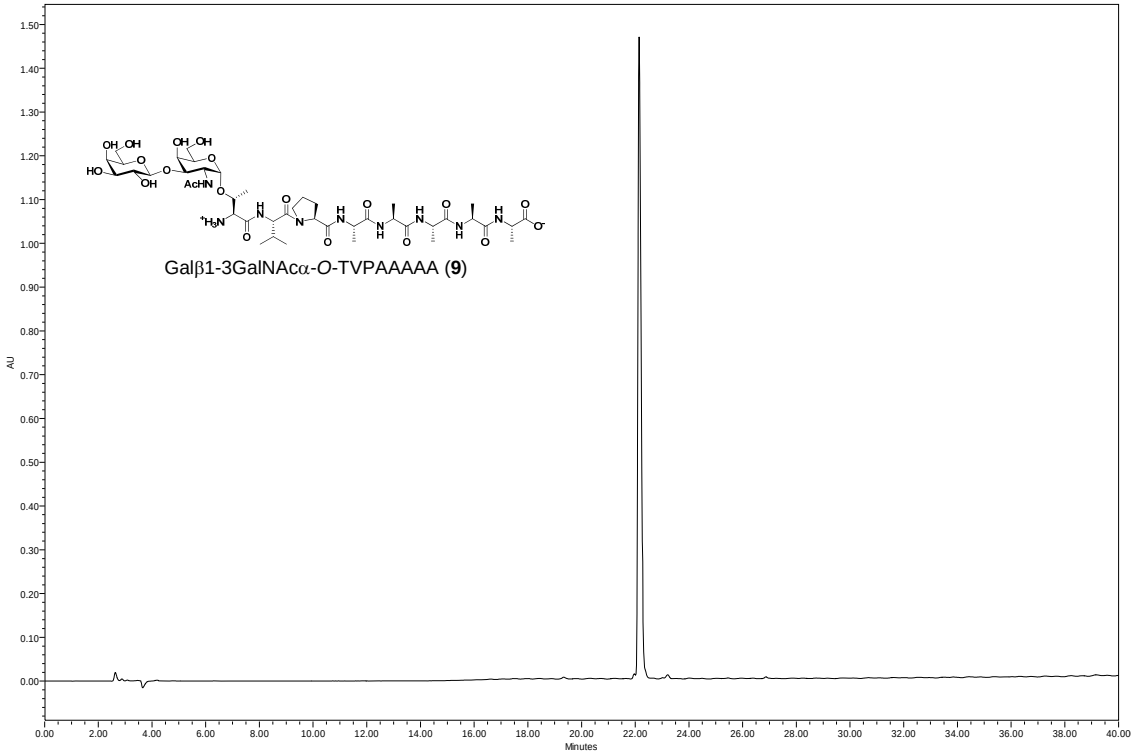

Galβ1-3GalNAcα-O-TVPAAVVV-NH2 (6)
AU
Minutes

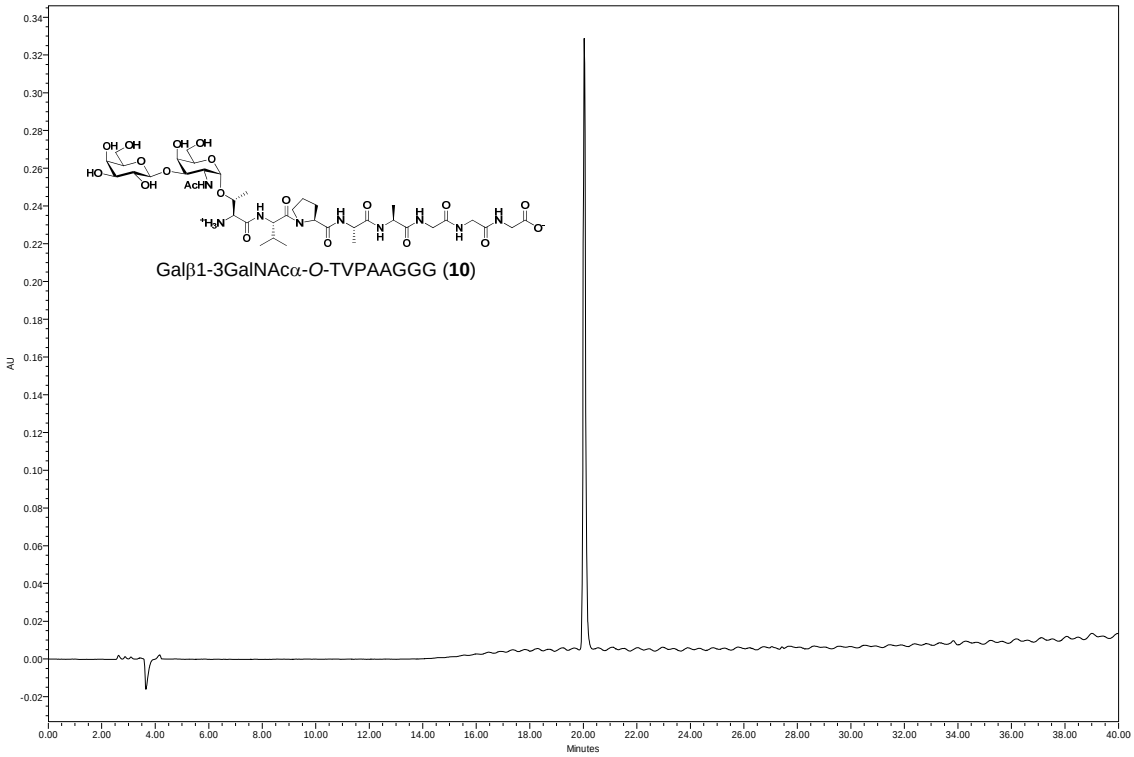


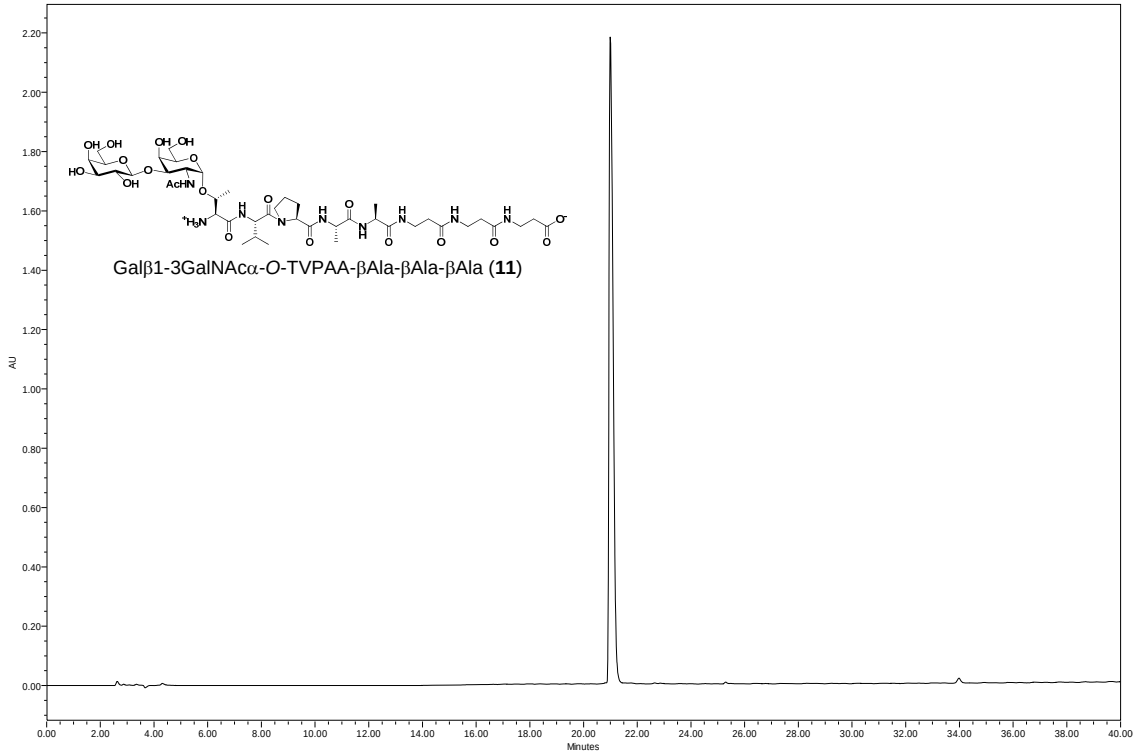
Galβ1-3GalNAcα-*O*-TVPAALLL (**7**)

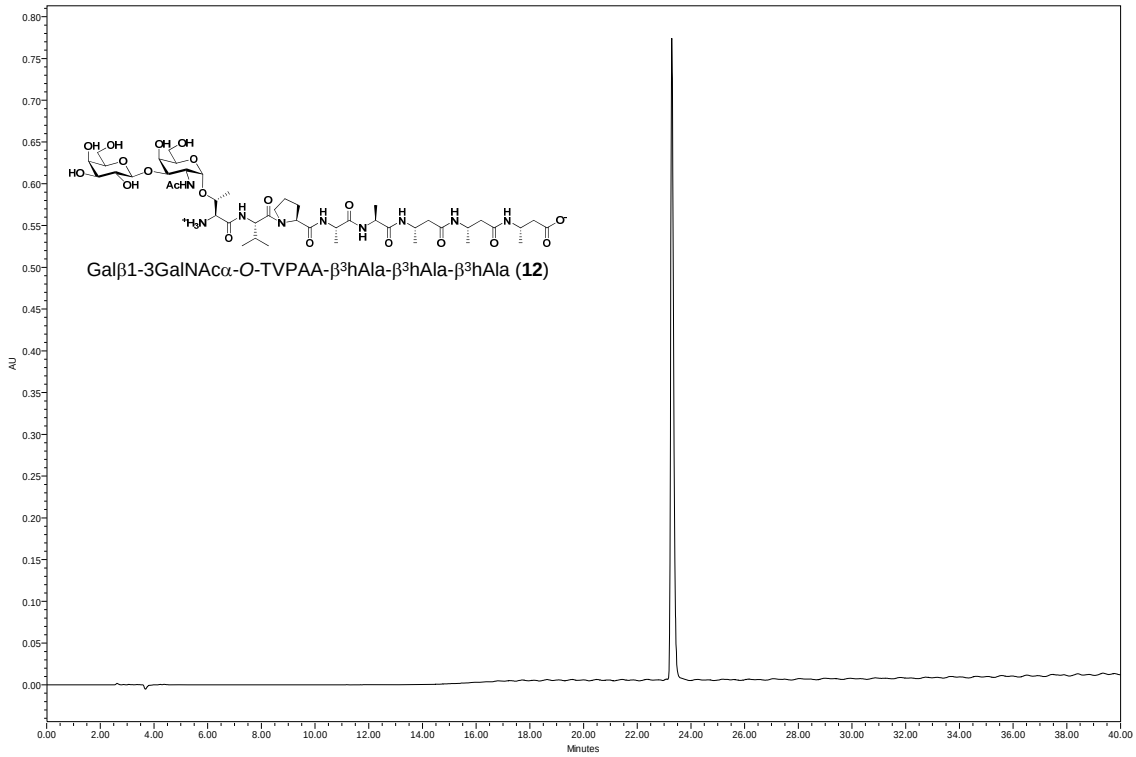


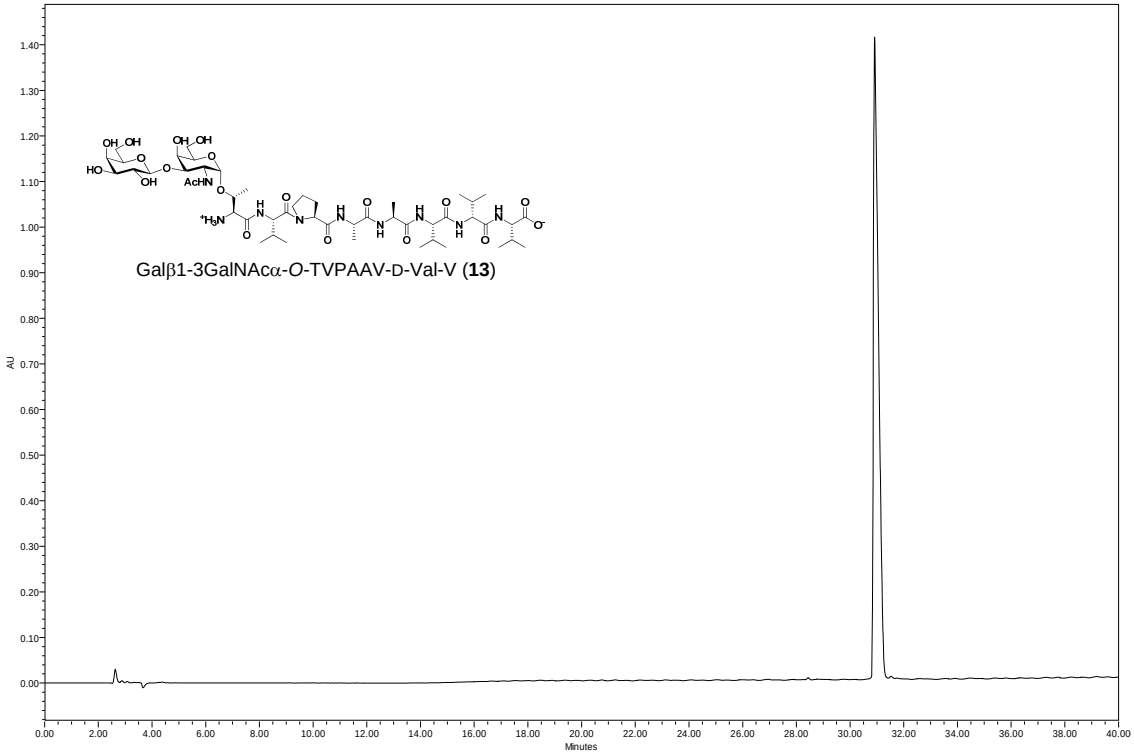
Galβ1-3GalNAcα-*O*-TVPAA-Abu-Abu-Abu (**8**)


Galβ1-3GalNAcα-*O*-TVPAAAAA (**9**)
AU
Minutes

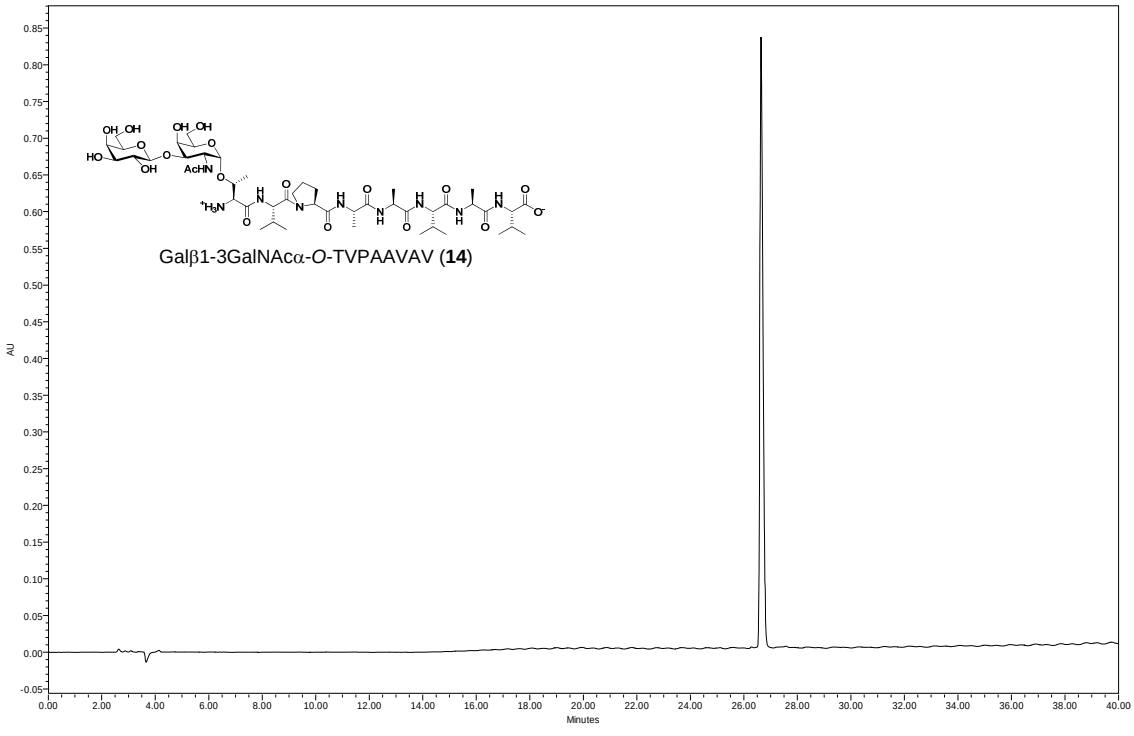

Galβ1-3GalNAcα-*O*-TVPAAGGG (**10**)
AU
Minutes


Galβ1-3GalNAcα-*O*-TVPAA-βAla-βAla-βAla (**11**)
AU
Minutes


Galβ1-3GalNAcα-*O*-TVPAA-β³hAla-β³hAla-β³hAla (**12**)
AU
Minutes



Galβ1-3GalNAcα-*O*-TVPAAV-D-Val-V (**13**)



Galβ1-3GalNAcα-*O*-TVPAAVAV (**14**)

Table S3: Chemical Shift Assignments for Galβ1-3GalNAcα-*O*-TVPAAVVV (**3**) and TVPAAVVV (**5**)

Galβ1-3GalNAcα-*O*-TVPAAVVV (3)

	^{1}H δ (ppm)		^{13}C δ (ppm)
Gal H1'	4.424	**Gal** C1'	107.47
Gal H2'	3.502	**Gal** C2'	73.48
Gal H3'	3.600	**Gal** C3'	75.45
Gal H4'	3.900	**Gal** C4'	71.52
Gal H5'	3.637	**Gal** C5'	77.80
Gal H6'	3.759	**Gal** C6'	63.99

	^{1}H δ (ppm)	$^{3}J_{NH-\alpha}$ (Hz)		^{15}N δ (ppm)
GalNAc N*H*	7.794	10.1	**GalNAc** *N*H	121.45

	^{1}H δ (ppm)		^{13}C δ (ppm)
GalNAc C*H*$_3$	2.022	**GalNAc** *C*H$_3$	25.21
GalNAc H1	4.776	**GalNAc** C1	*
GalNAc H2	4.231	**GalNAc** C2	50.99
GalNAc H3	3.963	**GalNAc** C3	79.67
GalNAc H4	4.205	**GalNAc** C4	71.68
GalNAc H5	4.023	**GalNAc** C5	74.15
GalNAc H6a	3.774	**GalNAc** C6	64.26
GalNAc H6b	3.753		

*indicates resonance could not be assigned due to overlap with the residual water signal

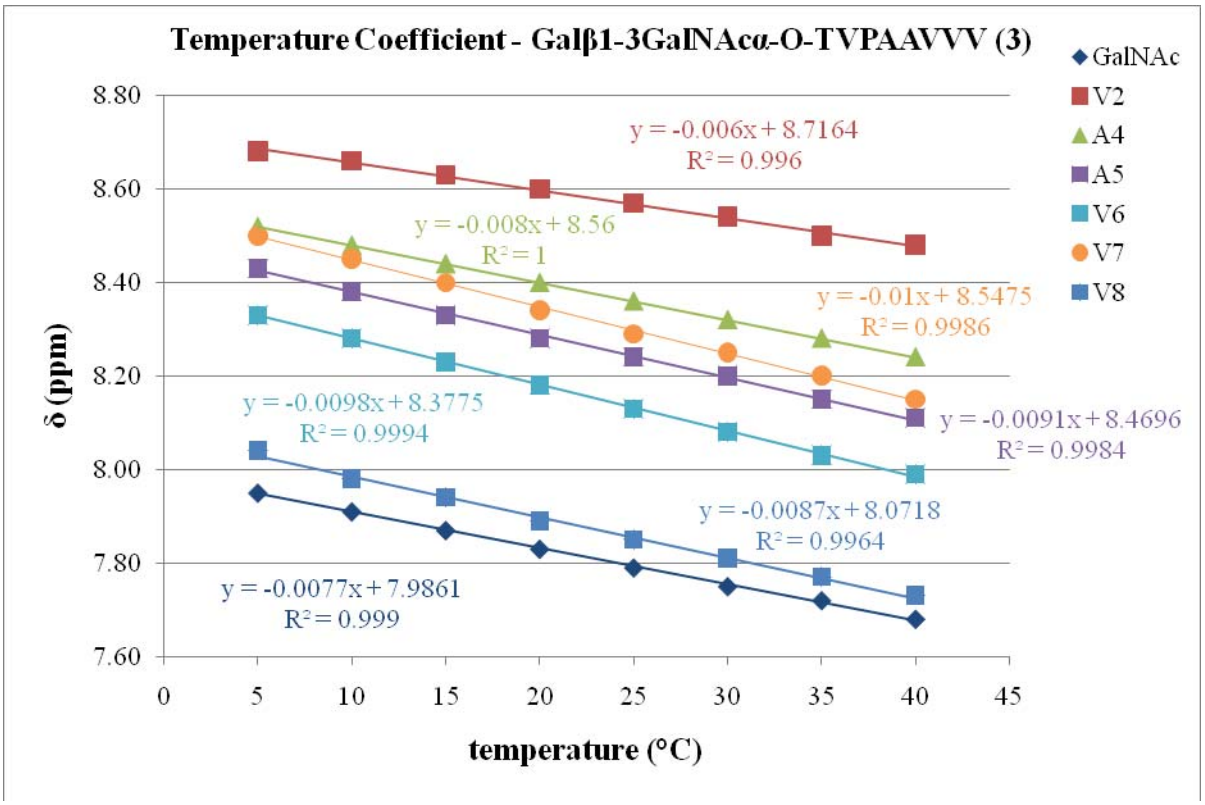
Residue	**H$_N$** ($^{3}J_{NH-H\alpha}$) (δ, ppm)	**N** δ (ppm)	**H$_\alpha$** δ (ppm)	**H$_\beta$** δ (ppm)	**C$_\alpha$** δ (ppm)	**C$_\beta$** δ (ppm)	**Others** δ (ppm)
Thr[1]	--	--	4.170	4.431	60.16	77.02	H$_\gamma$: 1.443 C$_\gamma$: 21.37 C': 170.62
Val[2]	8.567 (8.2 Hz)	119.70	4.528	2.149	59.61	32.39	H$^\gamma$: 0.922/1.012 C$_\gamma$: 19.73/21.09 C': 173.54

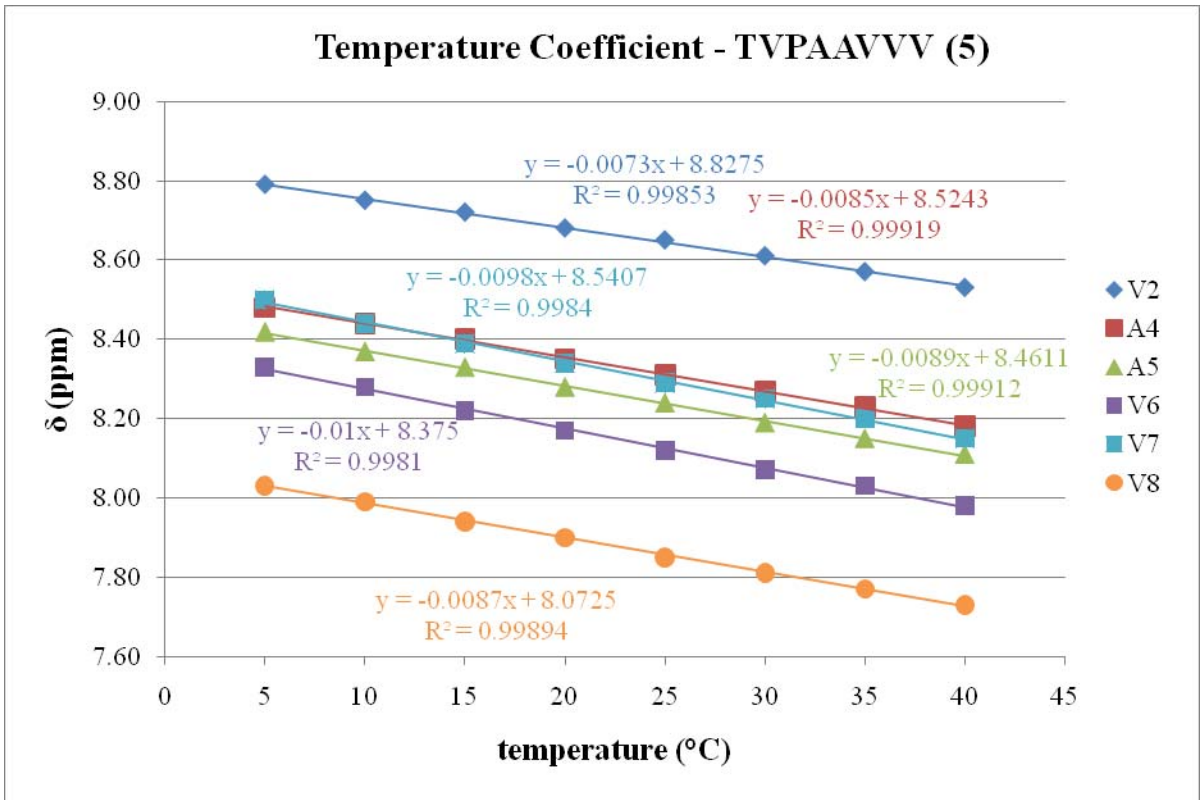
							H^{γ}: 2.067/1.992 C^{γ}: 27.63 H^{δ}: 3.721/3.700 C^{δ}: 50.88
Pro[3]	--	--	4.338	2.326/ 1.903	62.94	32.05	C': 176.52
Ala[4]	8.364 (5.3 Hz)	124.91	4.210	1.386	52.47	19.28	C': 177.51
Ala[5]	8.241 (5.8 Hz)	123.59	4.313	1.355	52.31	19.37	C': 177.58
Val[6]	8.129 (7.8 Hz)	120.44	4.099	2.023	62.40	32.95	H^{γ}: 0.915/0.938 C^{γ}: 21.22/21.58 C': 175.92
Val[7]	8.297 (8.5 Hz)	126.18	4.140	2.040	62.40	32.94	H^{γ}: 0.923 C^{γ}: 20.27/21.22 C': 175.34
Val[8]	7.851 (8.8 Hz)	128.72	4.084	2.060	62.58	33.28	H^{γ}: 0.894/0.909 C^{γ}: 20.75 C': 180.57

TVPAAVVV (5)

Residue	**H_N** ($^3J_{NH-H\alpha}$) δ (ppm)	**N** δ (ppm)	**H_α** δ (ppm)	**H_β** δ (ppm)	**C_α** δ (ppm)	**C_β** δ (ppm)	**Others** δ (ppm)
Thr[1]	--	--	3.928	4.103	61.44	69.26	H^{γ}: 1.280 C^{γ}: 21.65 C': 170.78
Val[2]	8.646 (8.1 Hz)	125.24	4.513	2.120	60.19	32.57	H^{γ}: 0.959/1.013 C^{γ}: 20.16/21.02 C': 174.07
Pro[3]	--	--	4.383	2.000/ 1.900	63.21	32.20	H^{γ}: 2.045/1.986 C^{γ}: 27.51 H^{δ}: 3.691/3.886 C^{δ}: 51.18 C': 176.42
Ala[4]	8.312 (5.3 Hz)	124.53	4.262	1.381	52.37	19.36	C': 177.42
Ala[5]	8.237 (5.8 Hz)	123.68	4.312	1.362	52.34	19.36	C': 177.51
Val[6]	8.120 (7.8 Hz)	120.39	4.092	2.023	62.41	32.94	H^{γ}: 0.923/0.938 C^{γ}: 20.74/20.74 C': 175.93
Val[7]	8.294 (8.5 Hz)	126.15	4.145	2.040	62.57	33.30	H^{γ}: 0.917/0.919 C^{γ}: 21.22 C': 175.36
Val[8]	7.854 (8.8 Hz)	128.65	4.056	2.062	63.55	33.29	H^{γ}: 0.890/0.892 C^{γ}: 20.27/21.58 C': 180.54

Figure S3. Temperature coefficient data for compounds **3** and **5**.





1. Chan, W. C.; White, P. D. *Fmoc solid phase peptide synthesis : a practical approach*. Oxford University Press: New York, 2000; p xxiv, 346 p.

2. Keay, S.; Zhang, C. O.; Trifillis, A. L.; Hise, M. K.; Hebel, J. R.; Jacobs, S. C.; Warren, J. W. Decreased H-3-thymidine incorporation by human bladder epithelial cells following exposure to urine from interstitial cystitis patients. *Journal of Urology* **1996,** 156, 2073-2078.

3. Keay, S. K.; Szekely, Z.; Conrads, T. P.; Veenstra, T. D.; Barchi, J. J.; Zhang, C. O.; Koch, K. R.; Michejda, C. J. An antiproliferative factor from interstitial cystitis patients is a frizzled 8 protein-related sialoglycopeptide. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** 101, 11803-11808.

4. Piotto, M.; Saudek, V.; Sklenar, V. Gradient-Tailored Excitation for Single-Quantum Nmr-Spectroscopy of Aqueous-Solutions. *Journal of Biomolecular Nmr* **1992,** 2, 661-665.

5. Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. Nmrpipe - a Multidimensional Spectral Processing System Based on Unix Pipes. *Journal of Biomolecular Nmr* **1995,** 6, 277-293.

6. Johnson, B. A. Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol Biol* **2004,** 278, 313-52.

7. Cheng, T. J.; Zhao, Y.; Li, X.; Lin, F.; Xu, Y.; Zhang, X. L.; Li, Y.; Wang, R. X.; Lai, L. H. Computation of octanol-water partition coefficients by guiding an additive model with knowledge. *Journal of Chemical Information and Modeling* **2007,** 47, 2140-2148.