Supplementary Information (SI) for Analyst.
This journal is © The Royal Society of Chemistry 2026

Supporting Information

Synthesis of Rhodamine B Hydrazine Derivatives and Their Application in Glycan Analysis

Yu-Nan Chen,[†a] Xiao-Long Hou,[†d] Ying Liu,[*a] Xu-Yang Shen,[a] Yi-Xuan Gu,[a] Yue Yu,[a,b] Xin Zheng,[c] Ying-Lin Zhou,[*a] Xin-Xiang Zhang[a]

a. Beijing National Laboratory for Molecular Sciences (BNLMS), Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China. E-mail: zhouyl@pku.edu.cn. liuying17@pku.edu.cn.

b. Institute of Biotechnology Development, Qilu Pharmaceutical, Jinan 250100, China.

c. Beijing Key Laboratory of Clinical PK & PD Investigation for Innovative Drugs, Clinical Pharmacology Research Center, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100032, China.

d. School of Investigation, People's Public Security University of China, Beijing 100038, China.

† These authors contributed equally to this work.

Contents

Synthesis of labeling reagents

Synthesis of Intermediate 1-1, 1-2, and Compound 1 (Tetramethyl rhodamine hydrazine, TM): 3-Aminophenol (1.1 g, 10 mmol) was mixed with K_2CO_3 (3 g, 22 mmol) and dissolved in 10 mL anhydrous THF in a glovebox. Then, CH_3I (3.1 g, 22 mmol) was added, and the mixture was sealed and taken out of the glovebox. The reaction was carried out at 40°C for 12 hours. The reaction mixture was diluted with water, extracted three times with EA (ethyl acetate) (3 × 25 mL), and the organic phase was collected. After drying with $MgSO_4$ and concentration by rotary evaporation, purification was performed by silica gel column chromatography. The eluent used was EA:PE = 1:5 (v/v), resulting in the formation of a light purple solid Intermediate 1-1. 0.42 g of Intermediate 1-1 was mixed with phthalic anhydride (0.68 g, 4.6 mmol) and dissolved in 2.2 mL o-dichlorobenzene (*o*-DCB). The reaction was continued by adding 0.28 g of Intermediate 1-1 and allowing it to react for an additional 6 hours at 175°C. The reaction mixture was directly loaded onto a silica gel column for purification (2-5% Et3N/EA), yielding a purplish-red solid, Intermediate 1-2. 20 mg of Intermediate 1-2 was dissolved in 1 mL anhydrous ethanol, and under nitrogen protection, 2 mL of an ethanol solution containing 50 μL of 85% hydrazine hydrate was added. The reflux reaction was carried out for 8 hours, followed by purification using thin-layer chromatography (1% Et_3N/EA). The product was dried under nitrogen gas and further subjected to vacuum drying at 50°C for 12 hours, resulting in the formation of a light purple Compound 1. 1H NMR (400 MHz, DMSO-d6) δ 7.89 – 7.69 (m, 1H), 7.57 – 7.34 (m, 2H), 7.04 – 6.86 (m, 1H), 6.56 – 6.14 (m, 6H), 4.29 (s, 2H), 2.90 (s, 12H). ESI-MS calculated: $[M+H]^+$ 401.1973, observed: 401.1970.

Synthesis of Intermediate 2-1, 2-2, and Compound 2 (Tetraethyl rhodamine hydrazine, TE, also known as RBH): 3-Aminophenol (1.1 g, 10 mmol) was mixed with K_2CO_3 (3 g, 22 mmol) and dissolved in 10 mL anhydrous THF in a glovebox. C_2H_5I (3.43 g, 22 mmol) was added, and the mixture was sealed and taken out of the glovebox. The reaction was carried out at room temperature for 12 hours. The reaction mixture was diluted with water and extracted three times with EA (3 × 25 mL), and the organic phase was collected. After drying with $MgSO_4$ and concentration by rotary evaporation, purification was performed by silica gel column chromatography. The eluent used was EA:PE = 1:5 (v/v), resulting in the formation of a brown oily Intermediate 2-1. 0.51 g of Intermediate 2-1 was mixed with phthalic anhydride (0.68 g, 4.6 mmol) and dissolved in 2.2 mL *o*-DCB. After 1 hour of reaction at 175°C, 0.34 g of Intermediate 2-1 was added, and the reaction was continued for an additional 10 hours. The reaction mixture was directly loaded onto a silica gel column for purification (2% Et_3N/EA), yielding a purplish-red solid, Intermediate 2-2. 20 mg of 2-2 was dissolved in 1 mL anhydrous ethanol, and under nitrogen protection, 2 mL of an ethanol solution containing 50 μL of 85% hydrazine hydrate was added. The reflux reaction was carried out for 8 hours, followed by purification using thin-layer chromatography (1% Et_3N/EA). The product was dried under nitrogen gas and further subjected to vacuum drying at 50°C for 12 hours, resulting in the

formation of a light purple Compound 2. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.78 (s, 1H), 7.58 – 7.38 (m, 2H), 7.00 (d, J = 5.5 Hz, 1H), 6.37 (dd, J = 17.4, 6.5 Hz, 6H), 4.29 (d, J = 6.4 Hz, 2H), 3.36 (dd, J = 13.7, 6.6 Hz, 8H), 1.16 (s, 0H), 1.09 (q, J = 7.1 Hz, 12H). ESI-MS calculated: $[M+H]^+$ 457.2599, observed: 457.2604.

Synthesis of Intermediate 3-1, 3-2, and Compound 3 (Tetrapropyl rhodamine hydrazine, TP): 3-Aminophenol (1.1 g, 10 mmol) was mixed with K_2CO_3 (3 g, 22 mmol) and dissolved in 10 mL anhydrous THF in a glovebox. n-C3H7I (3.74 g, 22 mmol) was added, and the mixture was sealed and taken out of the glovebox. The reaction was carried out at room temperature for 12 hours. The reaction mixture was diluted with water, extracted three times with EA (3 × 25 mL), and the organic phase was collected. After drying with $MgSO_4$ and concentration by rotary evaporation, purification was performed by silica gel column chromatography. The eluent used was EA:PE = 2:5 (v/v), resulting in the formation of a black oily Intermediate 3-1. 0.60 g of Intermediate 3-1 was mixed with phthalic anhydride (0.68 g, 4.6 mmol) and dissolved in 2.2 mL *o*-DCB. The reaction was continued by adding 0.40 g of Intermediate 3-1 and allowing it to react for an additional 3 hours at 175°C. The reaction mixture was directly loaded onto a silica gel column for purification (2% Et_3N/EA), yielding a deep purple solid, Intermediate 3-2. 20 mg of 3-2 was dissolved in 1 mL anhydrous ethanol, and under nitrogen protection, 2 mL of an ethanol solution containing 50 μL of 85% hydrazine hydrate was added. The reflux reaction was carried out for 8 hours, followed by purification using thin-layer chromatography (EA). The product was dried under nitrogen gas, and further subjected to vacuum drying at 50°C for 12 hours, resulting in the formation of a red Compound 3. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.88 – 7.66 (m, 1H), 7.54 – 7.32 (m, 2H), 7.07 – 6.91 (m, 1H), 6.33 (dd, J = 13.8, 1.4 Hz, 6H), 4.26 (s, 2H), 3.21 (t, J = 7.7 Hz, 8H), 1.52 (q, J = 7.5 Hz, 8H), 0.88 (t, J = 7.3 Hz, 12H). ESI-MS calculated: $[M+H]^+$ 513.3325; observed: 513.3321.

Synthesis of Intermediate 4-1, 4-2, and Compound 4 (d_{20}-TE, also named as d_{20}-RBH): 3-Aminophenol (0.74 g, 6.7 mmol) was mixed with K_2CO_3 (2.58 g, 7.3 mmol) and dissolved in 5 mL anhydrous THF in a glovebox. C_2D_5I (2 g, 14.6 mmol) was added, and the mixture was sealed and taken out of the glovebox. The reaction was carried out at 40°C for 7 days. The reaction mixture was diluted with water, extracted three times with EA (3 × 25 mL), and the organic phase was collected. After drying with anhydrous $MgSO_4$ and concentration by rotary evaporation, purification was performed by silica gel column chromatography using EA:PE = 1:5 as the eluent, resulting in the formation of a brownish-red oily Intermediate 4-1. 0.288 g of Intermediate 4-1 was mixed with phthalic anhydride (0.216 g, 1.5 g) and dissolved in 1.5 mL *o*-DCB. The reaction was carried out at 175°C for 1 hour, followed by the addition of 0.192 g of Intermediate 4-1 and further reaction for 3 hours. The reaction mixture was directly loaded onto a silica gel column for purification (2% Et_3N/EA). Slow crystallization of Intermediate 4-2 was achieved by allowing it to crystallize in the glovebox. The resulting deep purple solid Intermediate 4-2 was dissolved in 35 mg of 1 mL of chloroform, and under ice bath conditions, a solution of 200 μL of sulfur chloride in chloroform was slowly added. The temperature was

increased to reflux for 10 minutes, followed by solvent removal under reduced pressure at 60°C. In an ethanol-liquid nitrogen cold trap bath (173-193 K), 200 μL of Et_3N was added, followed by the slow addition of a 2 mL ethanol solution containing 100 μL of 85% hydrazine hydrate. The mixture was allowed to naturally warm up to room temperature and react for 10 hours, followed by purification using thin-layer chromatography (EA:PE = 2:1). The resulting product was dissolved in 0.5 mL of EA:MeOH = 10:1 (v/v), and upon addition of 5 mL of n-hexane, crystals were precipitated. The crystals were filtered, dried, resulting in a light gray Compound 4. ^1H NMR (600 MHz, DMSO) δ 7.77 (s, 1H), 7.49 (ddd, J = 7.1, 5.1, 1.5 Hz, 2H), 7.08 – 6.87 (m, 1H), 6.56 – 6.07 (m, 6H), 4.27 (s, 2H). ESI-MS calculated: [M+H]+ 477.3854, observed: 477.3842.

Table S1. MS response of labeled products under different conditions of liquid-liquid extraction

Intensity ($\times 10^6$)	10% NH_4OH/H_2O-DCM		10% HAc/H_2O-DCM		H_2O-DCM		H_2O-n-Hexane		H_2O-EA	
	Int	**RSD**	**Int**	**RSD**	**Int**	**RSD**	**Int**	**RSD**	**Int**	**RSD**
DP5	/	/	5.1	11.1%	0.46	8.1%	2.1	77.4%	7.2	24.0%
DP6	/	/	4.8	5.8%	0.53	7.2%	2.4	78.0%	6.3	22.1%
DP7	/	/	2.7	10.9%	0.22	59.6%	1.2	78.6%	3.3	21.0%

Table S2 Working curves, LOD and LOQ of DP5, DP6, and DP7 labeled by TE

Analyte	Equation	Linear range (nM)	R^2	LOD (amol)	LOQ (amol)
DP5	$y\times10^{-4}=(6.80\pm0.14)\,x+(3.19\pm1.15)$	0.5-100	0.993	115	375
DP6	$y\times10^{-4}=(3.78\pm0.21)\,x+(7.87\pm3.26)$	0.5-100	0.991	265	875
DP7	$y\times10^{-4}=(0.73\pm0.02)\,x+(1.47\pm0.30)$	0.5-100	0.997	765	2550

Table S3. Recovery and intra-day and inter-day precision of DP5, DP6, and DP7 labeled by TE in plasma samples

Analyte		Theoretical value (nM)	Mean measured value (nM)	Recovery	RSD
DP5	Intra-day (n=3)	1.00	0.99	99.0%	3.98%
		50.0	52.8	106%	1.78%
		100.0	89.2	89.2%	1.80%
	Inter-day (n=3)	1.00	1.04	104%	4.96%
		50.0	51.6	103%	4.55%
		100.0	87.8	87.8%	2.99%
DP6	Intra-day (n=3)	1.00	0.89	89.0%	6.84%
		50.0	44.8	89.6%	4.52%
		100.0	95.1	95.1%	4.75%
	Inter-day (n=3)	1.00	0.86	86.0%	9.68%
		50.0	46.9	93.9%	5.94%
		100.0	91.6	91.6%	3.98%
DP7	Intra-day (n=3)	1.00	0.95	95.0%	2.05%
		50.0	47.6	95.3%	3.11%
		100.0	93.5	93.5%	2.35%
	Inter-day (n=3)	1.00	0.97	97.0%	2.71%
		50.0	48.2	96.3%	3.37%
		100.0	92.0	92.0%	5.37%

Table S4. Glycotypes identified and determined by peak alignment

m/z of TE labeling	m/z of d_{20}-TE labeling	Glycan Mol. Weight	RT (min)	Predict	Mass delta
878.370	888.434	1317.495	11.98	$(HexNAc)_2+(Man)_3(GlcNAc)_2$	0.001
951.400	961.461	1463.555	12.69	$(HexNAc)_2(Deoxyhexose)_1+(Man)_3(GlcNAc)_2$	0.003
959.396	969.459	1479.547	12.94	$(Hex)_1(HexNAc)_2+(Man)_3(GlcNAc)_2$	0.000
979.910	989.972	1520.575	12.46	$(HexNAc)_3+(Man)_3(GlcNAc)_2$	0.002
1009.473	1029.599	571.235	6.14	$(HexNAc)_2(Deoxyhexose)_1$	0.000
1032.424	1042.486	1625.603	13.63	$(Hex)_1(HexNAc)_2(Deoxyhexose)_1+(Man)_3(GlcNAc)_2$	-0.001
1040.423	1050.484	1641.601	13.96	$(Hex)_2(HexNAc)_2+(Man)_3(GlcNAc)_2$	0.001
1052.938	1063.000	1666.631	13.08	$(HexNAc)_3(Deoxyhexose)_1+(Man)_3(GlcNAc)_2$	0.000
1105.469	1125.594	667.231	7.78	$(Hex)_4$	0.002
1113.451	1123.514	1787.657	14.58	$(Hex)_2(HexNAc)_2(Deoxyhexose)_1+(Man)_3(GlcNAc)_2$	0.000
1133.964	1144.027	1828.683	13.90	$(Hex)_1(HexNAc)_3(Deoxyhexose)_1+(Man)_3(GlcNAc)_2$	0.000
1177.972	1188.035	1916.699	7.53	$(Hex)_1(HexNAc)_2(Deoxyhexose)_1(NeuAc)_1+(Man)_3(GlcNAc)_2$	0.000
1185.971	1196.032	1932.697	7.70	$(Hex)_2(HexNAc)_2(NeuAc)_1+(Man)_3(GlcNAc)_2$	0.002
1214.990	1225.053	1990.735	14.74	$(Hex)_2(HexNAc)_3(Deoxyhexose)_1+(Man)_3(GlcNAc)_2$	-0.001
1258.998	1269.060	2078.751	8.07	$(Hex)_2(HexNAc)_2(Deoxyhexose)_1(NeuAc)_1+(Man)_3(GlcNAc)_2$	-0.001
1267.520	1287.646	829.282	9.24	$(Hex)_5$	0.000
1287.507	1297.570	2135.769	7.91	$(Hex)_2(HexNAc)_3(NeuAc)_1+(Man)_3(GlcNAc)_2$	-0.004
1360.537	1370.600	2281.829	8.25	$(Hex)_2(HexNAc)_3(Deoxyhexose)_1(NeuAc)_1+(Man)_3(GlcNAc)_2$	-0.002
1429.571	1449.697	991.3334	10.57	$(Hex)_6$	0.000

Note: Hex could be Man or Gal, HexNAc was GlcNAc，Deoxyhexose was Fuc.

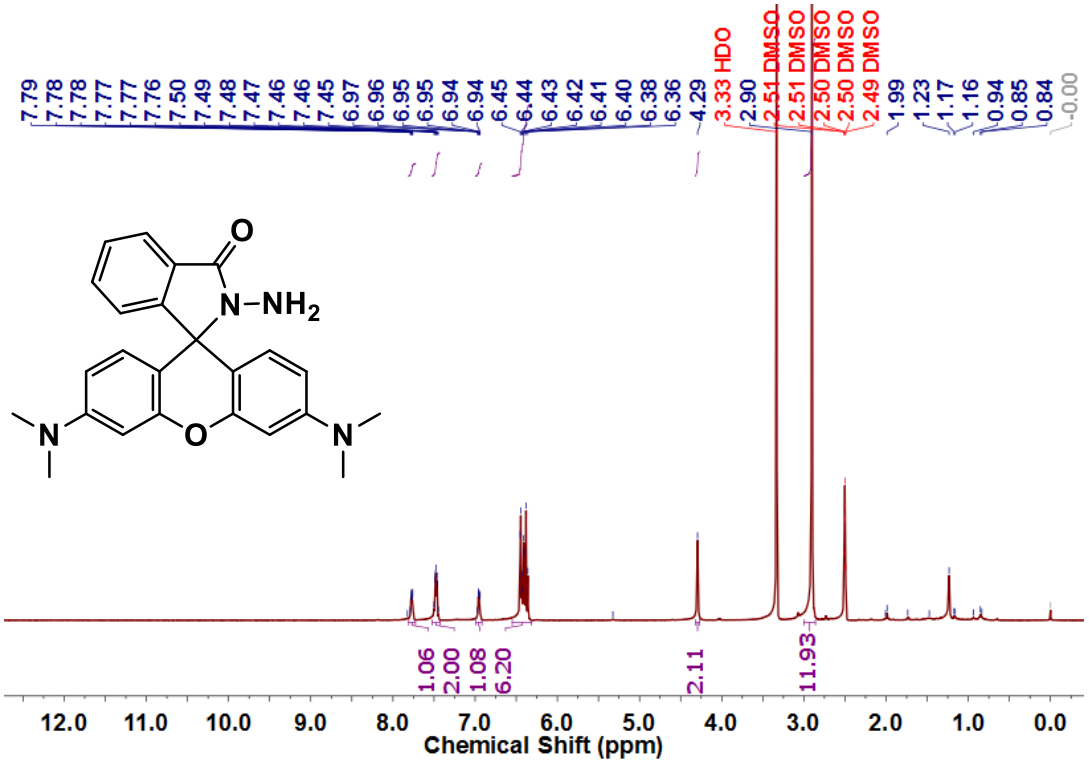

Figure S1. ^{1}H NMR spectrum of Compound 1

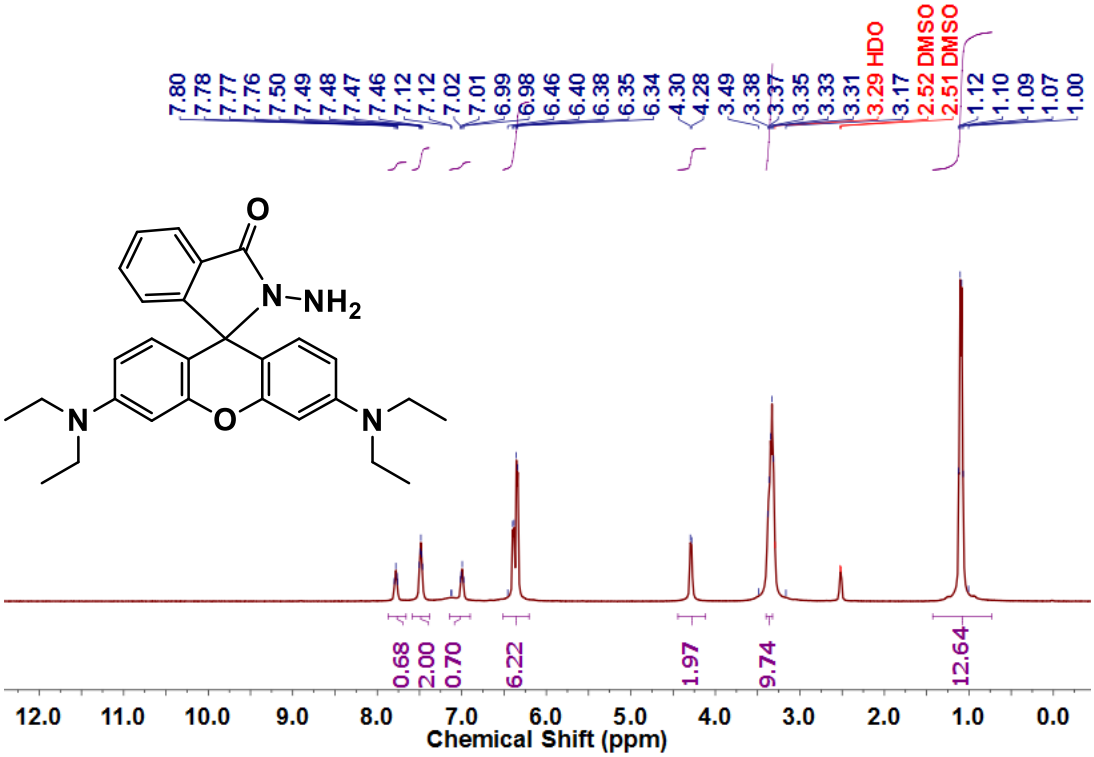

Figure S2. ^{1}H NMR spectrum of Compound 2

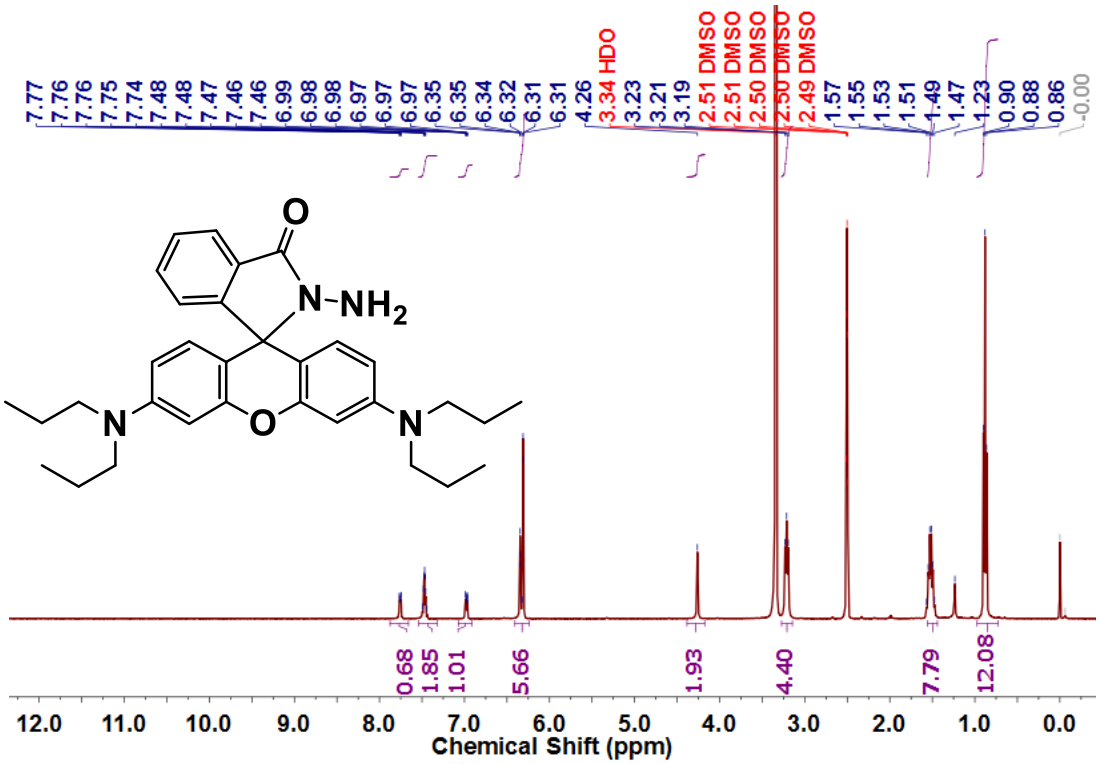

Figure S3. ^{1}H NMR spectrum of Compound 3

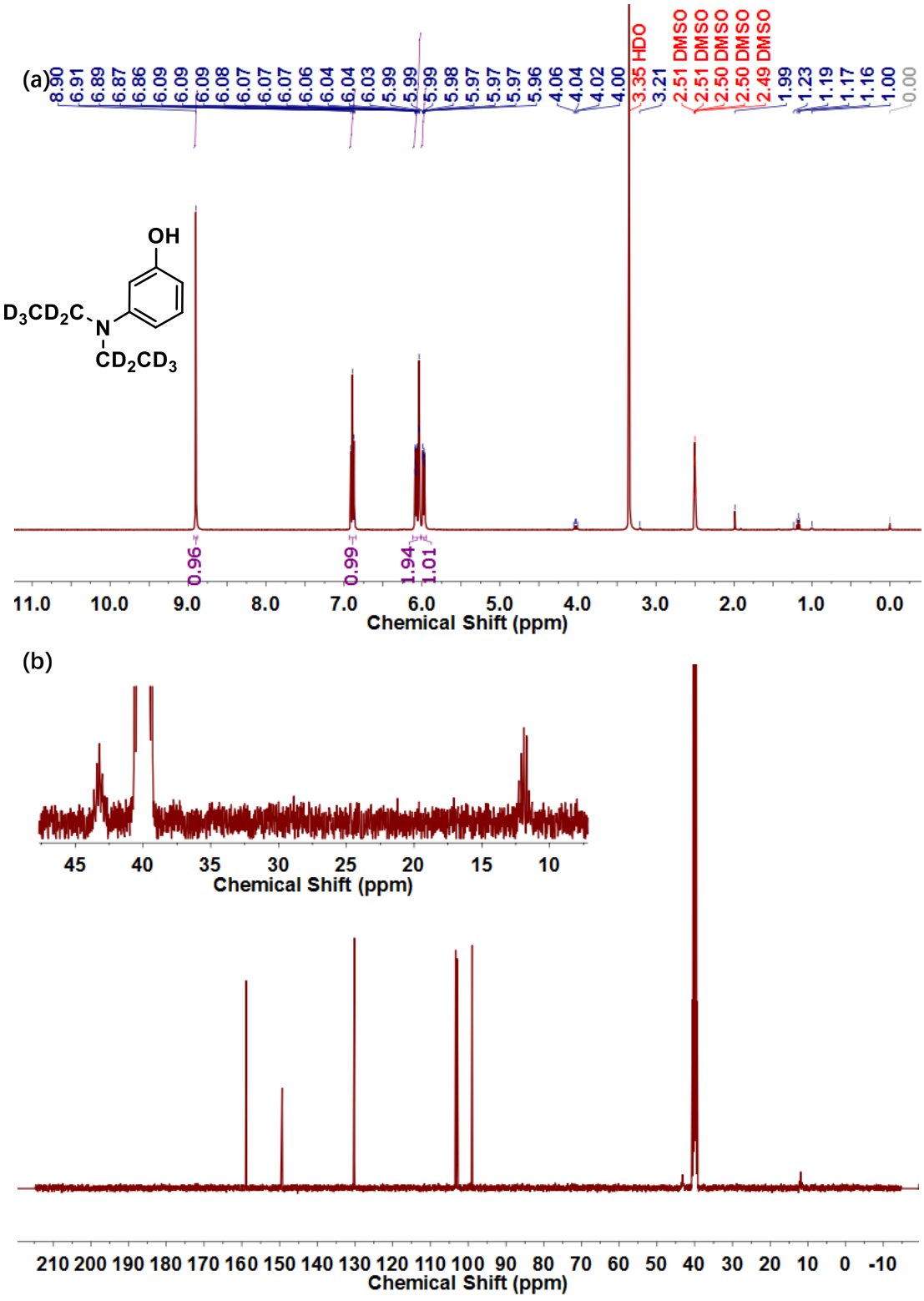

Figure S4. (a) ^{1}H NMR, (b) ^{13}C NMR spectra of Intermediate 4-1

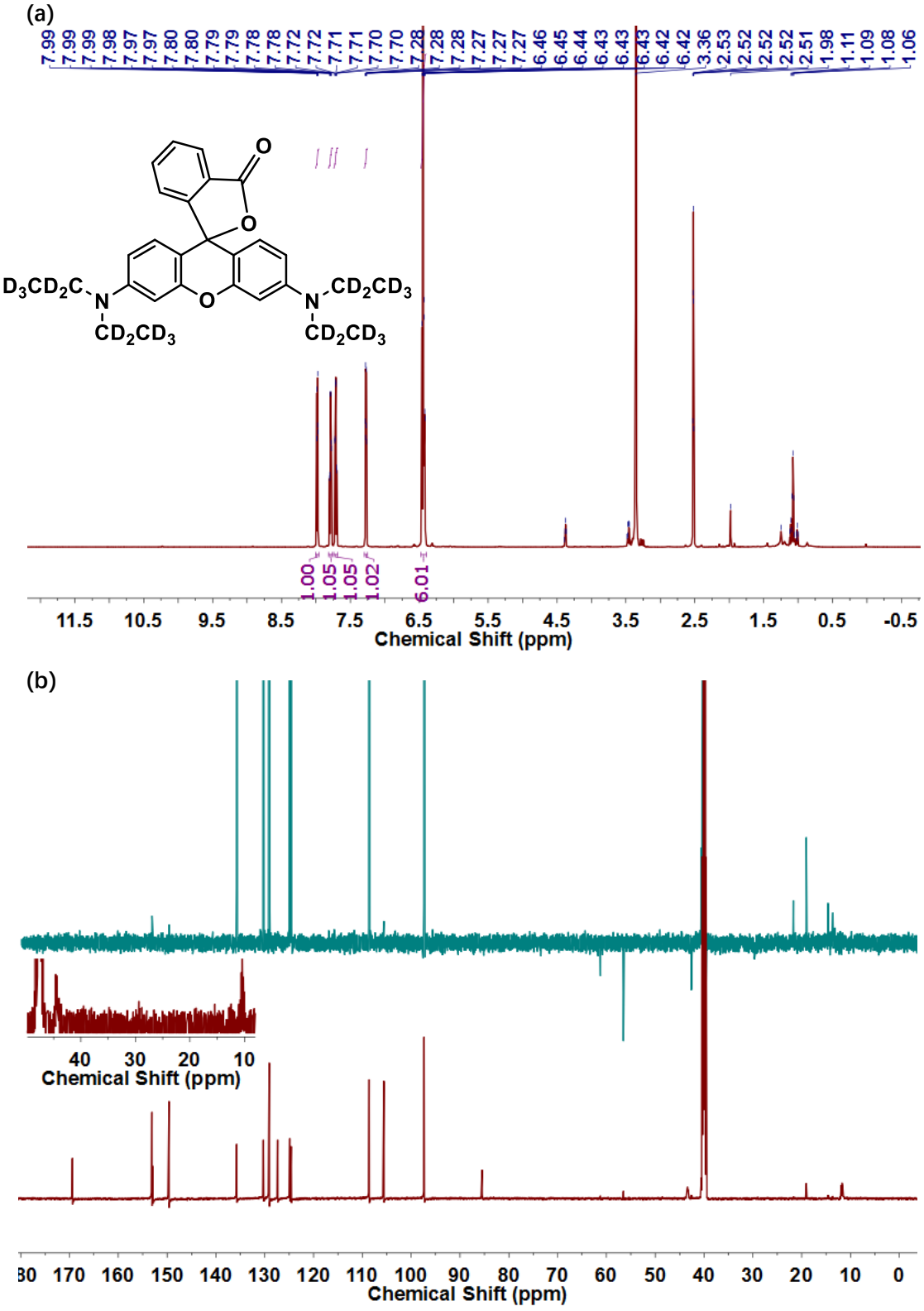

Figure S5. (a) ^{1}H NMR, (b) ^{13}C NMR (with DEPT) spectra of Intermediate 4-2

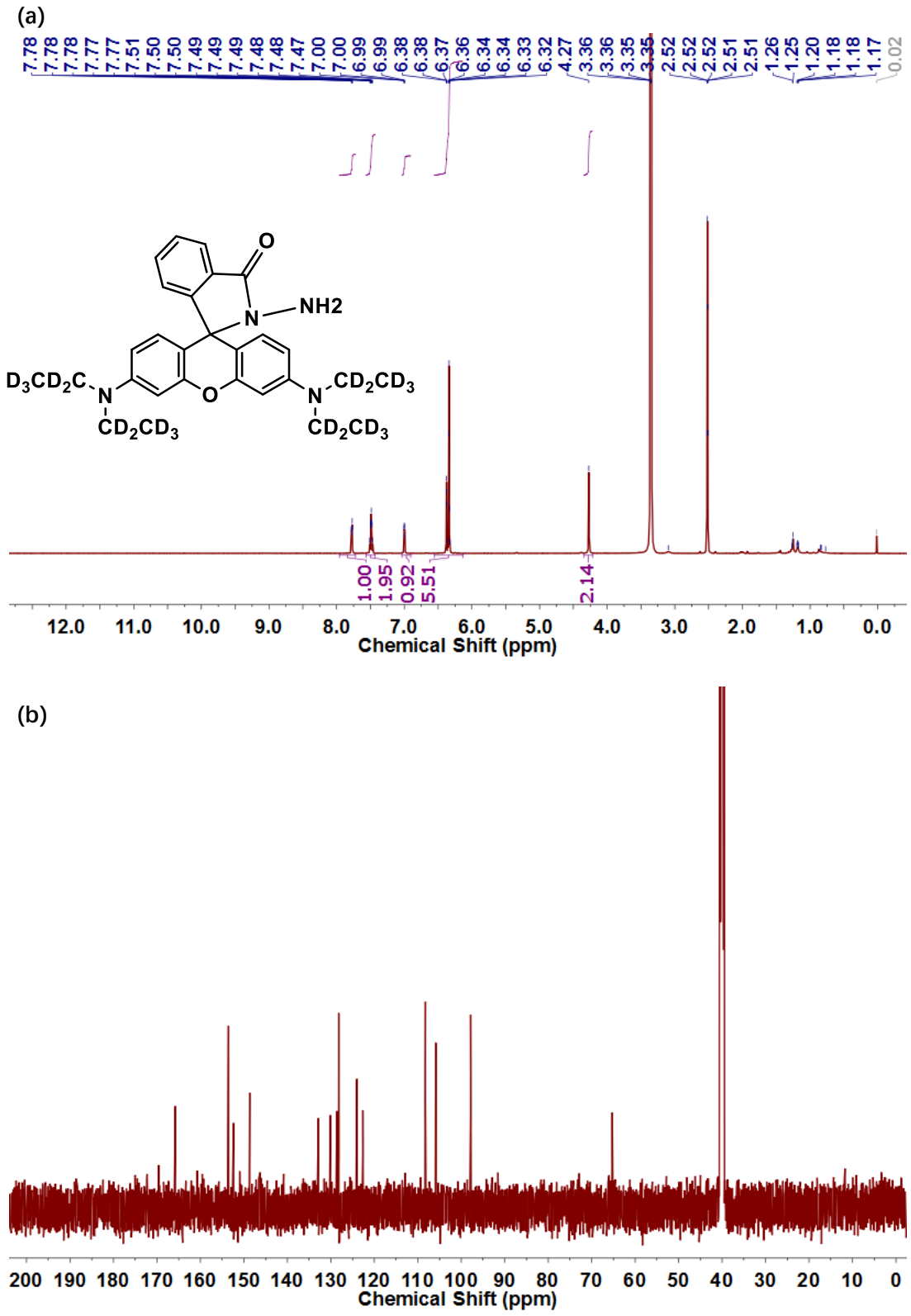

Figure S6. (a) ^{1}H NMR, (b) ^{13}C NMR spectra of Compound 4

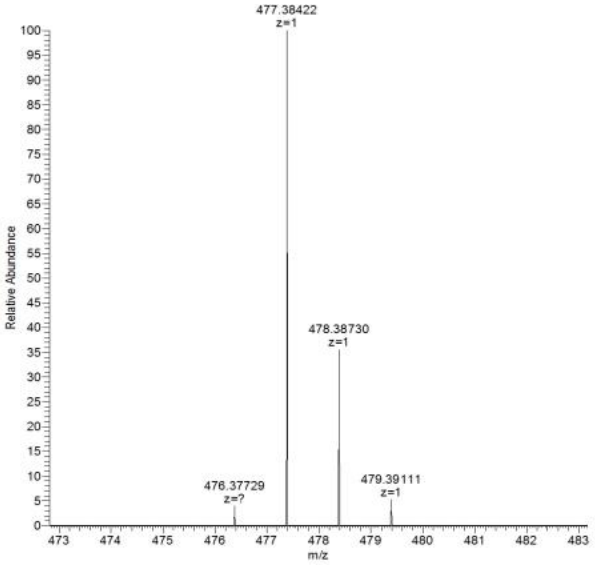

Figure S7. MS spectrum of Compound 4

(a)

Condition	HAc cont. (%)	H_2O cont. (%)	MeOH cont. (%)
A	10	10	80
B	20	10	70
C	30	10	60
D	20	1	79
E	20	5	75

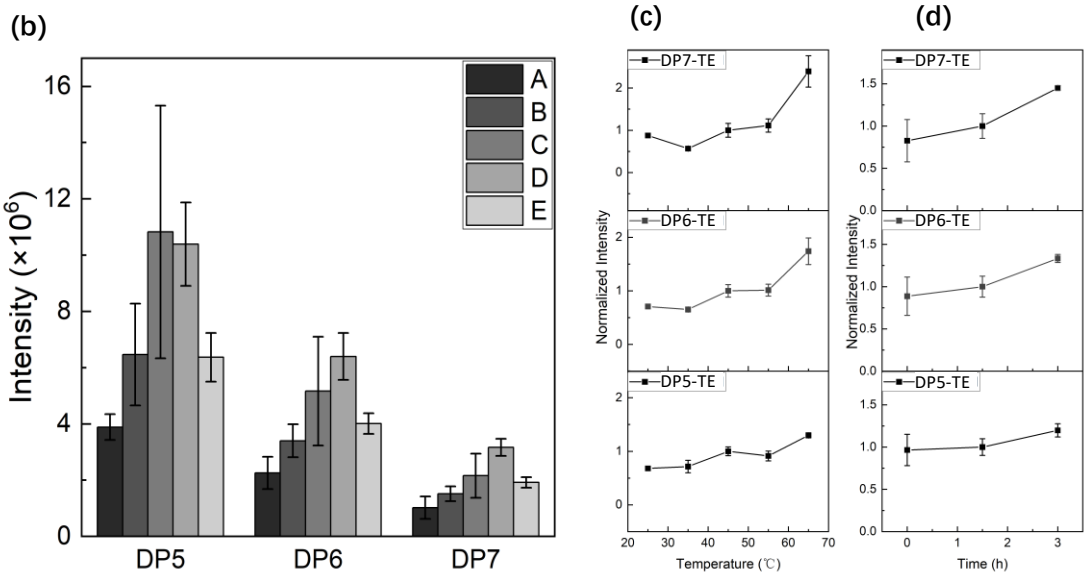

Figure S8. (a) Labeling condition of glycans with TE. (b-d) MS responses of TE labeled DP5, 6, 7 under different reaction conditions (b) composition of MeOH solution; (c) reaction temperature: 25-65℃; (d) reaction time: 0-3 h.

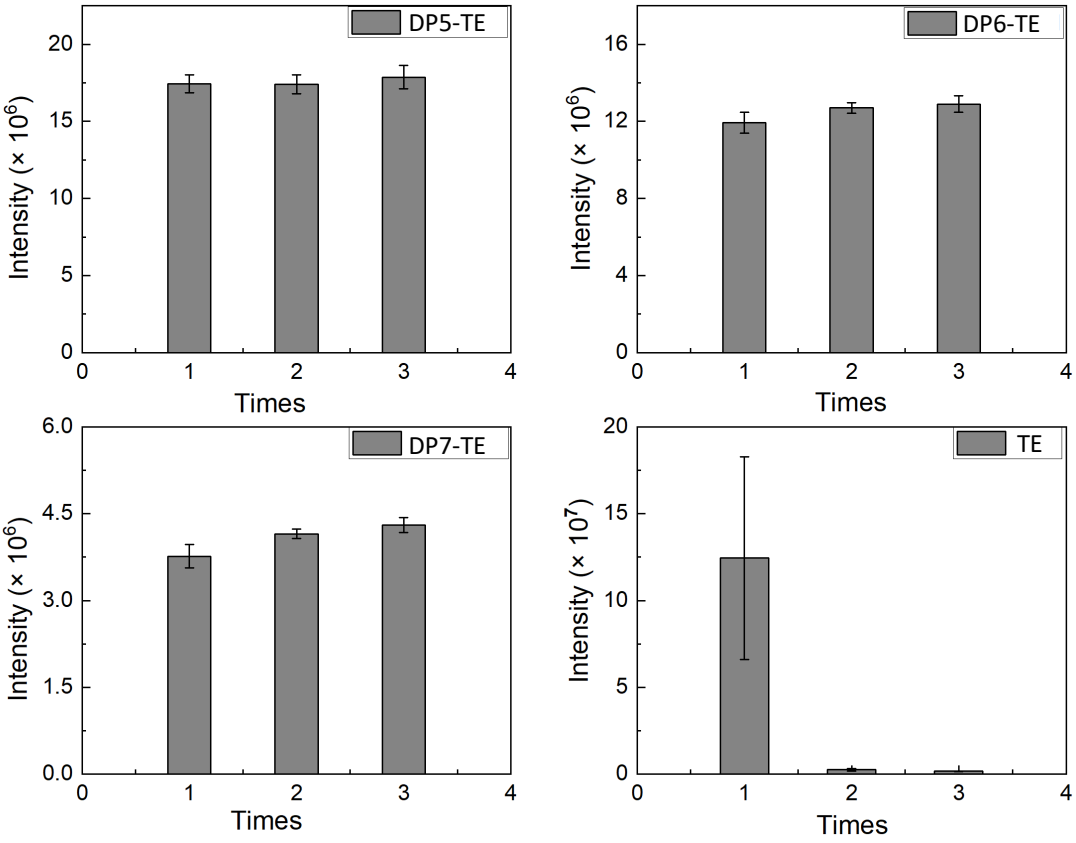

Figure S9. MS signals of DP-TE and TE after different times of EA/water extraction

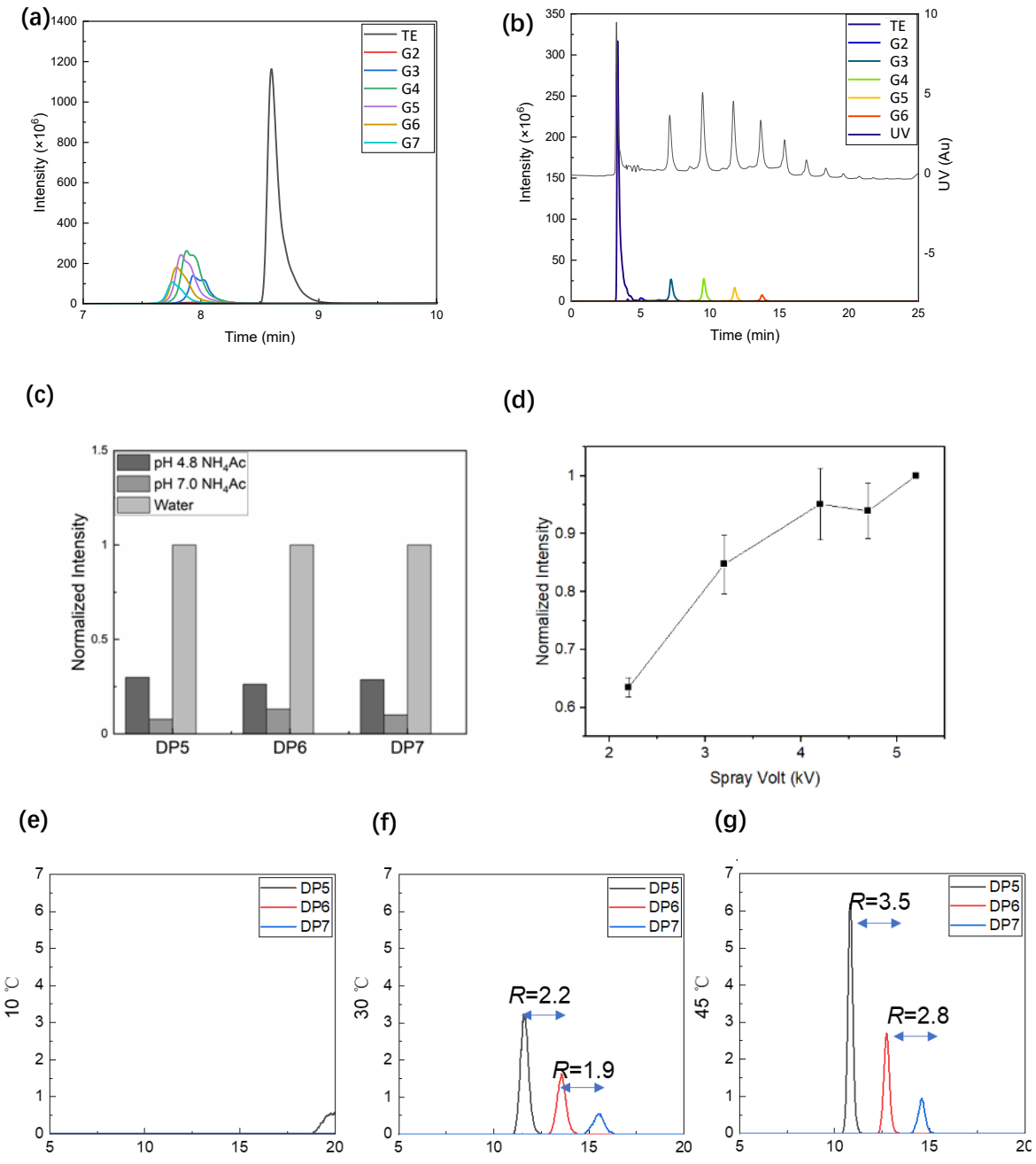

Figure S10. MS response of TE labeled dextran-1000 under different separation conditions (a) C18 column, (b) HILIC (with simultaneous UV detection at 280 nm), (c) different water phase, (d) different spray voltages. TE labeled DP5, 6, 7 showed varied EIC and separation under different column temperature (e-g).

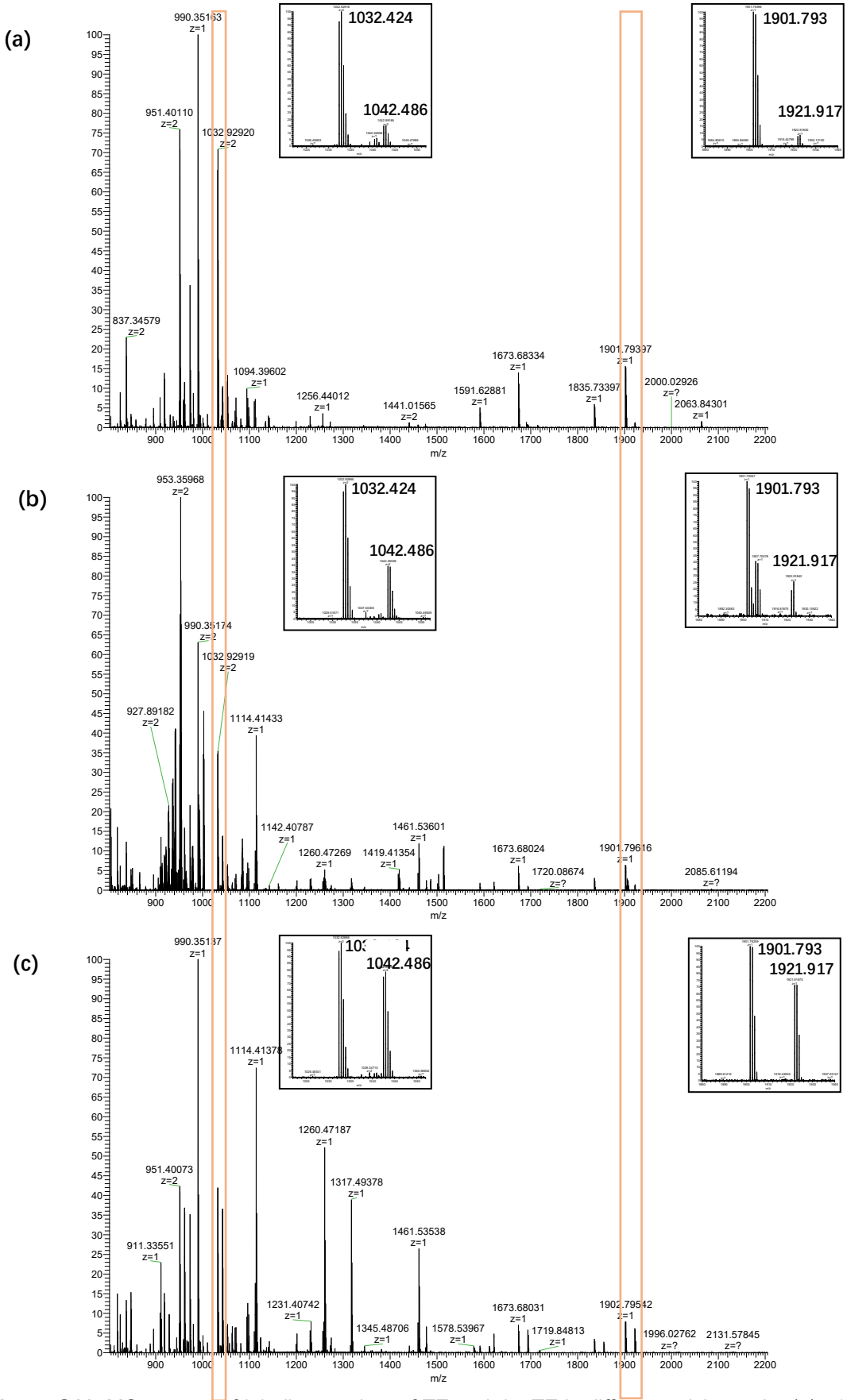

Figure S11. MS spectra of labeling product of TE and d_{20}-TE in different mixing ratio. (a)5:1; (b)2:1; (c)1:1