Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information for:

Reversing the undesirable pH-profile of doxorubicin *via* activation of di-substituted maleamic acid prodrug at tumor acidity

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Table S1. pH-dependent release of doxorubicin from Dox-DMA-GSH **8** at 25°C in phosphate buffer.

pH	Time	Mol. % of DMA 8	Mol. % of DMI 7 formed	Mol. % of Dox Released	Mol. % of Decomposition Products
pH 7.4	0 hr	94	0	0	6
	24 hr	45	23	19	13
	44 hr	29	29	28	14
	69 hr	18	32	27	23
	94 hr	16	30	31	23
	123 hr	11	27	23	39
	144 hr	10	27	23	40
pH 7.0	0.5 hr	75	7	8	10
	4 hr	71	11	8	10
	21 hr	31	24	34	11
	25 hr	26	24	35	15
	30 hr	22	25	38	15
	48 hr	12	30	43	15
pH 6.8	0.2 hr	79	8	4	9
	15 hr	31	18	42	9
	20 hr	26	21	43	10
	22 hr	22	21	47	10
	24 hr	16	25	49	10
	48 hr	6	22	54	18
pH 6.5	1 hr	69	7	10	14
	6 hr	32	11	43	14
	9 hr	24	11	50	15
	24 hr	5	13	67	15
pH 6.3	0.1 hr	74	9	9	8
	3.5 hr	36	9	47	8
	6 hr	23	8	59	10
	24 hr	4	16	68	12
pH 6.0	0.1 hr	67	12	16	5
	2 hr	28	10	57	5
	5 hr	12	10	68	10
	24 hr	0	10	80	10

Table S2. pH-dependent release of doxorubicin from Dox-DMA-GSH **8** at 37°C in phosphate buffer.

pH	Time	Mol. % of DMA 8	Mol. % of DMI 7 formed	Mol. % of Dox Released	Mol. % of Decomposition Products
pH 7.4	0 hr	73	10	0	17
	2 hr	53	17	10	20
	5 hr	23	24	33	20
	9 hr	19	26	33	22
	12 hr	18	26	34	22
	16 hr	14	27	34	25
	20 hr	12	27	36	25
	24 hr	12	24	36	28
	48 hr	8	18	29	45
	81 hr	1	11	28	60
pH 6.5	0 hr	94	6	0	0
	1 hr	56	8	35	1
	3 hr	20	10	69	1
	4 hr	16	10	73	1
	5 hr	13	12	74	1
	6.5 hr	6	12	76	6
	8 hr	2	12	78	8
	9 hr	2	16	74	8
	12 hr	1	16	73	10

Table S3. pH-dependent release of doxorubicin from Dox-DMA-GSH **8** at 37°C with 45% FBS.

pH	Time	Mol. % of DMA 8	Mol. % of DMI 7 formed	Mol. % of Dox Released	Mol. % of Decomposition Products
pH 7.4	0 hr	86	5	0	9
	1 hr	81	10	0	9
	4 hr	35	15	11	29
	8 hr	22	20	11	47
	24 hr	15	22	13	50
	32 hr	3	21	11	65
	45 hr	0	6	10	84
pH 6.5	0 hr	90	8	0	2
	1 hr	61	10	27	2
	2 hr	47	13	37	3
	3 hr	38	13	45	4
	5 hr	11	13	57	19
	6 hr	8	12	59	21
	7 hr	6	11	61	22
	8.5 hr	5	10	62	23
	12 hr	4	10	63	23
	24 hr	3	3	65	29
	28.5 hr	2	2	59	37
	32 hr	2	2	59	37
	48 hr	0	2	54	44

Table S4. Exact numerical values of the mean, the standard deviation, and the *P* values used to generate the cell viability graphs in Figure 3 and Figure S4-S6.

Dose Dependence

Figure 3a ES-2, 6hr (n=5)	pH 6.6-6.8 Cell Viability Average (Avg)	pH 6.6-6.8 Ave, Standard Deviation (SD)	pH 7.2-7.5 Avg	pH 7.2-7.5 SD	*P* value
Buffer (4% 25mM Pi)	96	3	100	5	
Dox-DMI-GSH 0.5 µM	90	4	97	5	
Dox-DMI-GSH 1 µM	79	3	80	3	
Dox-DMA-GSH 0.5 µM	64	12	61	14	
Dox-DMA-GSH 1 µM	**22**	4	**45**	8	pH 6.6-6.8 vs. pH 7.2-7.5: 0.00141
Dox 0.5 µM	**32**	2	**16**	1	pH 6.6-6.8 vs. pH 7.2-7.5: 0.0000225
***P* value**	Dox-DMA-GSH 1 µM vs. Dox 0.5 µM: 0.00331		Dox-DMA-GSH 1 µM vs. Dox 0.5 µM: 0.00127		

Figure 3b MDA-MB231, 2 hr (n=5)	pH 6.3-6.4 Avg	pH 6.3-6.4 SD	pH 7.6-7.7 Avg	pH 7.6-7.7 SD	*P* value
Buffer	92	11	100	12	
Dox-DMA-GSH 4 µM	53	6	68	2	
Dox-DMA-GSH 6 µM	24	2	68	2	
Dox-DMA-GSH 8 µM	**13**	1	**68**	7	pH 6.3-6.4 vs. pH 7.6-7.7: 0.0000311
Dox 3 µM	**30**	5	**15**	2	pH 6.3-6.4 vs. pH 7.6-7.7: 0.00213
***P* value**	Dox-DMA-GSH 8 µM vs. Dox 3 µM: 0.0015		Dox-DMA-GSH 8 µM vs. Dox 3 µM: 0.0000242		

Table S4. (continued)

Figure 3c OVCAR3, 2hr (n=5)	pH 6.3-6.4 Avg	pH 6.3-6.4 SD	pH 7.4-7.6 Avg	pH 7.4-7.6 SD	*P* value
Buffer	102	3	100	6	
Dox-DMA-GSH 4 µM	64	3	57	3	
Dox-DMA-GSH 6 µM	**18**	1	**38**	2	pH 6.3-6.4 vs. pH 7.4-7.6: 0.00000102
Dox 3 µM	**57**	13	**24**	2	pH 6.3-6.4 vs. pH 7.4-7.6: 0.00391
***P* value**	Dox-DMA-GSH 6 µM vs. Dox 3 µM: 0.00213		Dox-DMA-GSH 6 µM vs. Dox 3 µM: 0.00000177		

pH scan

Figure 3e, pH scan, ES2, 2.5 hr, n=3 for buffer, n=4 for others							
pH	**Buffer Avg**	**Buffer SD**	**Dox-DMA-GSH 2 µM Avg**	**Dox-DMA-GSH 2 µM SD**	**Dox 1 µM Avg**	**Dox 1 µM SD**	***P* value**
7.2-7.4	100	4	**80**	3	**23**	1	Dox-DMA-GSH 2 µM vs. Dox 1 µM: 0.00000485
7.2	105	2	**69**	8	**25**	3	Dox-DMA-GSH 2 µM vs. Dox 1 µM: 0.000676
6.8-6.9	106	3	**63**	2	**42**	2	Dox-DMA-GSH 2 µM vs. Dox 1 µM: 0.00000322
6.7-6.8	96	2	51	3	44	3	
6.5	101	4	50	5	49	4	
6.3-6.4	87	5	43	3	43	4	

Table S4. (continued)

Figure 3f, pH scan, MDA-MB231, 2.5 hr, n=3 for buffer, n=4 for others							
pH	**Buffer Avg**	**Buffer SD**	**Dox-DMA-GSH 6 µM Avg**	**Dox-DMA-GSH 6 µM SD**	**Dox 3 µM Avg**	**Dox 3 µM SD**	***P* value**
7.5-7.7	100	2	**84**	4	**31**	13	Dox-DMA-GSH 6 µM vs. Dox 3 µM: 0.00205
7.2-7.3	86	2	**66**	3	**29**	2	Dox-DMA-GSH 6 µM vs. Dox 3 µM: 0.00000342
6.9-7.0	78	4	**56**	2	**30**	6	Dox-DMA-GSH 6 µM vs. Dox 3 µM: 0.0012
6.6-6.7	77	2	**53**	4	**36**	5	Dox-DMA-GSH 6 µM vs. Dox 3 µM: 0.0011
6.5	77	3	32	3	29	2	
6.3-6.4	71	6	27	1	30	3	

Figure 3g, pH scan, OVCAR3, 2 hr, n=3 for buffer, n=4 for others							
pH	**Buffer Avg**	**Buffer SD**	**Dox-DMA-GSH 6 µM Avg**	**Dox-DMA-GSH 6 µM SD**	**Dox 2 µM Avg**	**Dox 2 µM SD**	***P* value**
7.2-7.5	100	2	52	8	48	7	
7.1-7.3	108	8	55	6	51	2	
6.8-6.9	102	4	46	2	46	3	
6.6-6.7	105	7	**34**	1	**58**	3	Dox-DMA-GSH 6 µM vs. Dox 2 µM: 0.0000856
6.4-6.5	102	3	**16**	1	**55**	3	Dox-DMA-GSH 6 µM vs. Dox 2 µM: 0.00000472
6.2-6.4	99	7	**15**	3	**60**	5	Dox-DMA-GSH 6 µM vs. Dox 2 µM: 0.0000287

Table S4. (continued)

HK2, toxicity

Figure 3h, HK2, effect on noncancerous cells				
Concentration (nM)	**Dox Avg (n=6)**	**Dox SD**	**Dox-DMA-GSH Avg (n=5)**	**Dox-DMA-GSH SD**
3	101	5	103	4
10	101	5	105	6
30	97	11	98	6
100	95	5	107	10
300	95	6	96	5
1000	71	2	106	5
3000	60	3	93	3
10000	50	6	78	5
30000	31	3	71	4

Figure 3i, ES2, Dox-DMA-GSH, FBS effect (n=3)					
Time	**pH 6.5-6.9 Avg**	**pH 6.5-6.9 SD**	**pH 7.2-7.7 Avg**	**pH 7.2-7.7 SD**	***P* value**
Buffer	92	9	100	1	
Dox-DMA-GSH 2μM	58	6	86	4	pH 6.5-6.9 vs. pH 7.2-7.7: 0.0046
Dox-DMA-GSH 4μM	21	2	32	6	
Dox-DMA-GSH 6μM	7	1	17	1	pH 6.5-6.9 vs. pH 7.2-7.7: 5.61E-4
Dox 1μM	77	1	48	16	
Buffer/FBS (50%)	89	1	94	3	
Dox-DMA-GSH/FBS(50%) 2μM	50	3	89	5	pH 6.5-6.9 vs. pH 7.2-7.7: 9.15E-4
Dox-DMA-GSH/FBS(50%) 4μM	19	5	46	6	pH 6.5-6.9 vs. pH 7.2-7.7: 0.00407
Dox-DMA-GSH/FBS(50%) 6μM	8	2	21	3	pH 6.5-6.9 vs. pH 7.2-7.7: 0.00406
Dox/FBS(50%) 1μM	71	1	36	7	pH 6.5-6.9 vs. pH 7.2-7.7: 0.0135

Table S4. (continued)

Free Dox Dose and Time

Figure S4a ES2 (n=3)					
[Dox] µM	**pH 6.6-6.7 Avg**	**pH 6.6-6.7 SD**	**pH 7.5-7.6 Avg**	**pH 7.5-7.6 SD**	***P* value**
0	100	4	100	11	
0.1	**87**	6	**62**	8	pH 6.6-6.7 vs. pH 7.5-7.6: 0.0147
0.3	**72**	8	**33**	6	pH 6.6-6.7 vs. pH 7.5-7.6: 0.00291
1	**35**	3	**15**	3	pH 6.6-6.7 vs. pH 7.5-7.6: 0.00128
3	**21**	4	**2**	1	pH 6.6-6.7 vs. pH 7.5-7.6: 0.01053

Figure S4b Free Dox time effect (from 4 different plates)					
Time	**pH 6.6-6.8 Avg**	**pH 6.6-6.8 SD**	**pH 7.2-7.6 Avg**	**pH 7.2-7.6 SD**	***P* value**
2hr, 1 µM (n = 4)	**44**	3	**23**	1	pH 6.6-6.8 vs. pH 7.2-7.6: 0.000256
6hr, 0.5 µM (n = 5)	**32**	2	**16**	1	pH 6.6-6.8 vs. pH 7.2-7.6: 0.0000225
12hr, 0.5 µM (n = 5)	**47**	2	**13**	1	pH 6.6-6.8 vs. pH 7.2-7.6: 0.0000000211
24hr, 1 µM (n = 3)	**35**	3	**15**	3	pH 6.6-6.8 vs. pH 7.2-7.6: 0.00128

Table S4. (continued)

PC3 and SKOV3

Figure S5b PC3, 2.5 hr (n=6)	pH 6.3-6.4 Avg	pH 6.3-6.4 SD	pH 7.5-7.6 Avg	pH 7.5-7.6 SD	*P* value
Buffer	103	6	100	4	
Dox 4 µM	**53**	7	**15**	3	pH 6.3-6.4 vs. pH 7.5-7.6: 0.00000742
Dox-DMA-GSH 10 µM	**56**	4	**70**	8	pH 6.3-6.4 vs. pH 7.5-7.6: 0.00617
Dox-DMI-GSH 10 µM	90	5	84	7	
***P* value**			Dox-DMA-GSH 10 µM vs. Dox 4 µM: 0.00000226		

Figure S5a SKOV3, 2.5 hr (n=6)	pH 6.3-6.5 Avg	pH 6.3-6.5 SD	pH 7.5-7.6 Avg	pH 7.5-7.6 SD	*P* value
Buffer	116	1	100	1	
Dox 4 µM	**39**	2	**22**	1	pH 6.3-6.5 vs. pH 7.5-7.6: 0.000000530
Dox-DMA-GSH 10 µM	**43**	2	**53**	2	pH 6.3-6.5 vs. pH 7.5-7.6: 0.00000870
Dox-DMI-GSH 10 µM	75	3	63	2	
***P* value**			Dox-DMA-GSH 10 µM vs. Dox 4 µM: 0.00000000160		

Table S4. (continued)

Longer Time Effects

Figure S6a OVCAR3, 6hr (n=5)	pH 6.5-6.7 Avg	pH 6.5-6.7 SD	pH 7.3-7.6 Avg	pH 7.3-7.6 SD	*P* value
Buffer (4% 25mM Pi)	94	4	100	4	
Dox-DMA-GSH 2 µM	76	4	79	2	
Dox-DMA-GSH 4 µM	**32**	5	**49**	3	pH 6.5-6.7 vs. pH 7.3-7.6: 0.000315
Dox 1 µM	**81**	4	**51**	7	pH 6.5-6.7 vs. pH 7.3-7.6: 0.000158
***P* value**	Dox-DMA-GSH 4 µM vs. Dox 1 µM: 0.000000181				

Figure S6b ES2, 12hr (n=5)	pH 6.6-6.7 Avg	pH 6.6-6.7 SD	pH 7.4-7.6 Avg	pH 7.4-7.6 SD	*P* value
Buffer (8% 25mM Pi)	106	11	100	8	
Dox-DMA-GSH 0.3µM	**100**	5	**73**	6	pH 6.6-6.7 vs. pH 7.4-7.6: 0.0000537
Dox-DMA-GSH 1µM	**50**	5	**23**	2	pH 6.6-6.7 vs. pH 7.4-7.6: 0.0000638
Dox 0.5µM	**47**	2	**13**	1	pH 6.6-6.7 vs. pH 7.4-7.6: 0.0000000211
***P* value**			Dox-DMA-GSH 1 µM vs. Dox 0.5 µM: 0.0000235		

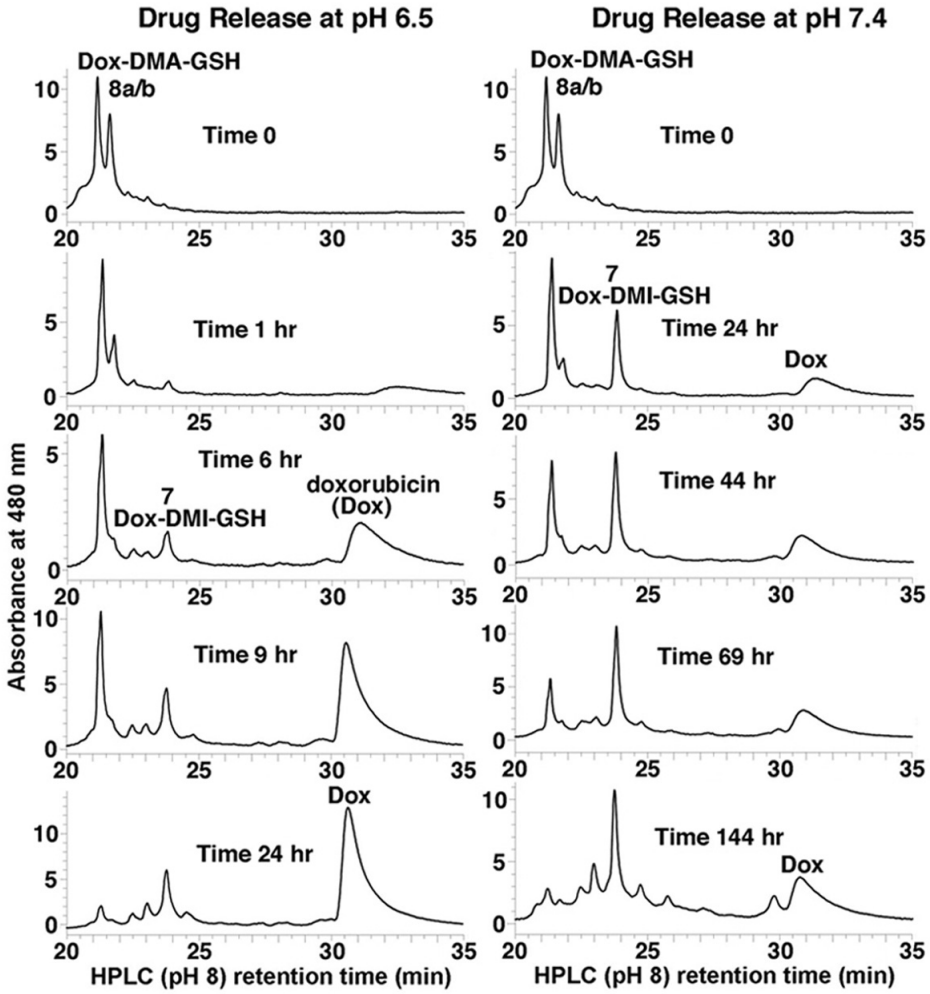


Figure S1. Dox release from Dox-DMA-GSH prodrug **8** in 25 mM aq. sodium phosphate buffer, at room temperature (~25°C), pH 7.4 vs. 6.5, as followed by HPLC (condition D). Dox release was followed by pH 8 HPLC at 480 nm (absorbance of Dox), and areas under the peaks were integrated to give the percentages reported in Table S1 in Supporting Information (S.I.), which are plotted in Figure 1. Dox peaks are broad likely due to equilibrating protonation states. The two peaks present at time 0 correspond to the two regioisomers of **8a/b**.

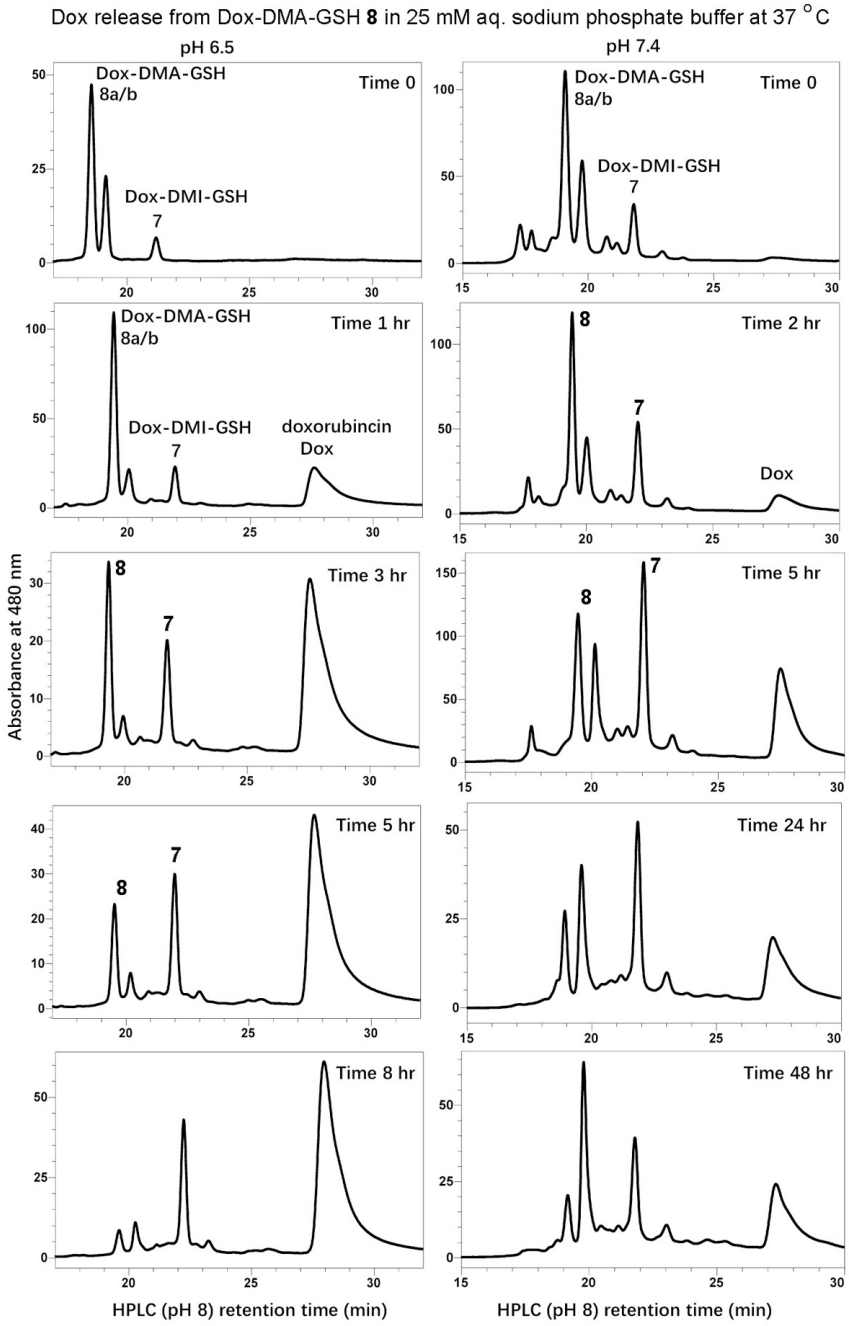


Figure S2. Dox release from Dox-DMA-GSH prodrug **8** in 25 mM aq. sodium phosphate buffer, at 37°C, pH 7.4 vs. 6.5, as followed by HPLC (condition E, pH 8, 480 nm is absorbance of Dox). Areas under the peaks were integrated to give the percentages reported in Table S2, which are plotted in Figure 2a-b. Minor shifts in retention times are due to small variations in pH of HPLC solvents. At pH 7.4, the peak with t_R ~ 20 min at 24 or 48 hr is mostly not DMA **8** but a decomposition byproduct (confirmed by MS, data not shown).

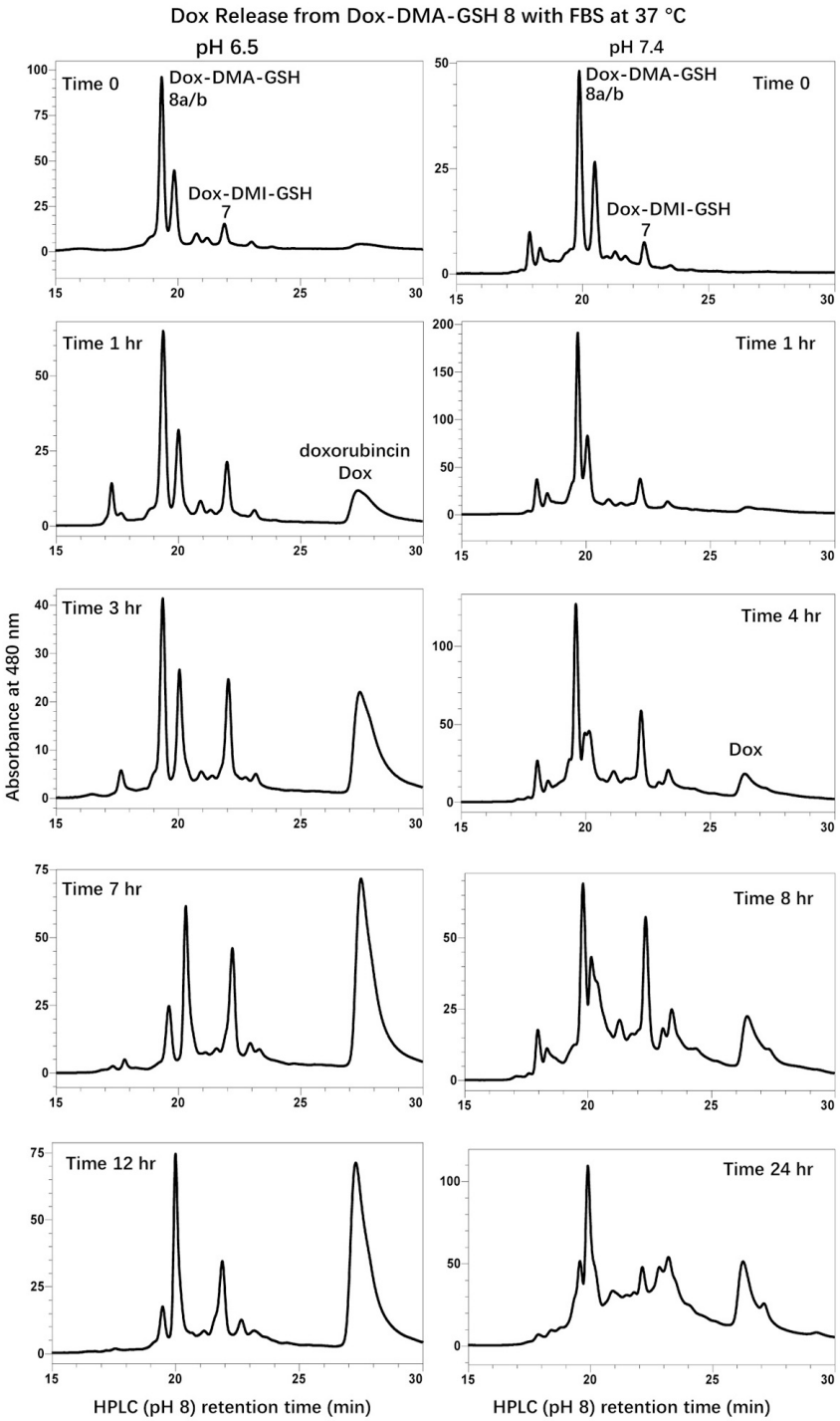


Figure S3. Dox release from Dox-DMA-GSH **8** in 10:45:45 aq. sodium phosphate (25 mM) buffer / fetal bovine serum (FBS) / DMEM-F12 cell medium, at 37°C, pH 7.4 vs. 6.5, as followed by HPLC (condition E, pH 8, 480 nm is absorbance of Dox). Areas under the peaks were integrated to give the percentages reported in Table S3, which are plotted in Figure 2c-d. The peak with t_R ~ 20 min after a few hours is mostly not DMA **8** but a decomposition byproduct (switch over can be seen ~ 4hr in the pH 7.4 traces).

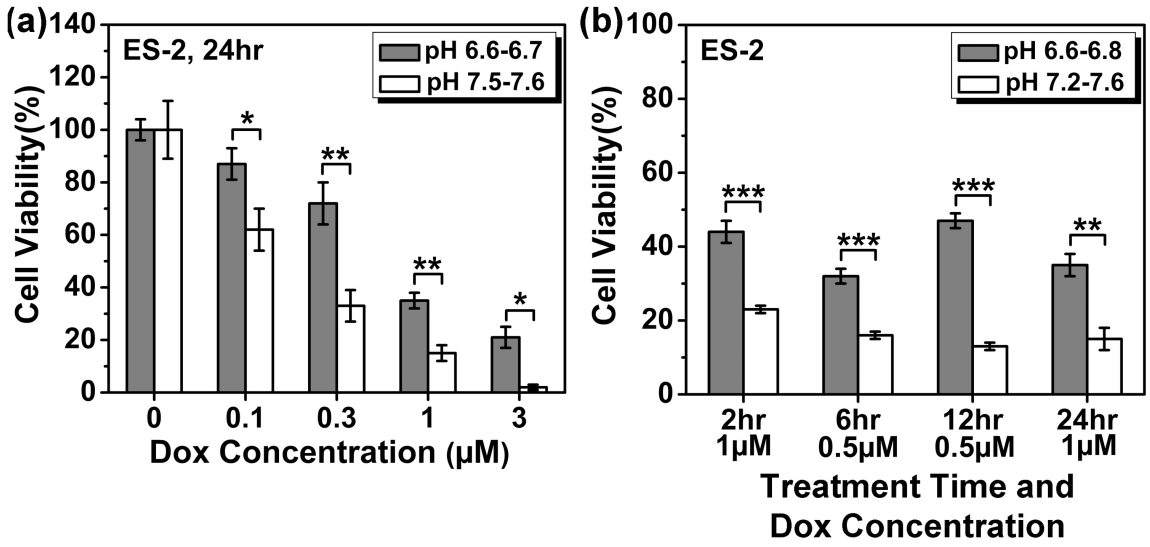


Figure S4. The undesirable pH-profile of free Dox — more cytotoxic at physiological pHe of 7.2-7.6 than tumor pHe of 6.6-6.8. In antiproliferation experiments with ES-2 cells, such undesirable pH-profile was seen over a wide range of PK-relevant concentrations (a, 0.1-3 μM) and time frames (b, 2-24 hr). ES-2 cells in 96-well plates were treated with Dox or buffer (negative control) at the given concentration and pH for the durations shown, then grown under normal conditions for 2-4 days before counted in MTS assays. Cell viability percentages shown are normalized to the buffer negative control at pH 7.4, which is shown as 0 μM in (a) and not shown in (b). The pH values of treatment solutions were measured before and after each experiment to give the pH ranges reported. Error bar is ± standard deviation (SD) (for (a): n = 3; for (b): 2 hr, n = 4; 6 hr and 12 hr, n = 5; 24 hr, n = 3). Statistical significance of the difference between the chosen data sets was evaluated using unpaired, two-tailed Student's t-test (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$). Exact numerical values of the mean, the SD, and the P values are reported in Table S4.

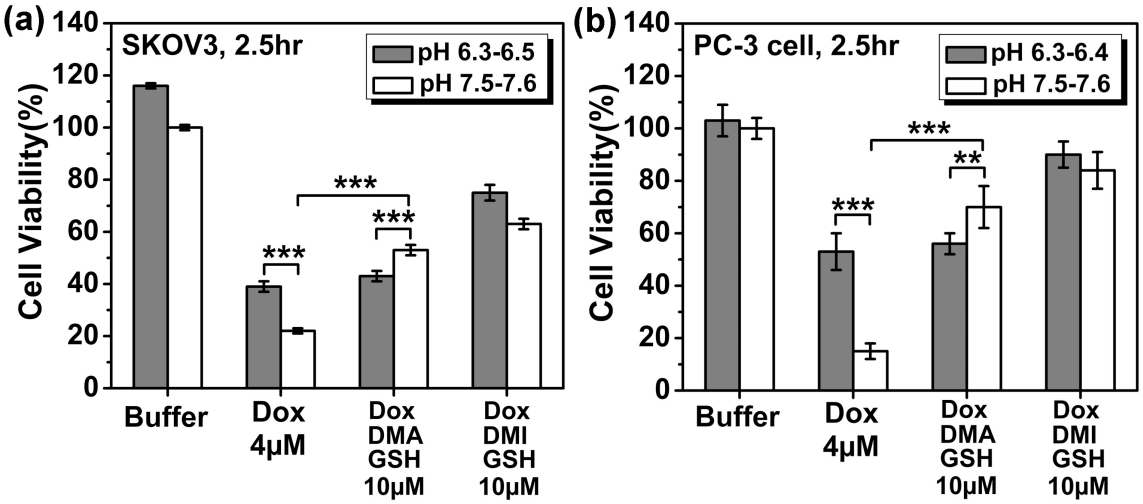


Figure S5. Antiproliferation effects of Dox-DMA-GSH **8** with SKOV3 and PC-3 cells. SKOV3 (a) and PC-3 (b) cells in 96-well plates were treated with Dox (positive control), DMA prodrug **8**, Dox-DMI-GSH **7**, or buffer vehicle (negative control) at the given concentration and pH for 2.5 hrs, then grown under normal conditions for 2-4 days before counted in MTS assays. Cell viability percentages shown are normalized to the buffer negative control at pH 7.4. The pH values of treatment solutions were measured before and after each experiment to give the pH ranges reported. Error bar is ± standard deviation (SD) (n = 6). Statistical significance of the difference between the chosen data sets was evaluated using unpaired, two-tailed Student's t-test (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$). Exact numerical values of the mean, the SD, and the P values are reported in Table S4.

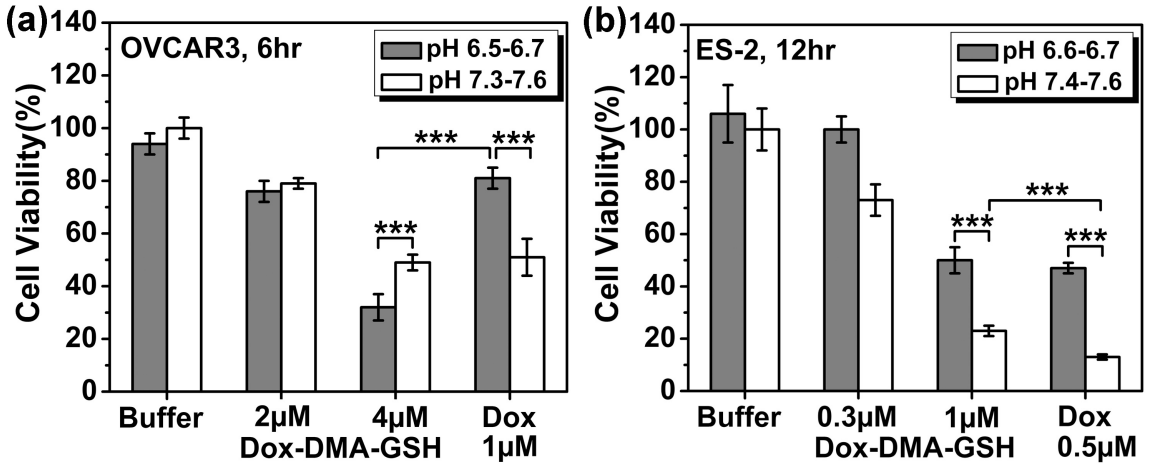


Figure S6. Antiproliferation effects of Dox-DMA-GSH **8** at longer treatment times. OVCAR3 cells (a) and ES-2 cells (b) were treated with Dox (positive control), prodrug **8**, or buffer vehicle (negative control) at the given concentration and pH for 6 hrs (a) and 12 hrs (b), respectively, then grown under normal conditions for 2-4 days before counted in MTS assays. Cell viability percentages shown are normalized to the buffer negative controls at pH 7.4. The pH values of treatment solutions were measured before and after each experiment to give the pH ranges reported. Error bar is ± standard deviation (SD) (n = 5). Statistical significance of the difference between the chosen data sets was evaluated using unpaired, two-tailed Student's t-test (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$). Exact numerical values of the mean, the SD, and the P values are reported in Table S4.

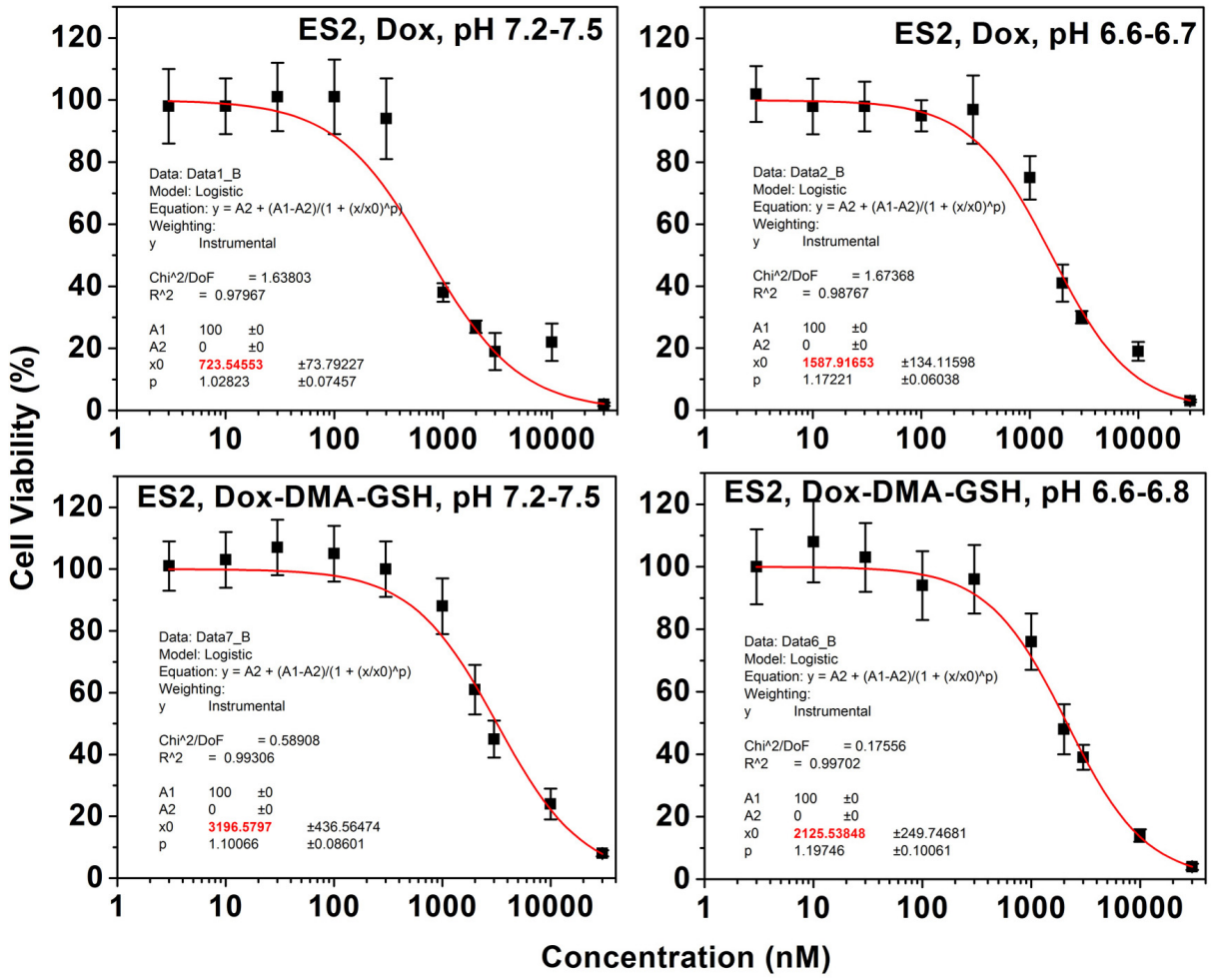


Figure S7. IC_{50} plots of Dox and Dox-DMA-GSH **8** with ES2 cells at pH 7.4 vs. pH 6.7. ES2 cells in 96-well plates were treated with Dox or DMA prodrug **8** at the given concentrations and pH values for 2.5 hrs, then grown under normal conditions for 2-4 days before counted in MTS assays. Cell viability percentages shown are normalized to the buffer negative control at a particular pH. The pH values of treatment solutions were measured before and after each experiment to give the pH ranges reported. IC_{50} plots are fitted with 0% and 100% inhibition at very low and very high concentrations (experimental data shown support such fitting, i.e. IC_{50} is approximately the same as EC_{50}). Error bar is ± 1 standard deviation (SD) (n = 6). The IC_{50} values extracted from the fittings are plotted in Figure 3d.

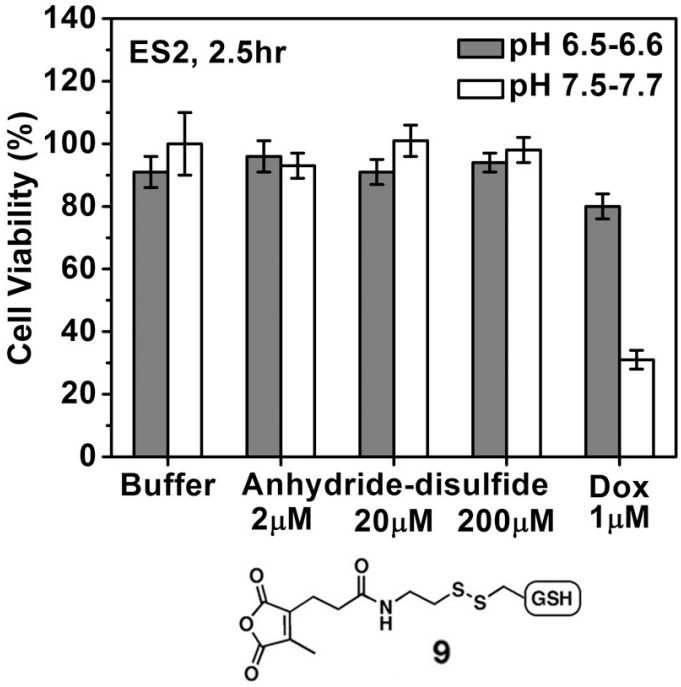


Figure S8. Drug release byproduct **9**, anhydride-disulfide of GSH, is not toxic to cells. Byproduct **9** was independently synthesized by facile disulfide exchange from cross-linker **4**: To a solution of cross-linker **4** (18 mg, 50 μmol, 1 eq.) in 500 μL of DMSO-d_6 was added glutathione (GSH) free acid (18 mg, 60 μmol, 1.2 eq.) and 500 μL of D_2O (reaction mixture pH: 2-3). The reaction mixture was vortexed until all components dissolved. The reaction mixture turned yellow immediately, signaling the release of 2-mercaptopyridine / thiopyridone (confirmed with ^{1}H-NMR). Color change stopped after a few minutes (i.e. disulfide exchange mostly finished). The reaction was kept at rt in the dark for 1 h, then at 4°C over night to ensure reaction reached completion (confirmed with ^{1}H-NMR). Subsequently, potential cytotoxic effect of byproduct **9** was evaluated in antiproliferation assays. The reaction mixture was used in cell experiments without purification: 2 μL of this stock of 50 mM of byproduct **9** (along with 10 mM free GSH, and 50 mM of 2-mercaptopyridine / thiopyridone) was diluted with DMEM F12 media of appropriate pH to final concentrations shown in Figure S8. ES-2 cells were treated with Dox (positive control), byproduct **9**, or buffer vehicle (negative control) at the given concentration (all had 1% DMSO) and pH for 2.5 hrs, then grown under normal conditions for 3 days before counted in MTS assays. Cell viability percentages shown are normalized to the buffer negative controls at pH 7.4. The pH values of treatment solutions were measured before and after each experiment to give the pH ranges reported. Error bar is ± standard deviation (SD) (n = 6). These data confirmed that byproduct **9** has no cytotoxic effect on cells up to 200 μM concentration (which is at least 20-fold higher than concentration of **9** in prodrug experiments).

METHODS / EXPERIMENTAL DETAILS.

Please note: No unexpected or unusually high safety hazards were encountered in this study.

Chemicals for Synthesis (General Information). Dimethylformamide (DMF), triethylamine (TEA), trifluoroacetic acid (TFA), and 2-aminoethanethiol hydrochloride (98% pure) were purchased from Acros Organics; triethyl-2-phosphonopropionate (98% pure), sodium hydride (57-63% dispersion in mineral oil, moist powder), pyridyl disulfide (98% pure), dimethylsulfoxide (DMSO), anhydrous acetonitrile, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU, 99% pure), 4-dimethylaminopyridine (DMAP, 99% pure), and oxalyl chloride (98% pure) from Alfa Aesar; dimethyl-2-oxo-glutarate (95% pure) from TCI America; tetrahydrofuran (THF), ethyl ether, anhydrous magnesium sulfate, ethyl acetate, sodium hydroxide, methanol, pyridine from Fisher Scientific; chloroform-d, and dimethyl sulfoxide-d6 from Cambridge Isotope Laboratories (CIL); doxorubicin (Dox) hydrochloride salt from LC Laboratories; hexanes from BDH; ethanol from Pharmaco-APPER; dichloromethane from Macron Fine Chemicals / Avantor Performance Materials; glutathione (free acid form, reduced) from EMD Millipore - Calbiochem; acetic acid, sodium phosphate (dibasic and monobasic) from JT Baker; SiliaFlash P60 silica gel, 40-63 μm, 60Å from SiliCycle; and acetonitrile (HPLC solvent) from Spectrum or JT Baker. Purity of each reagent and solvent is greater than 99% (unless noted otherwise) and used without further purification. Water was obtained from Millipore Milli-DI system connected to in-house deionized (DI) water source.

General Procedures for HPLC. High pressure/performance liquid chromatography (HPLC) is used to purify various compounds, monitor reaction progress, isolate desired products, and ascertain sample integrity over time. A Shimadzu HPLC system F (two LC-6AD pumps with a SPD-M20A Prominence 200-800 nm full-spectrum diode array detector) or a Shimadzu HPLC system P (one LC-20AT quaternary pump with a SPD-20A two-wavelengths UV-vis detector) connected to a semi-prep Agilent Zorbax 9.4 mm (width) x 250 mm (length) SB-C18 reverse phase semi-prep column (column Z) or a Hamilton 4.1 mm (w) x 150 mm (l) PRP-1 polystyrene-divinylbenzene (PS-DVB) polymer reverse phase analytical column (column H) is run with the following general method: flow rate, 3 mL / min for semiprep column Z, or 1 mL / min for analytical column H; solvent systems A/B will be defined in specific HPLC conditions below; 60-min method: at 99:1 A/B for 10 min, followed by 99:1 A/B to 1:99 A/B in 30 min (the gradient), then at 1:99 A/B for 5 min, 1:99 A/B to 99:1 A/B in 2 min, and then at 99:1 A/B for 13 min. Specific HPLC conditions that differ in column choice (Z or H), solvents A/B, mobile phase pH (pH 2-3 or pH 8-8.5) and specific HPLC set-up (system F or P) are described below:

HPLC condition A: C18 column Z; pH 8-8.5; HPLC system F; phase A is water (with 0.08% TEA and 0.04% TFA); phase B is acetonitrile (with 0.08% TEA and 0.04% TFA).

HPLC condition B: C18 column Z; pH 2-3; HPLC system P; phase A is water/acetonitrile 95:5 (with 0.01% TFA); phase B is acetonitrile (with 0.01% TFA).

HPLC condition C: C18 column Z; pH 2-3; HPLC system F; phase A is water/acetonitrile 95:5 (with 0.01% TFA); phase B is acetonitrile (with 0.01% TFA).

HPLC condition D: C18 column Z; pH 8-8.5; HPLC system P; phase A is water (with 0.08% TEA and 0.04% TFA); phase B is acetonitrile (with 0.08% TEA and 0.04% TFA).

HPLC condition E: PS-DVB column H; pH 8-8.5; HPLC system P; phase A is water/acetonitrile 95:5 (with 0.08% TEA and 0.04% Acetic Acid); phase B is acetonitrile (with 0.08% TEA and 0.04% Acetic Acid).

For purification runs, the collected eluents were concentrated under reduced pressure to remove acetonitrile after which the aqueous solution/suspension was lyophilized to dryness (solid or oily residue). The residue may contain small amounts of triethylammonium trifluoroacetate ionic liquid, depending on the specific solvent systems used. When doxorubicin-containing material was quantified using absorbance at 480 nm, the extinction coefficient of 11,500 $M^{-1}.cm^{-1}$ was used.[1] Since all Dox conjugations described in this paper took place on the amino group of the pyranose sugar daunosamine, which is not conjugated to the chromophore, we assumed that all Dox conjugates would also have the same extinction coefficient at 480 nm (11,500 $M^{-1}.cm^{-1}$).

Mass Spectrometry. All mass measurements were obtained by the Mass Spectrometry Laboratory at the University of Illinois, Urbana-Champaign,

HPLC Assay of pH-dependent DMA Cleavage (i.e. Doxorubicin Release from DMA 8). Release of doxorubicin from Dox-DMA-GSH **8a/b** was studied at different pH, temperature (rt ~ 25°C or 37°C) and environment (sodium phosphate buffer or 45% FBS in cell media) through monitoring by HPLC at ~ pH 8. DMI **7** was converted to DMA **8a/b** after 1-2 hr rt incubation at pH 9.6 - 9.9 in 25 mM aq. sodium phosphate buffer (see later synthesis sections for details). The pH of the DMA **8a/b** solution was adjusted to 7.4, 7.0, 6.8, 6.5, 6.3, or 6.0. Then the solution was allowed to sit at rt (~ 25°C) or placed in an incubator at 37°C in the dark for up to a few days (the pH did not change during this time). Release of doxorubicin was monitored using HPLC (condition D or E, 480 nm, ~ 5 nmol per HPLC run). Representative HPLC traces are shown in Figures S1-S3 (see these figures and their captions for more detailed information). Areas under the peaks were integrated using the HPLC program LabSolutions Lite to give the percentages reported in Table S1-S3 and plotted in Figure 1-2.

Cell Cultures. ***Materials.*** The following media, buffers, and cell culture reagents were purchased from Life Technologies – Invitrogen: DMEM/F-12 Medium without phenol red (Gibco 21041), DMEM/F-12 Medium (Gibco 11320), Ham's F-12K Medium (Kaighn's modification, with L-glutamine, Gibco 21127), Fetal Bovine Serum (FBS) (Gibco 26140), Antibiotic-Antimycotic (Anti-Anti) 100 x (Gibco 15240), Trypan blue stain 0.4% (for cell counting), and Trypsin-EDTA (for cell dissociation). Good's buffers ingredients 2-[4-(2-hydroxyethyl)-piperazin-1-yl]-ethane-sulfonic acid (HEPES, pKa = 7.5 at 25 °C) and 2-(*N*-morpholino)-ethane-sulfonic acid (MES, pKa = 6.2 at 25 °C) were purchased from EMD Chemicals - Cal-Biochem. RPMI-1640 Medium (ATCC 30-2001) was purchased from American Type Culture Collection (ATCC). McCoy's 5A Medium (Lonza 12-688) was purchased from Lonza. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma-Aldrich. MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay solution) was purchased from Promega Corp. AlamarBlue fluorescence dye cell viability assay reagent (10x) was purchased from Bio-Rad (BUF012B). Sterile cell culture flasks (25 cm^2 and 75 cm^2) were obtained from Corning Inc. MTS experiments were carried out in 96-well plates purchased from Greiner Bio-one.

Cell lines. NIH:OVCAR3 human ovarian cancer cells (adenocarcinoma, ovary, epithelial), MDA-MB-231 human breast cancer cells (adenocarcinoma, mammary gland / breast, derived from metastatic site of pleural effusion, epithelial), PC-3 human prostate cancer cells (adenocarcinoma, prostate, derived from metastatic site of bone, epithelial), and HK2 human kidney cortex / proximal tubule cells (epithelial, non-cancerous, human papilloma-virus 16 transformed) were obtained from the American Type Culture Collection (ATCC). SKOV3 human ovarian cancer cells (adenocarcinoma, ovary: ascites, epithelial) and ES-2 human ovarian cancer cells (clear cell carcinoma, ovary, fibroblast) were gifts from Dr. Juntao Luo (Department of Pharmacology, SUNY Upstate Medical University). OVCAR3 cells were cultured in RPMI-1640 Medium (ATCC 30-2001) supplemented with 1x Penicillin (100 IU) / Streptomycin (100 µg / ml) (Corning 30-002-CI); SKOV3 and ES-2 cells were cultured in McCoy's 5A Medium (Lonza 12-688) supplemented with 1x Penicillin (100 IU) / Streptomycin (100 µg / ml) (Corning 30-002-CI); MDA-MB-231 cells were cultured in DMEM (Sigma-Aldrich D6429) supplemented with Antibiotic-Antimycotic (Anti-Anti) (1x) (Gibco 15240); PC-3 cells were cultured in Ham's F-12K (Kaighn's modification, with L-glutamine, Gibco 21127) Medium supplemented with Anti-Anti (1x); HK2 cells were cultured in DMEM/F12 Medium (Gibco 11320) supplemented with Anti-Anti (1x). All cells were grown with media supplemented with 10% FBS (Gibco 26140). Cells were grown in an incubator (Thermo Electron Corp., Napco series 8000 WJ, model 3578, Revco Elite II) under a humidified atmosphere of air and 5% CO_2 at 37ºC.

Instruments. In the 96-well plate format, 490 nm absorbance readings for MTS cell viability assays were obtained using a BioTek ELx808 microplate reader. The pH values of treatment and incubation media were measured before and after the experiments using an Orion Ross Glass Combination Micro pH electrode connected to an Orion 3-Star Benchtop pH-meter with a BNC connector (both from Thermo Scientific).

pH-dependent anti-proliferation assays with cells. In 96-well plates, ~3,000 cells were seeded per well then grown for 24-48 hr until 30-40% confluency. After removing the growth media, cells were treated with DMA prodrug **8**, DMI **7**, byproduct **9**, free drug Dox (positive control, with 1% DMSO), or buffer (negative vehicle control, 10 or 25 mM sodium phosphate aq. buffer 3-8% by volume; the amount of DMSO and extra phosphate in all wells were kept constant) at a given concentration and pH in modified DMEM/F-12 media (without phenol red, with 0% or 50% FBS (Figure 3i only), supplemented with 60 mM (or 30 mM for Figure 3i FBS experiments) of MES for pHe 6.0-6.8 or HEPES for pHe 7.2-7.8) for 1-24 hr at 37°C in the incubator. After treatment solutions were removed, cells were incubated with normal growth media (pH 7.4, 10% FBS) and allowed to proliferate for additional 2-4 days until the buffer vehicle negative control wells have reached > 80% confluency, then MTS cell viability assays (except data shown in Figure 3i) were performed (i.e. growth media was replaced with 50 µL of pH 7.4 DMEM/F-12 media without phenol red, 10 µL of MTS reagent was added, absorbance at 490 nm was recorded 0.5-4 hr afterwards). For serum effect cell plate data shown in Figure 3i, cell viability was measured using alamarBlue assay (i.e. growth media was replaced with 45 µL of pH 7.4 DMEM/F-12 media without phenol red, 5 µL of alamarBlue reagent (10x) was added; fluorescence at 590 nm was recorded after excitation at 560 nm at ~ 1 hr afterwards). The pH values of treatment solutions were measured before and after each experiment to give the pH ranges reported. Results of these growth inhibition assays are shown in Figure 3 and S4-S7. In all of our experiments, 1% of DMSO in Dox

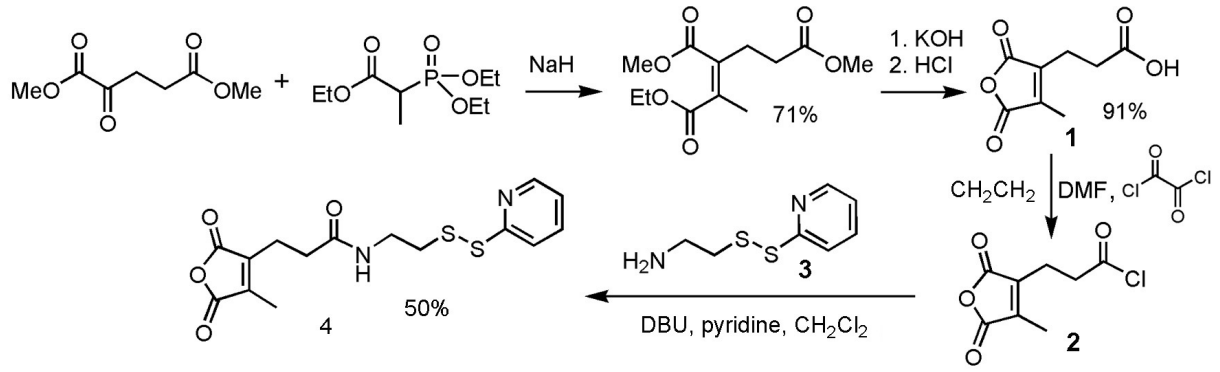
treatment solutions and mild acidic pHe in the range of 6.5 - 7.0 had no statistically significant effect on cell growth.

Statistical information for anti-proliferation data shown in Figure 3 and Figure S4-S7. Error bars shown in Figures 3 and S4-S7 reflect ± 1 standard deviation (SD). Statistical significance of the difference between the chosen data sets was evaluated using unpaired (unequal variance), two-tailed Student's *t*-test (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$). Exact numerical values of the mean ± SD and the *P* values are reported in Table S4. Errors of the mean at 95% confidence level (CL) can be estimated using the two-tailed confidence coefficient $t_{CL,\nu}$ in the Student's t-test distribution with ν degrees of freedom (ν = n-1), according to the following equation:

$$\text{estimate of true value} \approx \overline{X_n} \pm t_{CL,\nu} \frac{S_n}{\sqrt{n}}$$

where $\overline{X_n}$ is the mean, S_n is the sample standard deviation and n is the sample size. In our reported results, n varied from 3 to 6. These n values reflect repeats on a single 96-well plate. The critical $t_{CL,\nu}$ = 4.303, 3.182, 2.776, and 2.571 when n = 3, 4, 5 and 6, respectively. In other words, 95% confidence level standard error corresponds to 2.48, 1.59, 1.24 and 1.05 SD at n = 3, 4, 5 and 6, respectively. *All presented data were reproduced in at least one other plate.* However, due to plate-to-plate and week-to-week cell variabilities, only data from one representative plate was plotted.

Chemical Synthesis



Anhydride - activated disulfide bifunctional cross-linker 4.

Disubstituted maleic anhydride - acyl chloride **2** was prepared in situ from 2-propionic-3-methylmaleic anhydride **1**, which in turn was assembled via a Horner-Emmons reaction from dimethyl-2-oxo-glutarate and triethyl-2-phosphonopropionate, all following the procedures of Naganawa, Ichikawa, and Isobe.[2] Acyl chloride **2**: ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.31 (2H, t, ^{3}J = 6.9 Hz), 2.80 (2H, t, ^{3}J = 6.9 Hz), 2.13 (3H, s). ^{13}C NMR (151 MHz, $CDCl_3$): δ 173.0, 165.5, 165.4, 143.3, 140.4, 43.6, 20.3, 9.9. The ^{1}H NMR and ^{13}C NMR spectra of acyl chloride **2** are shown in Figures S9 and S10, respectively.

A solution of S-(2-pyridylthio)cysteamine HCl salt **3** (50 mg, 0.23 mmol, 1 eq., prepared following the method of Ebright et al.[3]), DBU (0.034 mL, 0.23 mmol, 1 eq.) and pyridine (0.027 mL, 0.34 mmol, 1.5 eq.) in 1 mL of dichloromethane was added to acyl chloride **2** (~ 45.6 mg, ~ 0.23 mmol, ~ 1 eq.). The resulting reaction mixture was kept at rt for 2 hr, and then directly loaded onto a silica gel column. The desired product disulfide **4** (40 mg) was isolated after chromatography (1:3 ethyl acetate / hexanes, followed by 1:1 diethyl ether / hexanes to elute the hydrolyzed carboxylic acid byproduct **1**, and then 1:1 ethyl acetate / hexanes to 5:2 ethyl acetate / hexanes) as oil in 50 % yield over two steps from 2-propionic-3-methylmaleic anhydride **1**. Anhydride – disulfide **4**: IR (KBr): 3413 (br), 3295 (br), 3065 (br), 2926, 1824, 1765, 1672, 1653, 1576, 1560, 1541, 1446, 1419, 1281, 1119, 908, 766, 733 cm^{-1}. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.50 (1H, d, J = 4.6 Hz), 7.67 - 7.64 (1H, m), 7.57 (1H, br), 7.52 (1H, d, J = 8.0 Hz), 7.20 - 7.18 (1H, m), 3.53 – 3.50 (2H, m), 2.90 (2H, t, J = 5.8 Hz), 2.78 (2H, t, J = 7.1 Hz), 2.58 (2H, t, J = 7.1 Hz), 2.09 (3H, s). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.7, 166.04, 165.96, 158.9, 149.4, 142.9, 142.2, 137.7, 121.8, 121.7, 38.9, 37.5, 33.1, 20.5, 9.7. MS (ESI+): Exact Mass Calcd for $C_{15}H_{16}N_2O_4S_2$ $[M+H^+]^+$: 353.1. Found: 352.9. The ^{1}H NMR and ^{13}C NMR spectra of anhydride – disulfide crosslinker **4** are shown in Figures S11 and S12, respectively. The MS data of crosslinker **4** is shown in Figure S13.

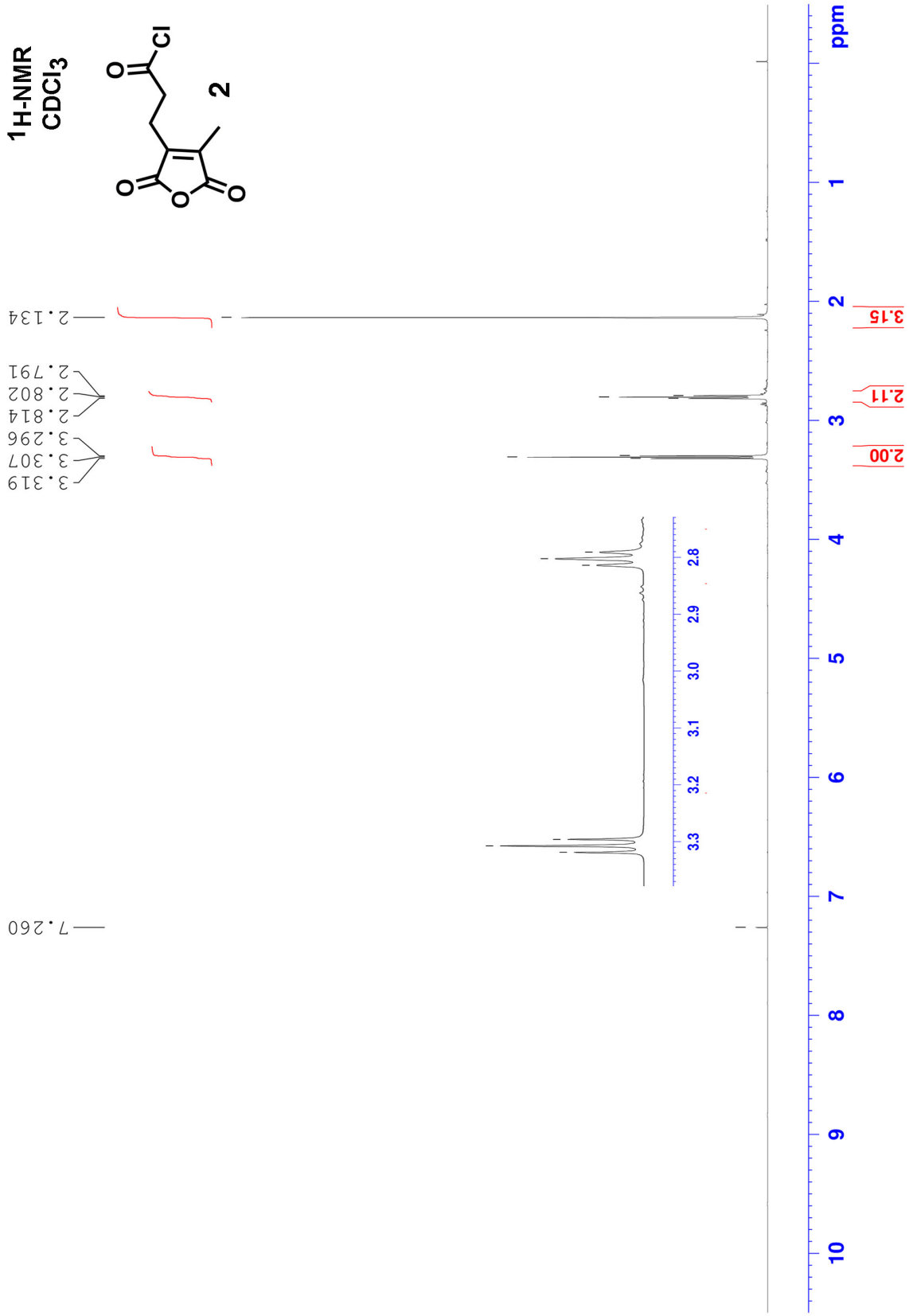


Figure S9. [1]H NMR spectrum of acyl chloride **2**.

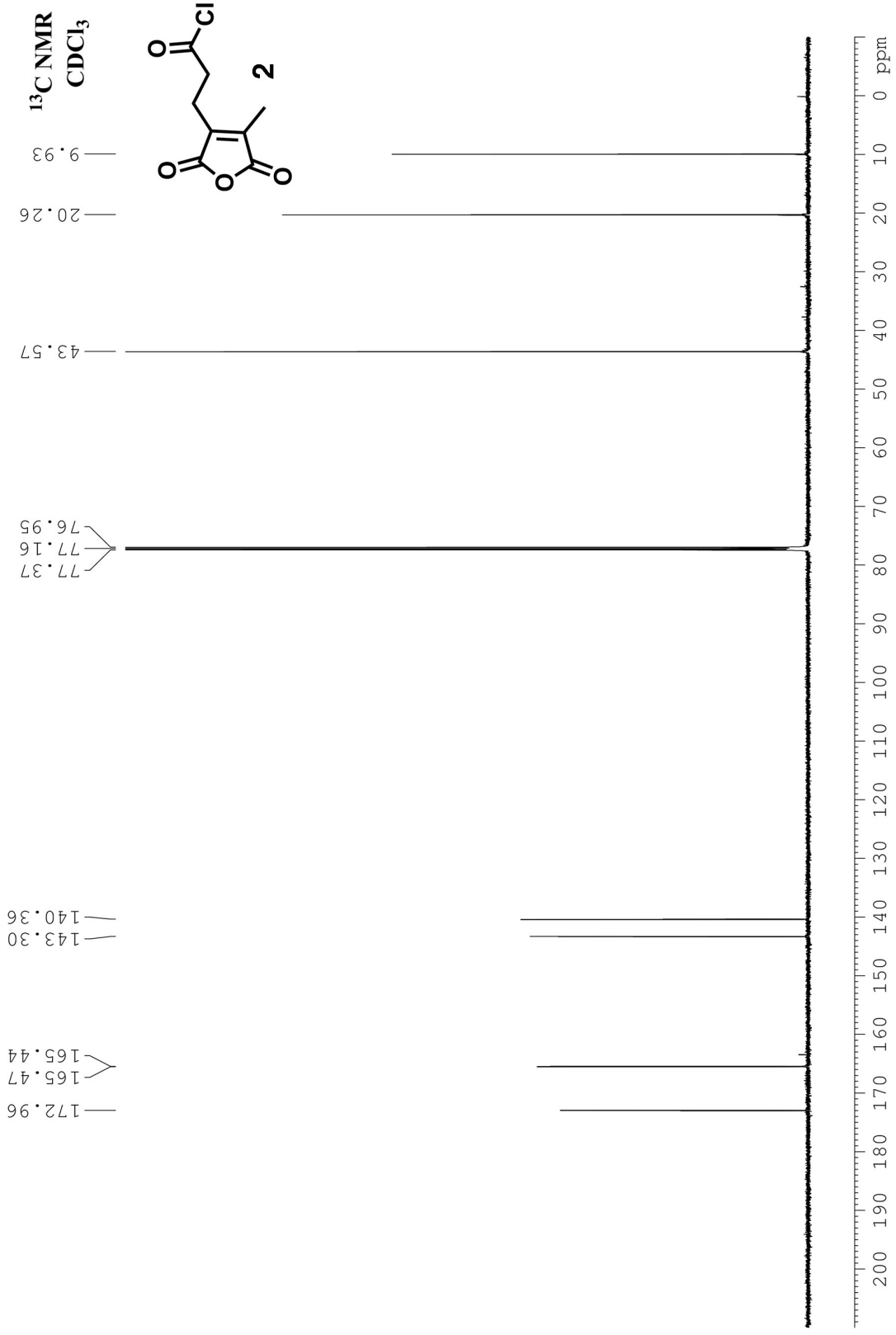


Figure S10. ^{13}C NMR spectrum of acyl chloride **2**.

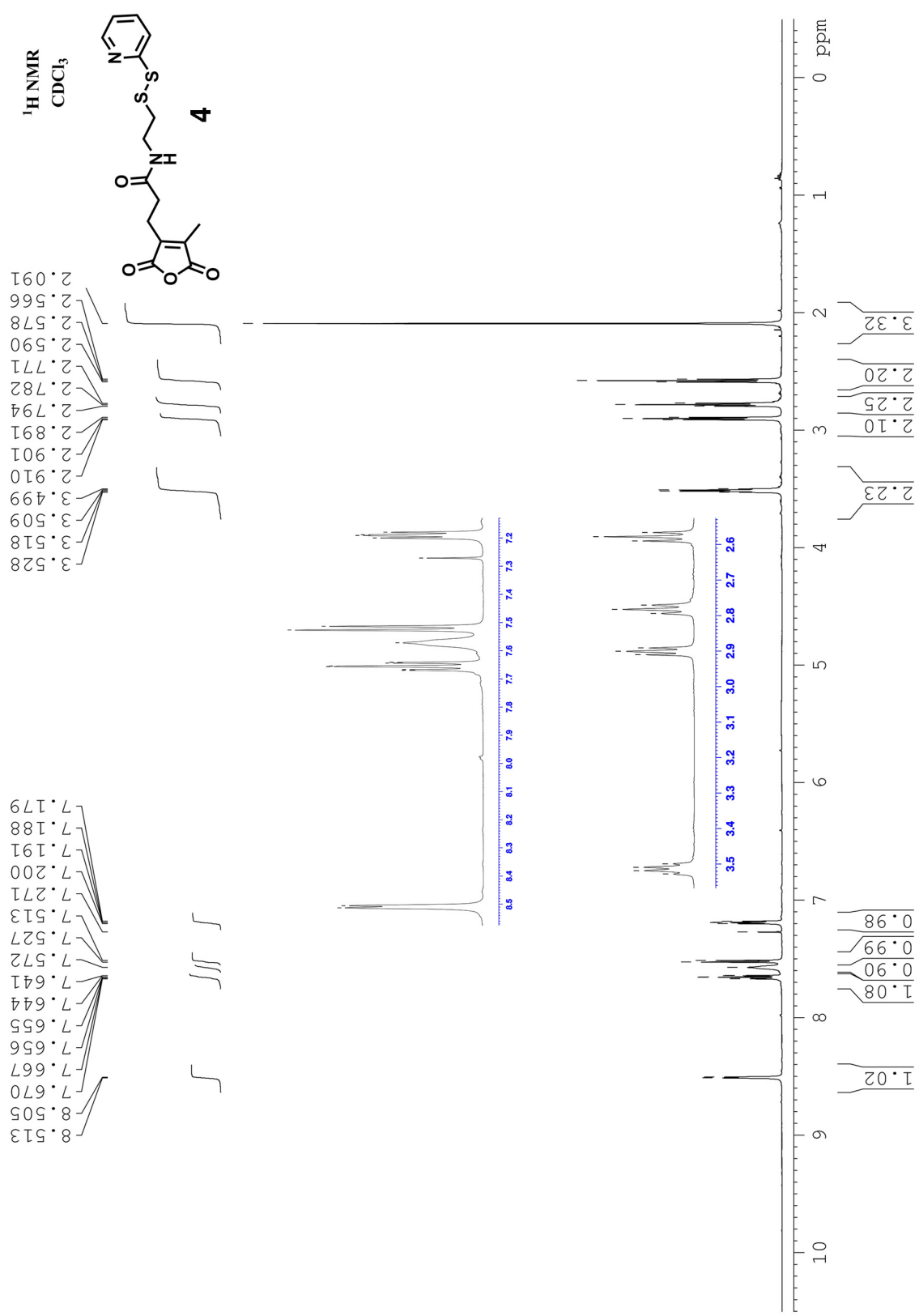


Figure S11. ^{1}H NMR spectrum of anhydride – disulfide crosslinker **4**.

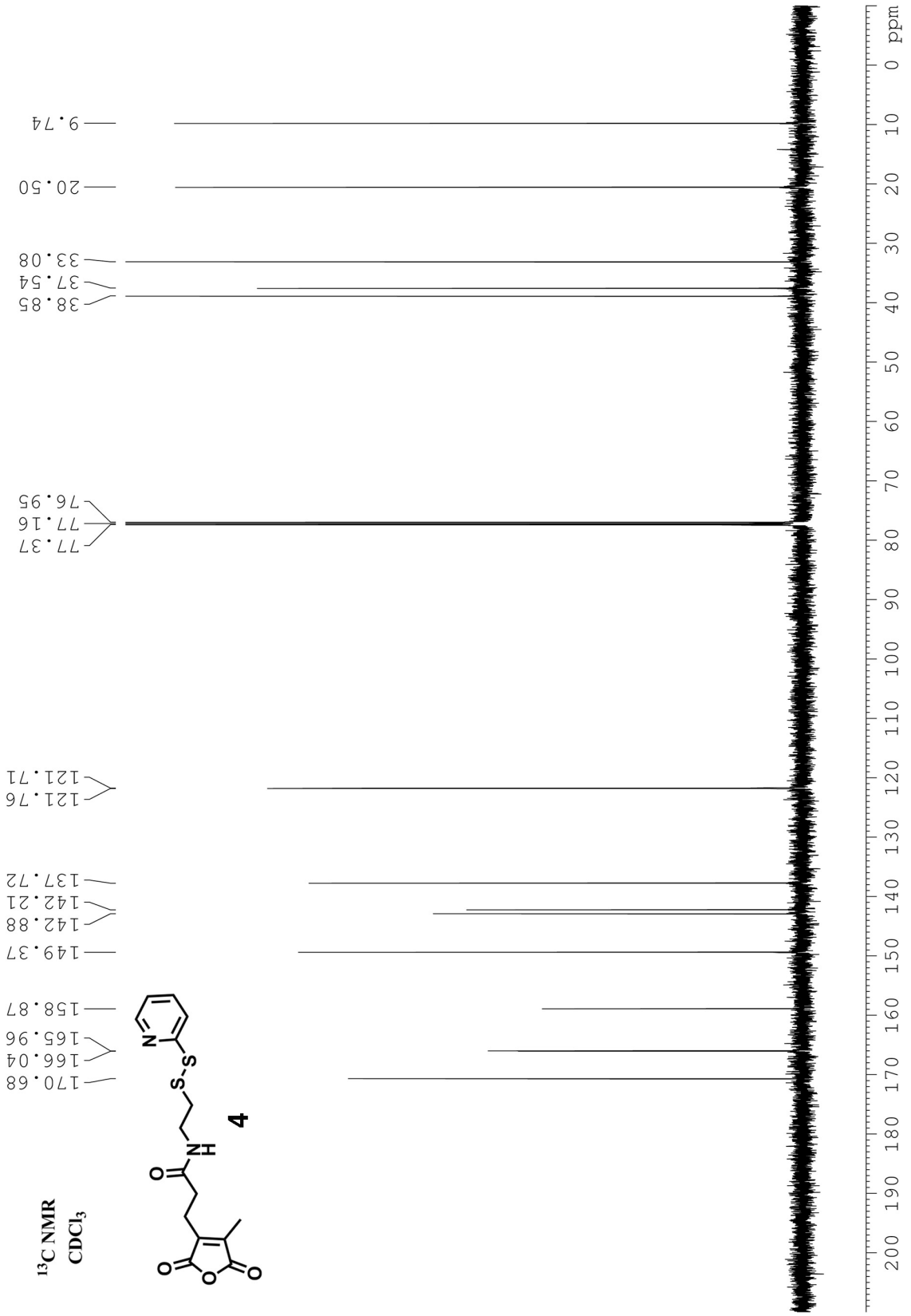


Figure S12. ^{13}C NMR spectrum of anhydride – disulfide crosslinker **4**.

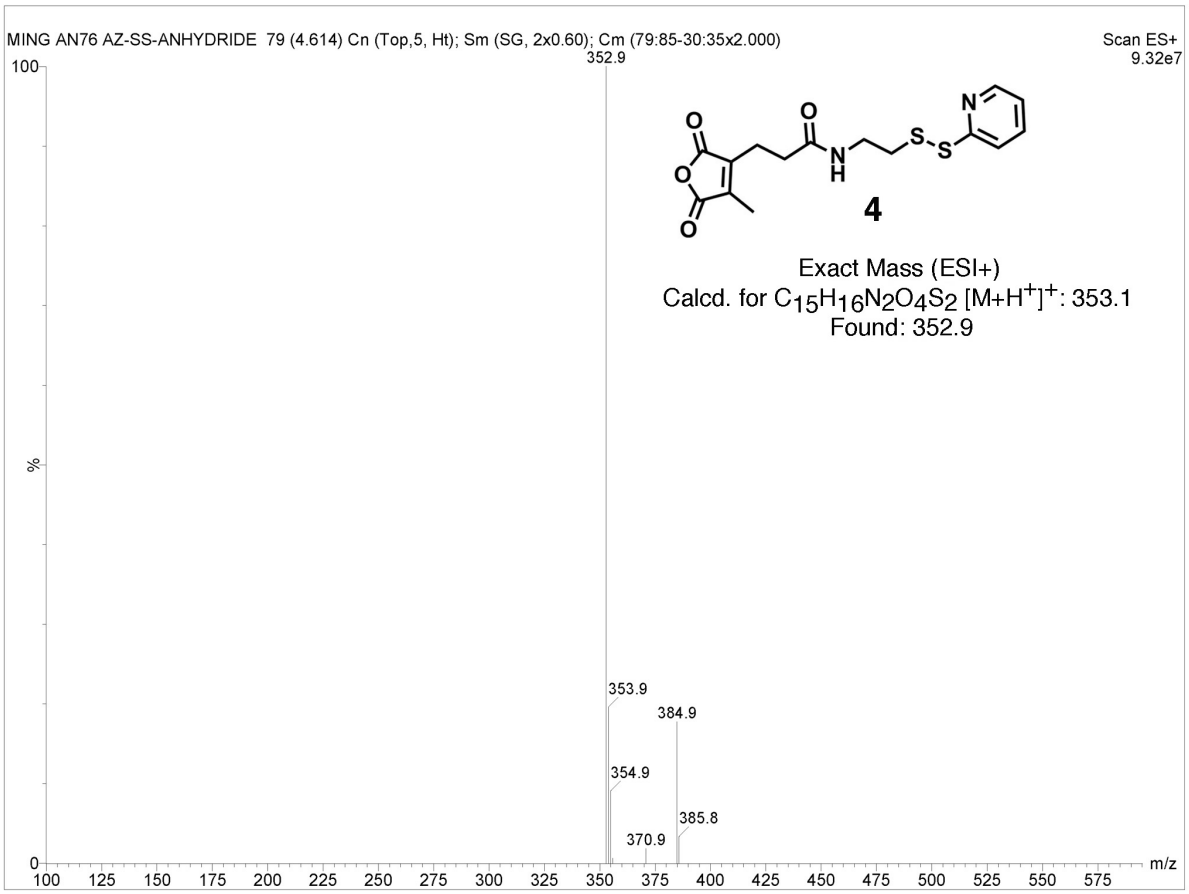
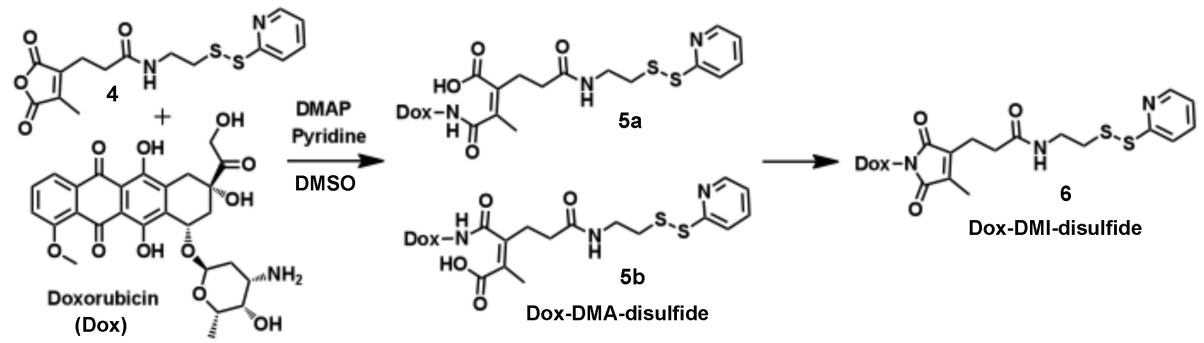


Figure S13. ESI+ MS data of anhydride – disulfide crosslinker **4**.



Doxorubicin disubstituted maleamic acid disulfide conjugate (Dox-DMA-disulfide) 5a/b and doxorubicin disubstituted maleimide disulfide conjugate (Dox-DMI-disulfide) 6.

To a solution of 33 mg of doxorubicin.HCl salt (58 µmol, 1 eq.), 14 mg of DMAP (114 µmol, 2 eq.) and 14 µL of pyridine (13 mg, 170 µmol, 3 eq.) in 500 µL of DMSO was added a solution of 20 mg of anhydride-disulfide cross-linker **4** (58 µmol, 1 eq.) in 250 µL of DMSO. The reaction mixture was kept in the dark at rt. HPLC condition A (pH 8-8.5, see general procedures of HPLC section for details) showed that Dox-DMA-disulfide regioisomers **5**a/b (t_R 24.6-25.9 min) were formed within 15 min, which over time (5 days) slowly converted to Dox-DMI-disulfide **6** (t_R 32.0-32.8 min). The product Dox-DMI-disulfide **6** (37 mg) was purified by HPLC condition B to give a yield of 74% from compound **4**.

Dox-DMA-disulfide **5a/b**: MS (ES^-), exact mass calculated for $C_{42}H_{45}N_3O_{15}S_2$ $[M\text{-}H^+]^-$: 894.2, found 894.6. (Dox-DMI-disulfide **6** also present, exact mass calculated for $C_{42}H_{43}N_3O_{14}S_2$ $[M\text{-}H^+]^-$: 876.2, found 876.6). The MS data of Dox-DMA-disulfide **5a/b** are shown in Figure S14.

Dox-DMI-disulfide **6**: ^{1}H NMR (600 MHz, DMSO) δ 13.91 (1H, s), 13.14 (1H, s), 8.39 – 8.35 (1H, m), 8.04 (1H, t, *J* = 5.6 Hz), 7.82 - 7.77 (2H, m), 7.77 – 7.72 (1H, m), 7.65 (1H, d, *J* = 8.1 Hz), 7.55 (1H, d, *J* = 8.3 Hz), 7.19 – 7.15 (1H, m), 5.28 (1H, s, br), 4.92 – 4.87 (1H, m), 4.59 (2H, s), 4.25 (1H, q, *J* = 6.4 Hz), 4.17 (1H, d, *J* = 13.8 Hz), 3.92 (3H, s), 3.40 (1H, s, br), 3.28 – 3.23 (2H, m), 3.11 - 3.04 (1H, m), 2.94 (1H, d, *J* = 17.9 Hz), 2.83 (1H, d, *J* = 17.9 Hz), 2.78 (2H, t, *J* = 6.7 Hz), 2.51 – 2.46 (2H, m), 2.27 – 2.18 (3H, m), 2.12 – 2.05 (1H, m), 1.80 (3H, s), 1.58 (1H, dd, *J* = 3.1, 12.1 Hz), 1.12 (3H, d, *J* = 6.4 Hz). ^{13}C NMR (151 MHz, $CDCl_3$) δ 213.8, 186.3, 186.1, 172.0, 171.7, 170.9, 160.7, 159.0, 156.1, 154.5, 149.5, 138.5, 137.7, 137.0, 136.1, 135.3, 134.5, 134.0, 121.1, 119.8, 119.6, 119.3, 118.9, 110.6, 110.5, 100.5, 74.8, 70.2, 68.7, 66.6, 63.7, 56.5, 49.5, 37.7, 37.3, 36.5, 33.1, 31.9, 26.7, 19.4, 17.0, 8.4. MS (ES^+): exact mass calculated for $C_{42}H_{43}N_3O_{14}S_2$ $[M+H^+]^+$: 878.2, found 878.4. The ^{1}H NMR and ^{13}C NMR spectra of Dox-DMI-disulfide **6** are shown in Figures S15 and S16, respectively. The MS data of Dox-DMI-disulfide **6** is shown in Figure S17.

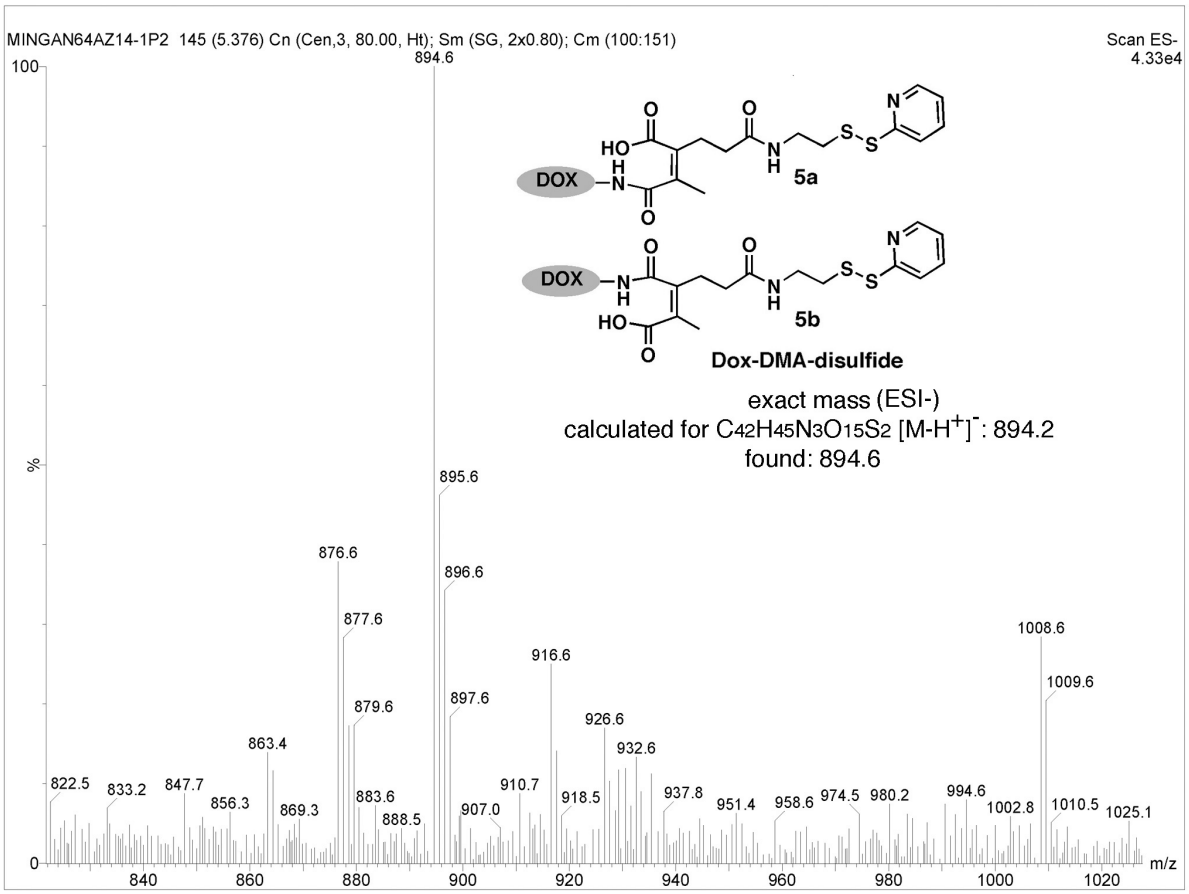


Figure S14. ESI- MS data of Dox-DMA-disulfide **5a/b**.

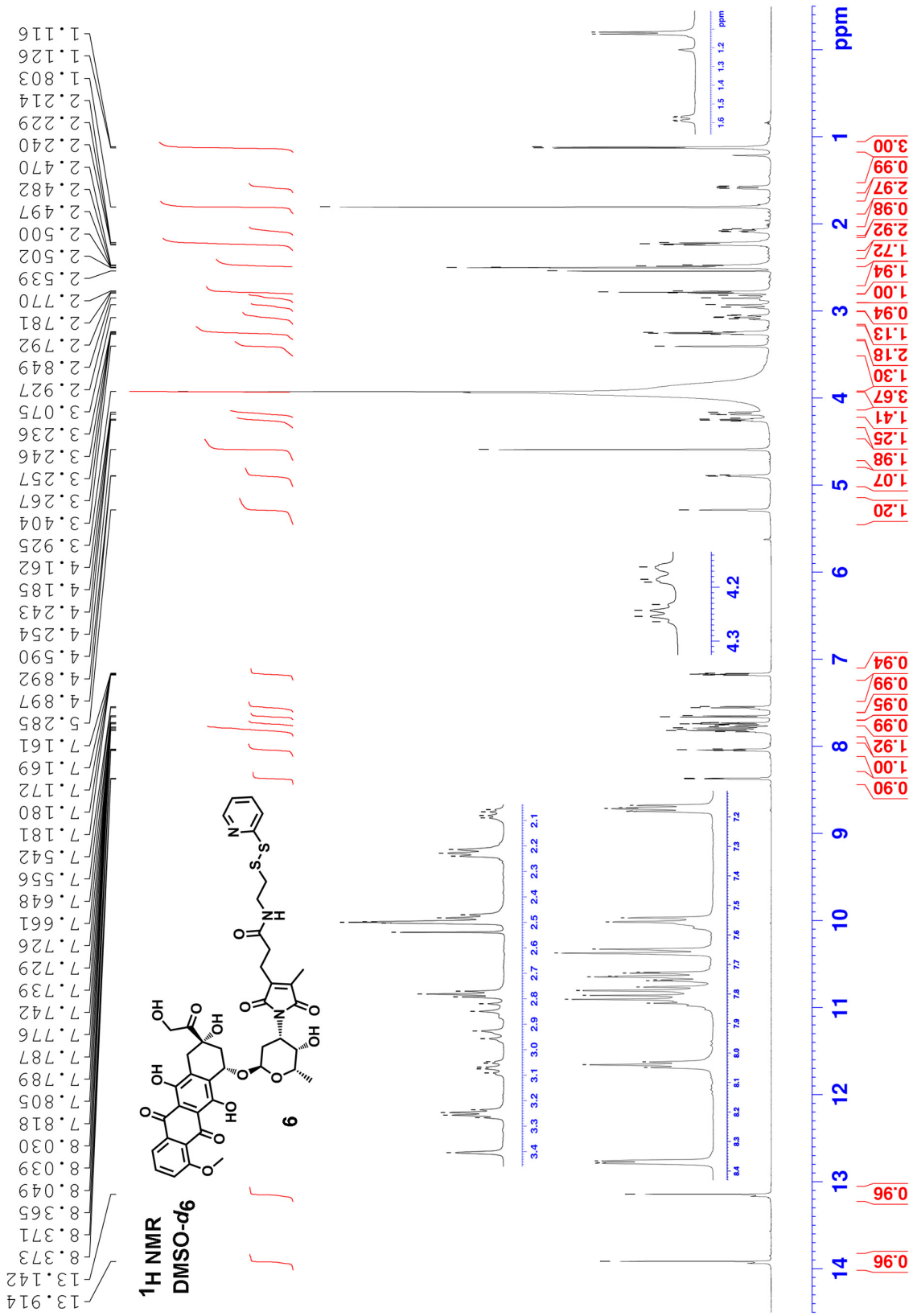


Figure S15. ^{1}H NMR spectrum of Dox-DMI-disulfide **6**.

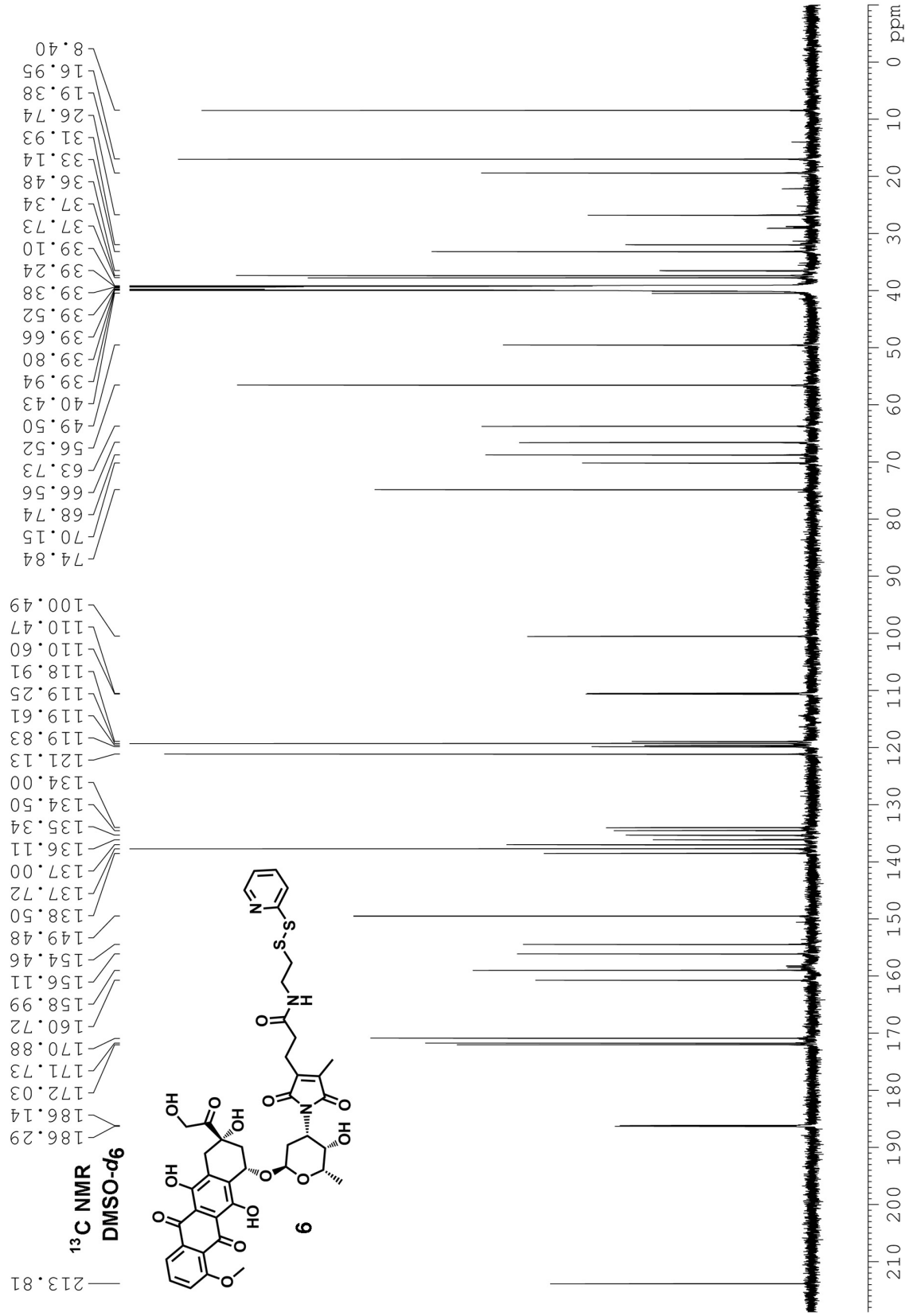


Figure S16. ^{13}C NMR spectrum of Dox-DMI-disulfide **6**.

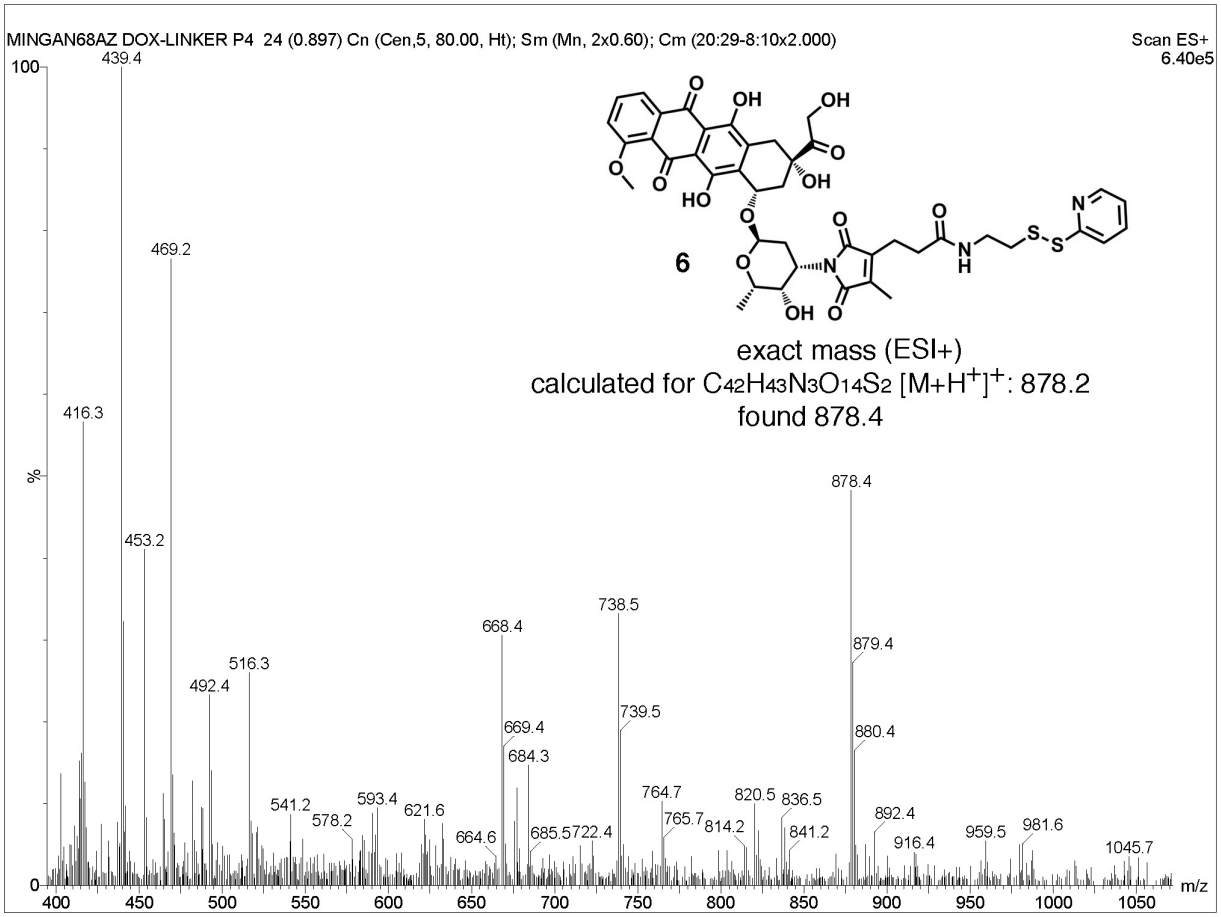
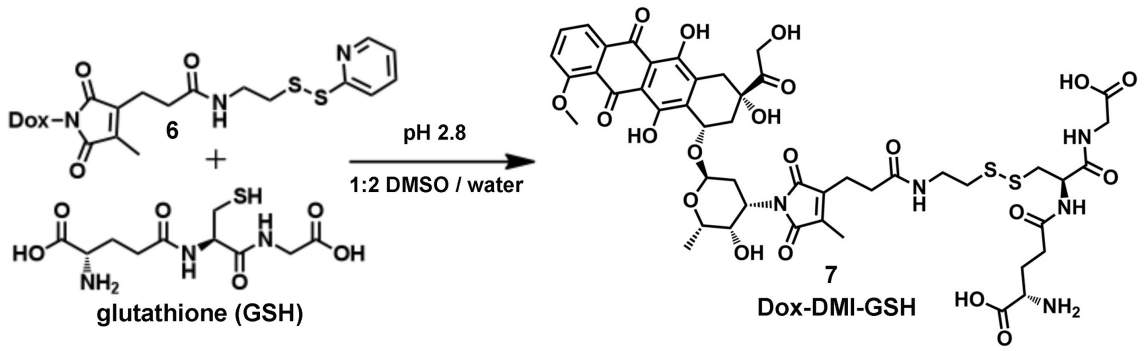


Figure S17. ESI+ MS data of Dox-DMI-disulfide **6**.



Doxorubicin disubstituted maleimide glutathione conjugate (Dox-DMI-GSH) 7.

To a solution of Dox-DMI-disulfide **6** (10 mg, 11.4 µmol, 1 eq.) in 287 µL of DMSO was added glutathione free acid (7 mg, 22.8 µmol, 2 eq.) and 574 µL of water. The mixture was vortexed until all components dissolved, and the resulting pH 2.8 solution (pH was measured on a sample prepared by diluting 20 µL of reaction mixture with 30 µL of water) was kept at rt in the dark for 1 hr. The reaction progress was monitored (s.m. Dox-DMI-disulfide **6** t_R 31.1 min, product Dox-DMI-GSH **7** t_R 25.0 min), and the desired product (8.6 mg, 70% yield) was isolated using HPLC condition C (see Figure S21 for HPLC traces). Dox-DMI-GSH **7**: ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.91 (1H, s), 13.15 (1H, s), 8.39 – 8.32 (2H, m), 8.29 (3H, s, br), 8.03 (1H, t, J = 5.6 Hz), 7.88 – 7.78 (2H, m), 7.56 (1H, d, J = 8.4 Hz), 5.28 (1H, s, br), 4.92 – 4.86 (1H, m, br), 4.59 (2H, s), 4.54 (1H, dt, J = 4.5, 9.0 Hz), 4.24 (1H, q, J = 6.5 Hz), 4.17 (1H, d, J = 13.9 Hz), 3.98 – 3.90 (1H, m), 3.93 (3H, s), 3.74 (2H, d, J = 5.9 Hz), 3.40 (1H, s), 3.30 – 3.23 (2H, m), 3.11-3.03 (2H, m), 2.98 – 2.91 (1H, m), 2.87 – 2.77 (2H, m), 2.70 (2H, t, J = 6.6 Hz), 2.48 (2H, t, J = 7.4 Hz), 2.42 – 2.34 (1H, m), 2.34 - 2.27 (1H, m), 2.23 (2H, t, J = 7.6 Hz), 2.26 – 2.18 (1H, m), 2.10 – 1.92 (3H, m), 1.82 (3H, s), 1.59 – 1.53 (1H, m), 1.12 (3H, d, J = 6.4 Hz). ^{13}C NMR (151 MHz, DMSO) δ 213.8, 186.4, 186.3, 172.1, 171.8, 171.2, 171.0, 170.9, 170.8, 170.3, 160.8, 156.1, 154.5, 138.6, 137.0, 136.2, 135.4, 134.6, 134.1, 119.9, 119.7, 119.0, 110.7, 110.6, 100.5, 74.8, 70.1, 68.8, 66.6, 63.7, 56.6, 51.8, 51.7, 49.5, 40.8, 40.6, 37.9, 37.0, 36.5, 33.1, 32.0, 30.7, 26.7, 25.9, 19.4, 16.9, 8.4. MS (ES+): exact mass calculated for $C_{47}H_{55}N_5O_{20}S_2$ $[M+H^+]^+$: 1074.3, found 1074.4; calculated for $[M+Na^+]^+$: 1096.3, found 1096.4. The ^{1}H NMR and ^{13}C NMR spectra of Dox-DMI-GSH **7** are shown in Figures S18 and S19, respectively. The MS data of Dox-DMI-GSH **7** is shown in Figure S20.

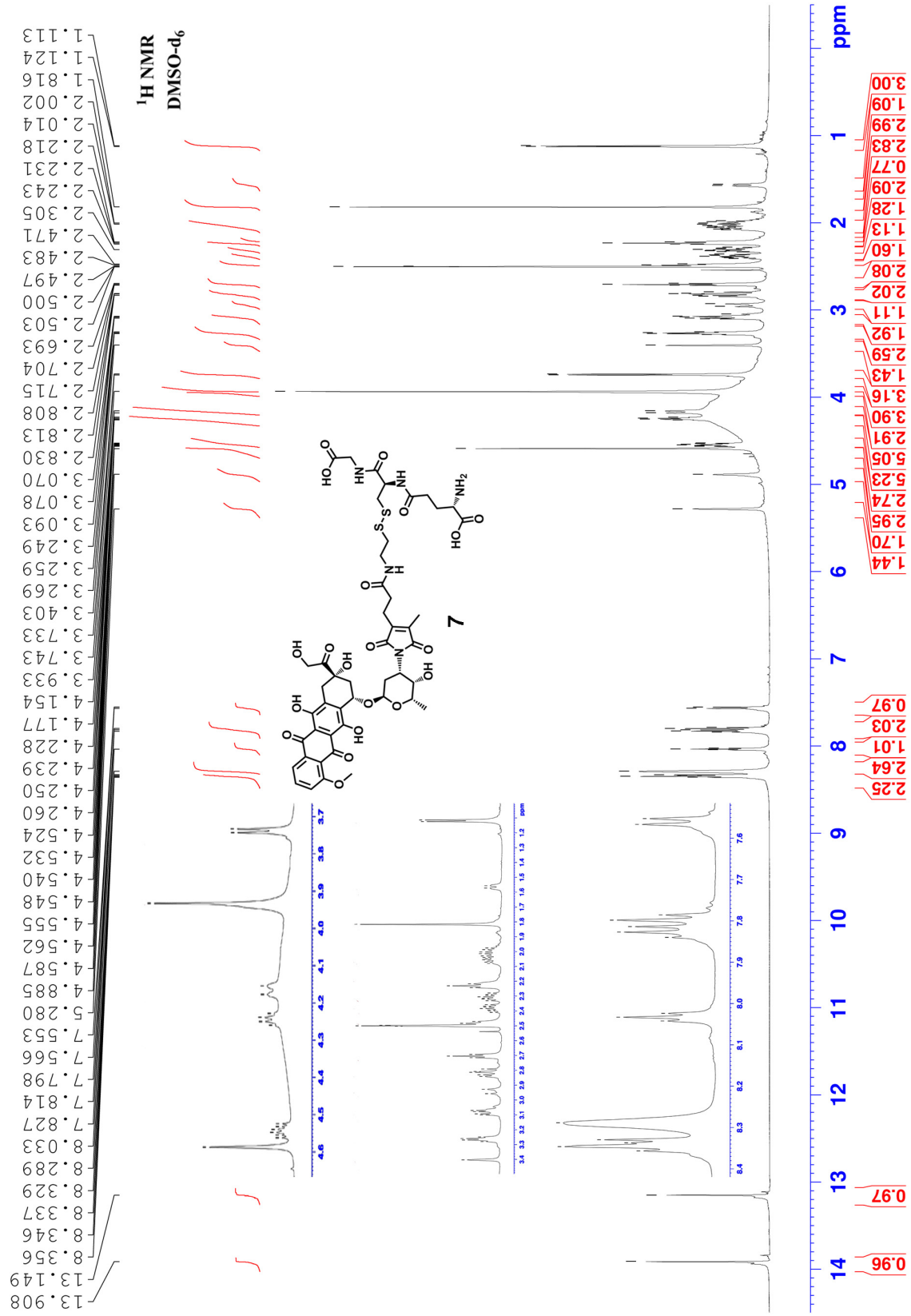


Figure S18. ^{1}H NMR spectrum of Dox-DMI-GSH **7**.

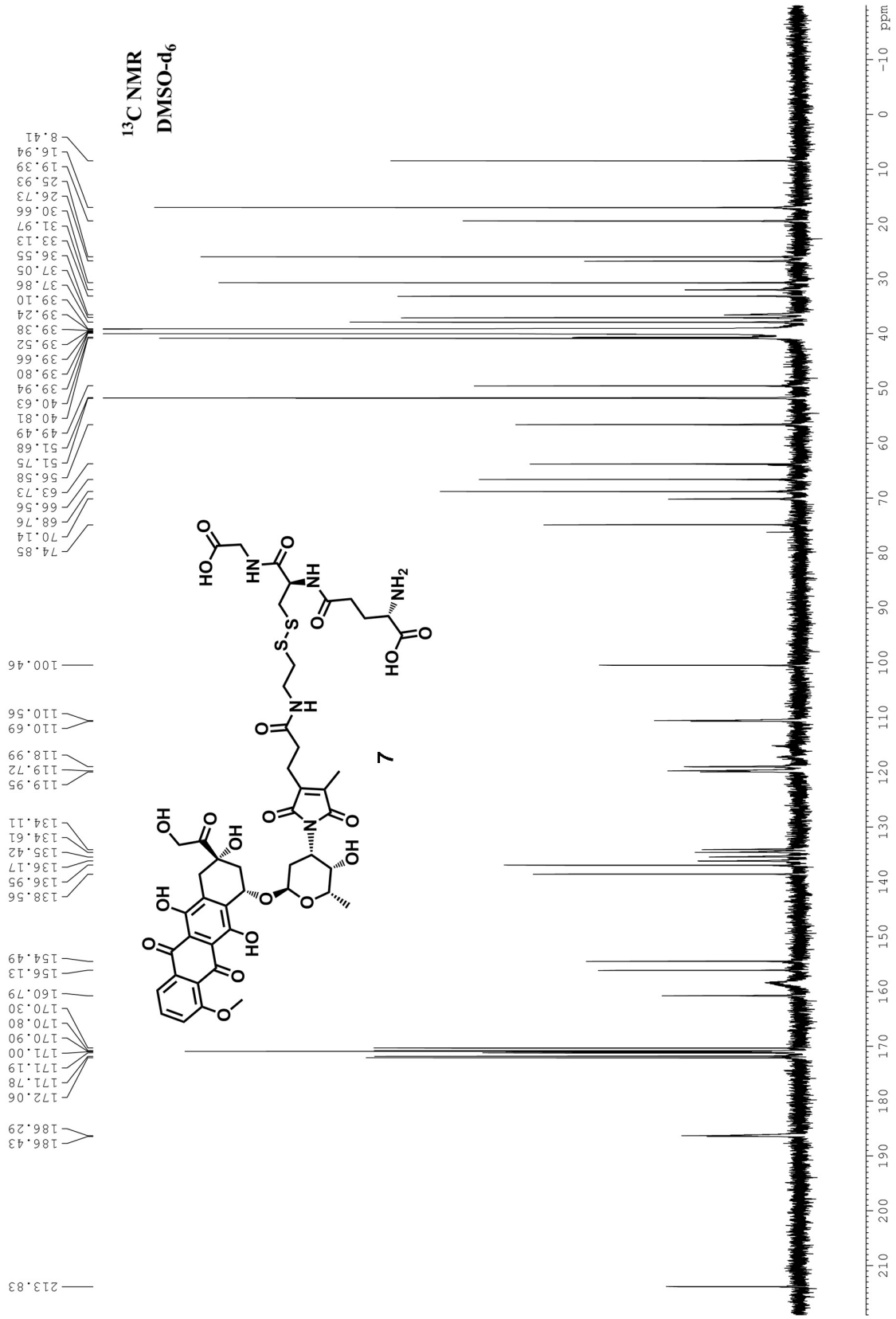


Figure S19. ^{13}C NMR spectrum of Dox-DMI-GSH **7**.

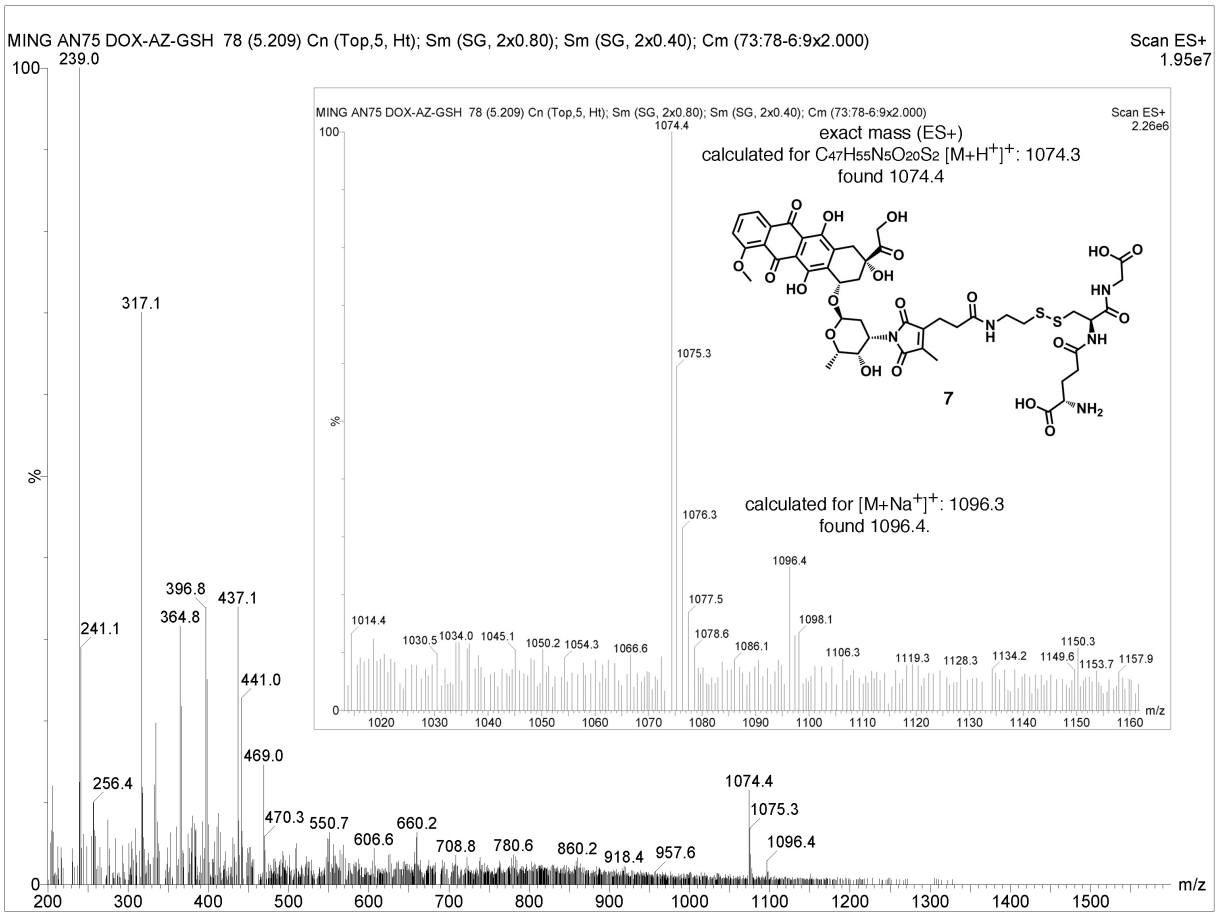


Figure S20. ES+ MS data of Dox-DMI-GSH **7**.

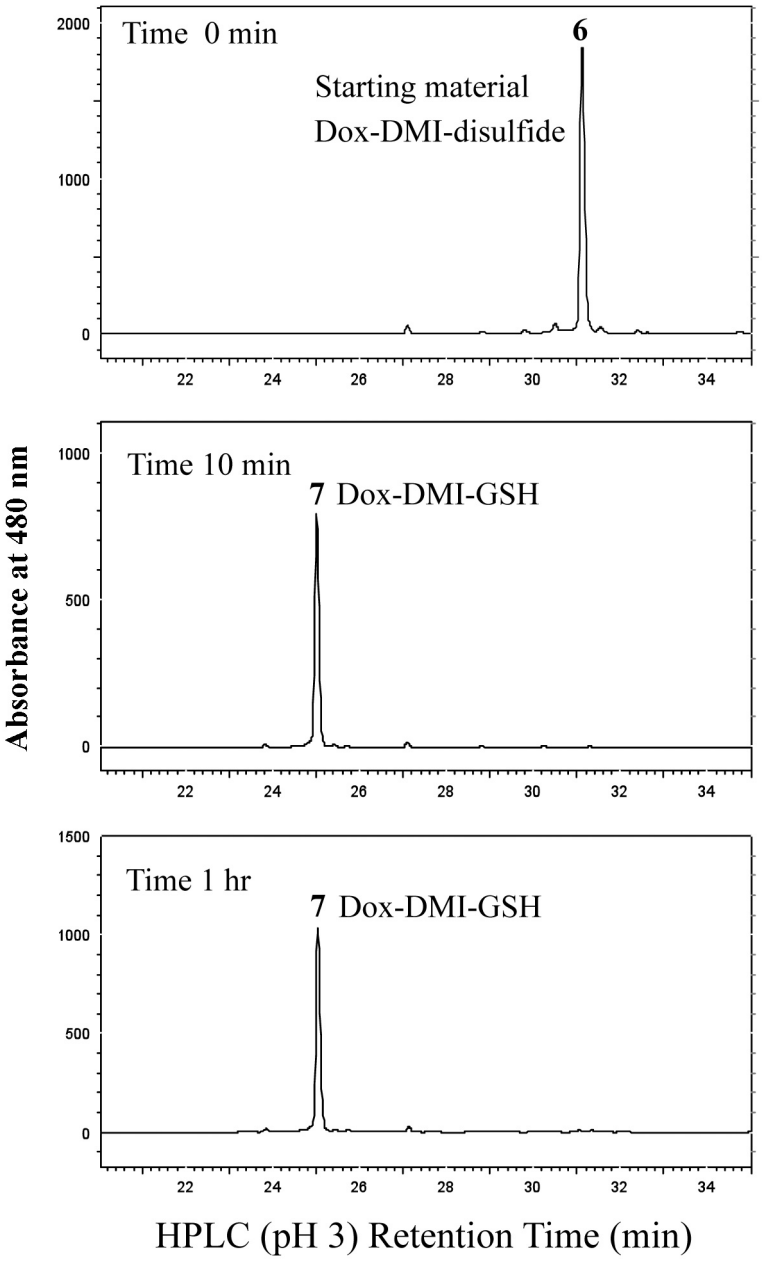
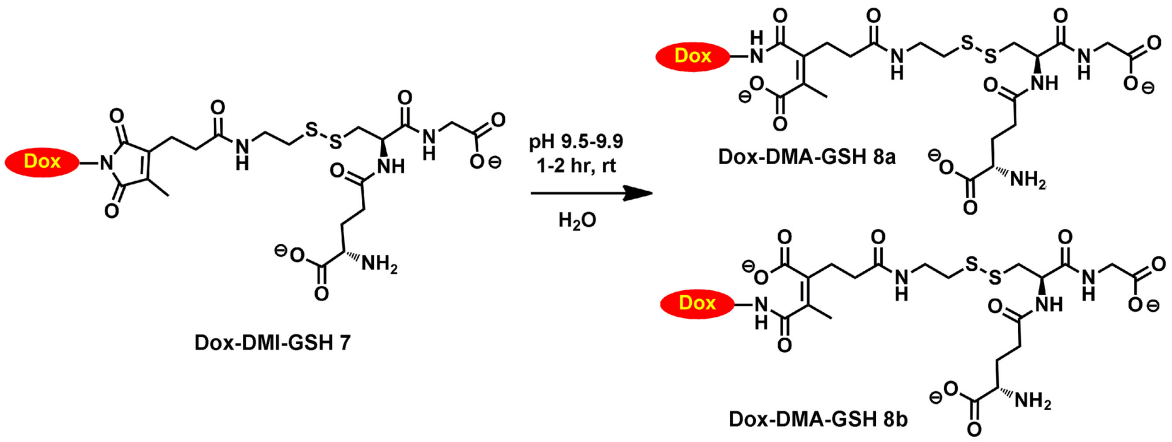


Figure S21. Formation of Dox-DMI-GSH **7** via disulfide exchange as followed by HPLC (pH 3). The starting material (s.m.) Dox-DMI-disulfide **6** had a retention time (t_R) of 31.1 min, while the desired product Dox-DMI-GSH **7** had a t_R of 25.0 min. The facile activated disulfide exchange reaction was essentially complete within 10 min, (likely catalyzed by the acidic condition and protonation of the 2-mercaptopyridyl leaving group on the N atom). No other side-product was detected (the reaction was peak-to-peak). The 25 min peak was isolated to give the desired product **7** (8.6 mg) in 70% yield (the rest of the material is likely lost due to normal process of HPLC because the reaction scale is small).



Doxorubicin disubstituted maleamic acid glutathione conjugate (Dox-DMA-GSH) 8a/b.

Sample preparation for mass analysis. A solution of Dox-DMI-GSH **7** (53.7 µg, 50 nmol, 357 µM) in 140 µL of aq. triethyl ammonium acetate (3.57 mM) / triethyl amine (3.57 mM) buffer with final pH of 9.9 was allowed to react at rt in the dark. After 10 min, pH of reaction mixture decreased to 9.6; thus, 25 µL of 10 mM triethyl ammonium acetate / 10 mM triethyl amine aq. buffer (solution A) was added to adjust pH back to 9.9. After 1 hr, HPLC at pH 2-3 (condition C) showed that > 70 % of doxorubicin (t_R 23.7 min) was released during HPLC, suggesting > 70% DMI **7** (t_R 25.0 min) had been converted to DMA **8a/b** (which are not stable at pH 2-3). At 1.5 hr, the reaction mixture pH again decrease to 9.5, and 30 µL of solution A was added to adjust pH back to 9.9. After 2 hr, 2 µL of 100 mM aq. acetic acid solution was added to the reaction mixture to adjust pH to 8.2. The resulting solution was submitted for mass analysis (~ 24 hr at rt en route). MS (ES^-): exact mass calculated for Dox-DMA-GSH **8a/b** $C_{47}H_{57}N_5O_{21}S_2$ $[M\text{-}H^+]^-$: expected 1090.3, found 1090.5; $[M\text{-}2H^+]^{-2}$: expected 544.7, found 545.2. Dox-DMI-GSH **7** was also present in the solution (exact mass calculated for Dox-DMI-GSH **7** $C_{47}H_{55}N_5O_{20}S_2$ $[M\text{-}H^+]^-$: expected 1072.3, found 1072.4). The MS data of Dox-DMA-GSH **8a/b** is shown in Figure S22.

Sample preparation for HPLC Dox release assay in phosphate buffer at rt. To a solution of Dox-DMI-GSH **7** (26.9 µg, 25 nmol, 25 µM) in 1 mL of 25 mM aq. sodium phosphate buffer (pH 8) was added small aliquots of 1 M aq. NaOH to adjust the pH to 9.9. The reaction solution was allowed to stand at rt for 1 hr in the dark, during which the pH decreased to ~ 9.6. Reaction progress was monitored using pH 8 HPLC (condition D): s.m. Dox-DMI-GSH **7** t_R 23.8 min, desired product Dox-DMA-GSH **8a/b** t_R 21.4 min and 21.8 min. After 1 hr, the pH of Dox-DMA-GSH **8a/b** solution was adjusted to desired pH for doxorubicin release assays at rt (Figure 1, S1; Table S1).

Sample preparation for HPLC Dox release assay in phosphate buffer at 37°C. To a solution of Dox-DMI-GSH **7** (86 µg, 80 nmol, 100 µM) in 800 µL of 25 mM aq. sodium phosphate buffer (pH 8) was added small aliquots of 1 M aq. NaOH to adjust the pH to 9.9. The reaction solution was allowed to stand at rt for 1 hr in the dark, during which the pH decreased to ~ 9.6. Reaction progress was monitored using pH 8 HPLC (condition E): s.m. Dox-DMI-GSH **7** t_R 21-22 min, desired product Dox-DMA-GSH **8a/b** t_R 18-20 min. After 1 hr, the pH of Dox-DMA-GSH **8a/b** solution was adjusted to desired pH and kept at 37 °C for doxorubicin release assays (Figure 2a-b, S2; Table S2).

Sample preparation for HPLC Dox release assay with 45% FBS at 37℃. To a solution of Dox-DMI-GSH **7** (107.4 μg, 100 nmol, 1 mM) in 100 μL of 25 mM aq. sodium phosphate buffer (pH 8) was added small aliquots of 1 M aq. NaOH to adjust the pH to 9.9. The reaction solution was allowed to stand at rt for 1 hr in the dark, during which the pH decreased to ~ 9.6. Reaction progress was monitored using pH 8 HPLC condition E. After 1 hr, the pH of Dox-DMA-GSH **8a/b** solution was adjusted to ~ pH 8, then mixed with 900 μL of 0.2 micron-filtered 1:1 FBS / DMEM-F12 with 1 % Anti-Anti. The pH of Dox-DMA-GSH **8a/b** solution in 10:45:45 aq. 25 mM phosphate / FBS / DMEM-F12 was adjusted to desired pH and kept at 37 °C for doxorubicin release assays (Figure 2c-d, S3; Table S3).

Sample preparations for pH-dependent anti-proliferation assays with cells.

General Procedure: To a 300 μM solution of Dox-DMI-GSH **7** (30 nmol) in 100 μL of 25 mM aq. sodium phosphate buffer (pH 8) was added small aliquots of 0.1 M aq. NaOH to adjust the pH to 9.9. The reaction solution was allowed to stand at rt for 1 hr in the dark, during which the pH decreased to ~ 9.6. After 1 hr, the reaction solution was split into two 50-μL portions (for pH 7.4 vs. pH 6.2-6.8 cell experiments). The pH of one portion was adjusted to pH 8 (for pH 7.4 cell experiments) or pH 7 (for pH 6.2-6.8 cell experiments). Subsequently, the solution was diluted 30-fold (to 10 μM) with pH 7.4 or pH 6.2-6.8 cell media (final volume 1.5 mL) and then filtered (0.2 micron) before used in cell experiments.

For data shown in Figure 3i (50% serum effect experiment), a similar procedure was used except the following: (a) A 100 μM solution of Dox-DMI-GSH **7** (22 nmol) in 220 μL of 10 mM aq. sodium phosphate buffer (pH 8) was used for conversion to DMA **8** at pH 9.9; (b) Subsequently, the DMA **8** solution was adjusted to pH 8, filtered (0.2 micron), and then diluted 17- to 50-fold (to ~ 2 - 6 μM final [DMA **8**] concentration) with pH 7.4 or pH 6.7 cell media (DMEM-F12 without phenol red or 1:1 DMEM-F12 without phenol red / FBS) (final volume 250 μL per specific pH and dose). Small amounts of 10 mM aq. sodium phosphate buffer (pH 8) was added to the more diluted sample solutions to keep the final concentration of sodium phosphate constant across samples of different DMA dose.

For data shown in Figure 3d and 3h (concentration scan from 3 nM to 30 μM), a similar procedure was used except the following: (a) A 187 μM solution of Dox-DMI-GSH **7** (75 nmol) in 400 μL of 10 mM aq. sodium phosphate buffer (pH 8) was used for conversion to DMA **8**; (b) Subsequently, the DMA **8** solution was adjusted to pH 8, filtered (0.2 micron), and then diluted to 10 μM and 30 μM stocks with pH 7.4 or pH 6.7 cell media (DMEM-F12 without phenol red), followed by serial dilutions to give the final concentrations (final volume 450 μL per specific pH and dose).

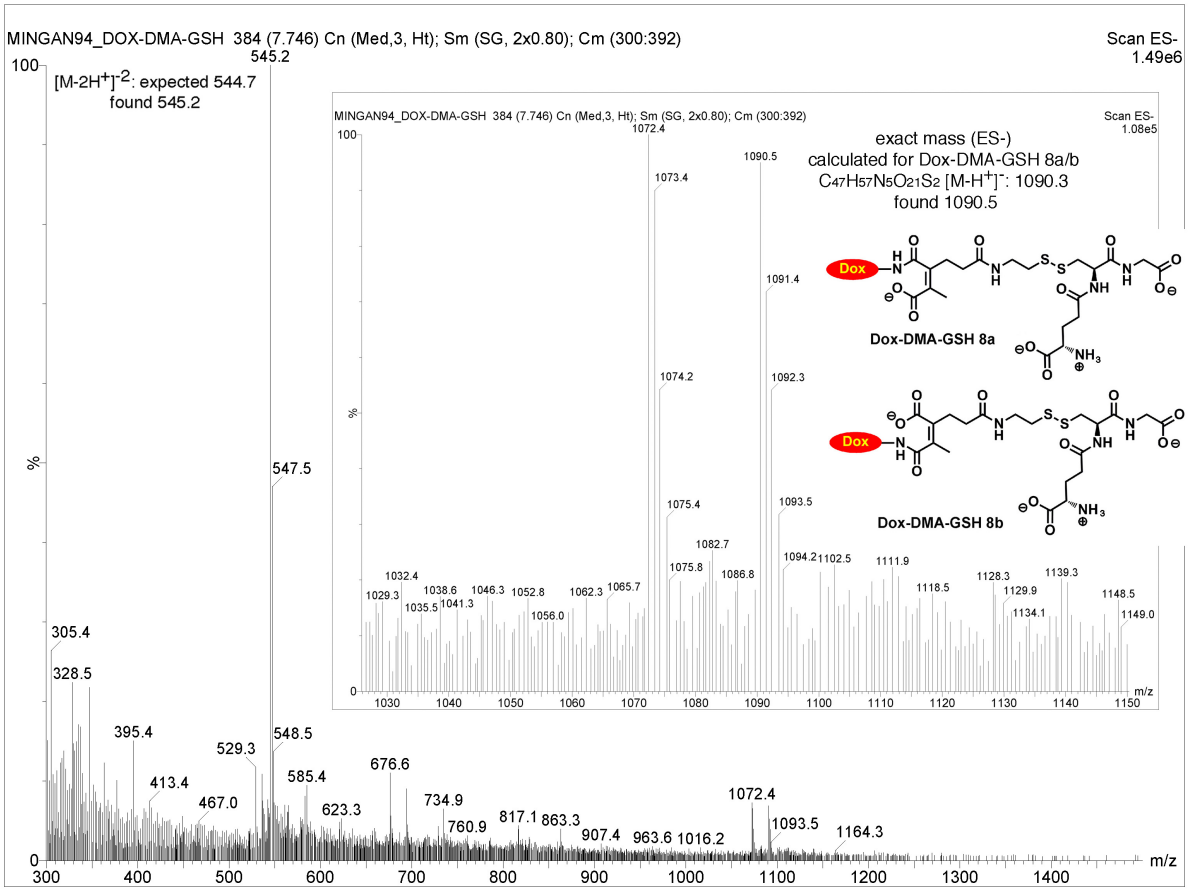


Figure S22. ES- MS data of Dox-DMA-GSH **8a/b**.

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