Vinylboronic Acid-Caged Prodrug Activation using Click-to-Release Tetrazine Ligation

Lianne Lelieveldt, Selma Eising, Abel Wijen, Kimberly Bonger

† Department of Biomolecular Chemistry and Synthetic Organic Chemistry, Radboud University Nijmegen, the Netherlands

email: k.bonger@science.ru.nl

Materials and Methods

All reagents were of commercial grade and used as received unless indicated otherwise. If no further details are given, the reaction was performed under a nitrogen atmosphere and at room temperature. Analytical thin layer chromatography (TLC) was performed on silica gel-coated plates (Merck, 60 F254) with the indicated solvent mixture, visualization was done using ultraviolet (UV) irradiation (λ = 254 nm) and/or staining with aqueous $KMnO_4$ or ninhydrin. Purification by column chromatography was carried out using silica gel 60 (Merck, 0.040-0.063 mm).

^{1}H NMR spectra were recorded on a Bruker Advance III 400 (400 MHz) or 500 (500 MHz) spectrometer. TMS (δH 0.00) or the NMR solvent residual peak of $CDCl_3$ (($CHCl_3$) δH 7.26), CD_3OD ((CHD_3O) δH 3.31) or $(CD_3)_2SO$ ((C_2HD_5SO) δH 2.50) were used as the internal reference. ^{13}C NMR spectra were recorded on a Bruker Advance III 400 (100 MHz) or 500 (125 MHz) spectrometer in $CDCl_3$ (δC 77.2) or $(CD_3)_2SO$ (δC 39.5) using their central resonance as the internal reference. All ^{13}C NMR spectra were proton decoupled. Peak assignments are based on 2D ^{1}H COSY, NOESY and ^{13}C HSQC and HMBC NMR experiments.

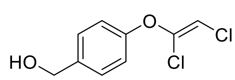
Low-resolution mass spectra (LRMS) were recorded on a Thermo LCQ Advantage Max (Electrospray Ionization (ESI)) or a Thermo Finnigan LCQ Fleet ESI ion-trap mass spectrometer, which is equipped with a Shimadzu HPLC (C18-column, 150 × 3 mm, particle size 3 µm) and a PDA detector.

High-resolution mass spectra (HRMS) of small molecules were recorded on a JEOL AccuTOF JMS-T100CS (ESI).

Analytical HPLC measurements were performed on a Shimadzu LC-20A Prominence system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a Gemini NX-C18 column, 150 × 3 mm, particle size 3 µm (Phenomenex, Utrecht, The Netherlands). The linear gradient used is 5% to 100% acetonitrile in H_2O, both with 0.1% TFA,in 30 minutes and a flow of 0.4 ml/min.

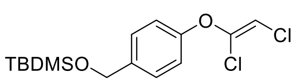
Synthetic procedures

***(Z)*-(4-((1,2-Dichlorovinyl)oxy)phenyl)methanol (2)**. 4-Hydroxybenzyl alcohol **1** (2.0 g, 16 mmol,



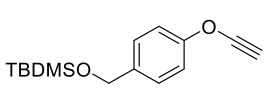
1.0 equiv.) was dissolved in DMF (16 mL) under ambient atmosphere and K_2CO_3 (2.7 g, 19 mmol, 1.2 equiv.) was added. Next, the mixture was heated at 70 °C and trichloroethylene (1.8 mL, 19 mmol, 1.2 equiv.) was added dropwise. After the mixture was heated at 70 °C overnight, it was cooled down to r.t., H_2O (20 mL) was added and the mixture was extracted with EtOAc (3 × 15 mL). The combined organic layers were washed with brine and dried over Na_2SO_4, and the volatiles were removed under reduced pressure. As there was still water present, the product was resuspended in H_2O and extracted with Et_2O (3 × 15 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. The volatiles were removed under reduced pressure yielding vinyl ether **2** (3.1 g, 87%) as a dark orange oil. R_f = 0.57 (50% EtOAc in heptane). ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.40 – 7.34 (m, 2H), 7.09 – 7.04 (m, 2H), 5.96 (s, 1H), 4.67 (s, 2H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 153.3, 140.0, 137.1, 128.6, 117.2, 103.8, 64.7. GCMS 6.68 min, *m/z* = 218 (M^+, calcd. for $C_9H_8Cl_2O_2$ = 218, 100%), 107 [$(M-C_7H_7O)^+$, 55%], 77 [$(M-C_6H_5)^+$, 85%].

***(Z)-tert*-Butyl((4-((1,2-dichlorovinyl)oxy)benzyl)oxy)dimethylsilane (3)**. Alcohol **2** (2.0 g, 9.1



mmol, 1.0 equiv.) was dissolved in dry DMF (16 mL) and the mixture was cooled to 0 °C. Then, imidazole (750 mg, 11 mmol, 1.2 equiv.) and TBDMSCl (1.7 g, 11 mmol, 1.2 equiv.) were added and the reaction was stirred for 2 h at r.t. The mixture was diluted with Et_2O (20 mL), washed with sat. NH_4Cl and brine and dried over Na_2SO_4. The volatiles were removed under reduced pressure yielding TBDMS ether **8** (3.1 g, quant.) as a slightly yellow oil. R_f = 0.32 (10% EtOAc in heptane). ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.36 – 7.29 (m, 2H), 7.06 – 6.99 (m, 2H), 5.94 (s, 1H), 4.72 (s, 2H), 0.94 (s, 9H), 0.10 (s, 6H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 152.7, 140.2, 137.8, 127.4, 116.9, 103.5, 64.4, 25.9, 18.4, -5.3. GCMS 8.36 min, *m/z* = 332 (M^+, calcd. for $C_{15}H_{22}Cl_2O_2Si$ = 332, <1%), 275 [$(M-C_{11}H_{13}O_2Si)^+$, 65%].

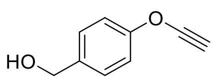
***tert*-Butyl((4-(ethynyloxy)benzyl)oxy)dimethylsilane (4)**. Dichlorovinyl ether **3** (2.0 g, 6.0 mmol,



1.0 equiv.) was dissolved in dry Et_2O (50 mL) and *n*-BuLi (15 mL of 1.6 M in hexanes, 23.8 mmol, 4.0 equiv.) was added dropwise to the mixture. The solution was then stirred for 1 h at -78 °C, before the mixture was warmed to -40 °C over the course of 1 h and stirred for another 1 h at -40 °C. Next, the mixture was quenched with H_2O (10 mL) and extracted with Et_2O (3× 15mL). The combined organic layers were washed with sat. NH_4Cl and brine and dried over Na_2SO_4. The volatiles were removed under reduced pressure and the crude product was purified using column chromatography (1% EtOAc in heptane), yielding alkyne **4** (1.4 g, 87%) as a dark brown oil. R_f = 0.33 (1% EtOAc in heptane). ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.35 – 7.29 (m, 2H), 7.26 – 7.23 (m, 2H), 4.71 (s, 2H), 2.07 (s, 1H), 0.94 (s, 9H), 0.09 (s, 6H).

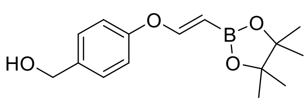
^{13}C NMR (100 MHz, chloroform-*d*) δ 154.5, 137.9, 127.4, 114.7, 84.7, 64.3, 33.2, 25.9, 18.4, -5.2. GCMS 6.71 min, *m/z* = 262 (M^+, calcd. for $C_{15}H_{22}O_2Si$ = 262, < 1%), 205 [$(M-C_{11}H_{13}O_2Si)^+$, 100%], 131 [$(M-C_9H_7O)^+$, 100%], 77 [$(M-C_6H_5)^+$, 30%].

(4-(Ethynyloxy)phenyl)methanol (5). TBMDS ether **4** (1.0 g, 3.8 mmol, 1.0 equiv.) was dissolved



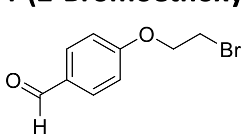
in dry THF (20 mL) and the mixture was cooled to 0 °C. 1 M TBAF in THF (4.2 mL, 4.2 mmol, 1.1 equiv.) was added and the mixture was stirred for 30 mins at 0 °C before the reaction was quenched with H_2O (15 mL). The product was extracted with EtOAc (3 × 15mL) and the combined organic layers were washed with brine and dried over $MgSO_4$. The volatiles were removed under reduced pressure and the crude product was purified using column chromatography (30% EtOAc in heptane), yielding alcohol **5** (506 mg, 91%) as a dark green solid. R_f = 0.3 (30% EtOAc in heptane). ^1H NMR (400 MHz, chloroform-*d*) δ 7.40 – 7.36 (m, 2H), 7.30 – 7.27 (m, 2H), 4.68 (s, 2H), 2.10 (s, 1H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 155.0, 137.3, 128.5, 115.1, 84.5, 64.6, 33.5. GCMS 4.64 min, *m/z* = 148 (M^+, calcd. for $C_9H_8O_2$ = 148, 100%), 77 [$(M- C_6H_5)^+$, 70%].

***(E)*-(2-(4-(Hydroxymethyl)phenoxy)vinyl)boronic acid pinacol ester (6)**. Alkyne **5** (100 mg, 0.68



mmol, 1.0 equiv.) was dissolved in dry toluene (2 mL) and sparged with N_2 for 10 minutes. Pinacolborane (490 µL, 3.4 mmol, 5.0 equiv.) and $RuHClCO(PPh_3)_3$ (38 mg, 34 µmol, 0.05 equiv.) were added and the mixture was stirred at 50 °C overnight. The mixture was cooled down to r.t. before the volatiles were removed under reduced pressure. The crude product was dissolved in Et_2O (10 mL) and washed with sat. $NaHCO_3$ and brine, and dried over Na_2SO_4. The volatiles were removed under reduced pressure and the crude product was purified using column chromatography (30 to 40% EtOAc in heptane), yielding VBA **6** (141 mg, 76%) as a dark brown oil. R_f = 0.33 (40% EtOAc in heptane). ^1H NMR (400 MHz, chloroform-*d*) δ 7.36 – 7.31 (m, 2H), 7.23 (d, *J* = 13.8 Hz, 1H), 7.07 – 7.02 (m, 2H), 4.88 (d, *J* = 13.9 Hz, 1H), 4.66 (d, *J* = 5.7 Hz, 2H), 1.60 (t, *J* = 5.9 Hz, 1H), 1.27 (s, 12H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 159.4, 136.6, 128.5, 118.4, 83.0, 64.8, 24.7, 21.1. No signal was observed for the carbon attached to boron. GCMS 8.90 min, *m/z* = 275 (M^+, calcd. for $C_{15}H_{21}BO_4$ = 275, 100%), 77 [$(M-C_6H_5)^+$, 28%].

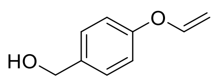
4-(2-Bromoethoxy)benzaldehyde (8). 4-Hydroxybenzaldehyde **7** (1.0 g, 8.2 mmol, 1.0 equiv.) was



dissolved in MeCN (63 mL) under ambient atmosphere. Dibromoethane (7.1 mL, 82 mmol, 10.0 equiv.) and K_2CO_3 (2.1 g, 14.9 mmol, 1.8 equiv.) were added and the mixture was stirred at 80 °C overnight. The mixture was cooled down to r.t., H_2O (50 mL) was added and the product was extracted with Et_2O (3 × 20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and the volatiles were removed under reduced pressure. The crude product was purified using column chromatography

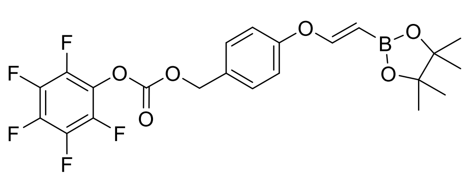
(30% EtOAc in heptane), yielding ether **8** (1.5 g, 79%) as a white solid. R_f = 0.38 (30% EtOAc in heptane). ^{1}H NMR (400 MHz, chloroform-*d*) δ 9.90 (s, 1H), 7.92 – 7.81 (m, 2H), 7.06 – 6.98 (m, 2H), 4.38 (t, *J* = 6.2 Hz, 2H), 3.67 (t, *J* = 6.2 Hz, 2H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 190.7, 163.0, 132.0, 130.5, 114.9, 68.0, 28.4. GCMS 6.87 min, *m/z* = 228 (M^+, calcd. for $C_9H_9BrO_2$ = 228.0, 67%), 201 [$(M-C_8H_8BrO)^+$, 70%], 107 [$(M-C_2H_4Br)^+$, 5%]. The data agrees with the reported literature values. [1]

(4-(Vinyloxy)phenyl)methanol (9). Benzaldehyde **8** (400 mg, 1.8 mmol, 1.0 equiv.) was dissolved



in dry DMSO (7 mL) and purged with N_2 for 10 minutes. *t*-BuOK (239 mg, 2.1 mmol, 1.2 equiv.) was slowly added in portions and the mixture was stirred for 10 minutes. Then, the mixture was diluted with EtOAc (35 mL) and quenched with ice water (1 mL). The layers were separated and the organic layers was washed with H_2O (3 × 10 mL), brine and dried over $MgSO_4$. The volatiles were removed under reduced pressure and the crude mixture was dissolved in MeOH (20 mL). $NaBH_4$ (132 mg, 3.5 mmol, 2.0 equiv.) was added in portions and the mixture was stirred for 1.5 h. The reaction was quenched with H_2O (20 mL) and the pH was adjusted with 0.1 M HCl until pH = 7. The mixture was extracted with EtOAc (3 × 15 mL), washed with brine and dried over $MgSO_4$. The volatiles were removed and the crude product was purified using column chromatography (20 to 30% EtOAc in heptane) yielding benzyl alcohol **9** (48 mg, 18%) as a colorless oil. R_f = 0.56 (30% EtOAc in heptane). ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.36 – 7.30 (m, 2H), 7.03 – 6.97 (m, 2H), 6.64 (dd, *J* = 13.7, 6.1 Hz, 1H), 4.76 (dd, *J* = 13.7, 1.7 Hz, 1H), 4.65 (d, *J* = 4.2 Hz, 2H), 4.44 (dd, *J* = 6.1, 1.7 Hz, 1H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 156.3, 148.2, 135.7, 128.6, 117.2, 95.2, 64.9. GCMS 4.55 min, *m/z* = 150 (M^+, calcd. for $C_9H_{10}O_2$ = 150, 100%), 107 [$(M-C_7H_7O)^+$, 70%], 77 [$(M-C_6H_5)^+$, 50%]. The data agrees with the reported literature values. [1]

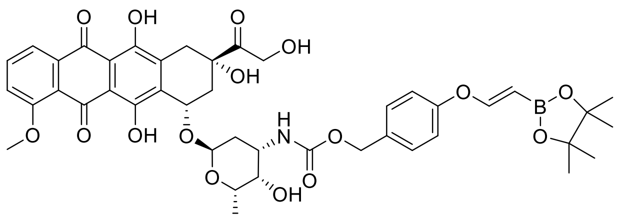
***(E)*-(2-(4-(Perfluorophenyl carbonate)phenoxy)vinyl)boronic acid pinacol ester (13)**. Alcohol **6**



(100 mg, 0.36 mmol, 1.0 equiv.) was dissolved in DCM (3 mL) under ambient atmosphere. Bis(pentafluorophenyl)carbonate (214 mg, 0.54 mmol, 1.5 equiv.) and Et_3N (252 µl, 1.8 mmol, 5.0 equiv.) were added and the mixture was stirred for 1 h. The volatiles were removed under reduced pressure and the crude product was purified using column chromatography (1 to 5% EtOAc in heptane), yielding VBA **13** (111 mg, 84%) as a colorless oil. R_f = 0.13 (5% EtOAc in heptane). ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.40 (d, *J* = 8.6 Hz, 2H), 7.23 (d, *J* = 13.8 Hz, 1H), 7.08 (d, *J* = 8.6 Hz, 2H), 5.29 (s, 2H), 4.93 (d, *J* = 13.8 Hz, 1H), 1.28 (s, 12H). ^{13}C NMR (126 MHz, chloroform-*d*) δ 158.7, 156.8, 151.3, 142.3, 140.8, 140.2, 138.8, 136.9, 130.5, 130.3, 129.3, 118.4, 83.1, 71.6, 24.7. No signal was observed for the carbon attached to boron. ^{19}F NMR (471 MHz, chloroform-*d*) δ -152.93 – -153.03 (m), -157.41 (t, *J* = 21.7 Hz), -161.90 – -162.05 (m). ^{11}B NMR (160 MHz, chloroform-*d*) δ

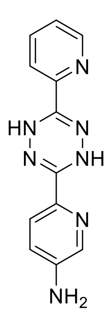
30.59. GCMS 12.91 min, *m/z* = 486 (M^+, $C_{22}H_{20}BF_5O_6$ = 486, 0%), 427 [$(M-C_{19}H_{13}BF_5O_5)^+$, 2%], 259 [$(M-C_{15}H_{20}BO_3)^+$, 50%].

***(E)*-(2-(4-(Doxorubicin carbamate)phenoxy)vinyl)boronic acid pinacol ester (14).** Doxorubicine HCl (20 mg, 34 µmol, 1.0 equiv.) was dissolved in dry DMF (1.5 ml) under N_2. Et_3N (5.3 µL, 38 µmol, 1.1 equiv.) and compound **13** (19 mg, 38 µmol, 1.1 equiv) were added. The reaction was stirred for 24 h in the dark. The solvents were removed under reduced pressure. Purification by flash chromatography (2-3% MeOH in DCM) yielded the desired **14** (12 mg, 40%) as a dark red solid. R_f = 0.18 (4% MeOH in DCM). ^{1}H NMR (500 MHz, chloroform-*d*) δ 13.99 (s, 1H), 13.25 (s, 1H), 8.06 (d, *J* = 7.6 Hz, 1H), 7.80 (t, *J* = 8.1 Hz, 1H), 7.41 (d, *J* = 8.1 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 2H), 7.21 (d, *J* = 13.8 Hz, 1H), 7.01 (d, *J* = 8.0 Hz, 2H), 5.52 (d, *J* = 3.7 Hz, 1H), 5.32-5.30 (m, 1H), 5.14 (d, *J* = 8.4 Hz, 1H), 5.00 (s, 2H), 4.88 (d, *J* = 13.8 Hz, 1H), 4.78 (s, 2H), 4.56 (s, 1H), 4.16 (d, *J* = 6.4 Hz, 1H), 4.10 (s, 3H), 3.93 – 3.84 (m, 1H), 3.69 (s, 1H), 3.66 (t, *J* = 6.7 Hz, 1H), 3.29 (d, *J* = 18.6 Hz, 1H), 3.03 (d, *J* = 18.8 Hz, 1H), 2.36 (d, *J* = 14.7 Hz, 1H), 2.19 (dd, *J* = 14.7, 4.0 Hz, 1H), 1.90 (dd, *J* = 13.5, 5.0 Hz, 1H), 1.79 (td, *J* = 13.3, 4.1 Hz, 1H), 1.27 (s, 12H), 1.26 (s, 3H). ^{13}C NMR (126 MHz, chloroform-*d*) δ 214.0, 187.2, 186.8, 161.2, 159.3, 156.3, 156.1, 155.8, 155.6, 135.9, 133.7, 133.7, 132.2, 130.0 (2C), 121.0, 120.0, 118.6, 118.4 (2C), 111.8, 111.6, 100.9, 83.2 (2C), 76.8, 69.9, 69.7, 67.4, 66.4, 65.7, 56.8, 47.1, 35.8, 34.2, 29.8, 24.9 (4C), 16.99. No signal was observed for the carbon attached to boron. ^{11}B NMR (160 MHz, chloroform-*d*) δ 30.15. HRMS (ESI+) *m/z* calcd for $C_{43}H_{48}BNNaO_{16}^+$ $[M+Na]^+$ 868.29638, found: 868.30001.



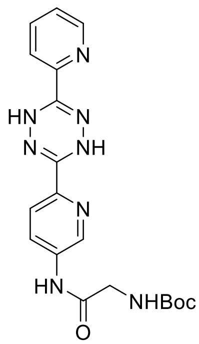
Dipyridyl-*s*-tetrazine **12** is synthesized as previously described.[2,3] Synthetic procedures are provided below.

6-(6-(Pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridine-3-amine (S1).



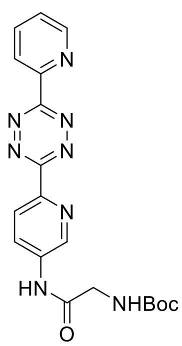
5-Amino-2-pyridinecarbonitrile (1.0 g, 8.39 mmol, 1.0 equiv.) and 2-cyanopyridine (0.87 g, 8.39 mmol, 1.0 equiv.) were heated in hydrazine monohydrate (1.6 mL, 33.57 mmol, 4.0 equiv.) overnight at 90 °C under nitrogen. The solvent was evaporated and the mixture was purified twice using column chromatography, first a column using 40-80% EtOAc in heptane and then 0-5% MeOH in DCM yielding dihydrotetrazine **S1** as a yellow solid (520 mg, 2.05 mmol, 24%). R_f = 0.25 (50% EtOAc in heptane). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (s, 1H), 8.65 (s, 1H), 8.62 (ddd, *J* = 4.9, 1.7, 1.0 Hz, 1H), 7.98-7.88 (m, 3H), 7.64 (dd, *J* = 8.6, 0.7 Hz, 1H), 7.51 (ddd, *J* = 7.2, 4.9, 1.5 Hz, 1H), 6.99 (dd, *J* = 8.6, 2.7 Hz, 1H), 5.88 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.6, 147.5, 146.7, 146.64, 146.60, 137.3, 134.2, 134.1, 125.2, 121.8, 120.8, 120.3. LRMS (ESI+) *m/z* calcd for $C_{12}H_{12}N_7^+$ $[M+H]^+$ 254.1, found: 254.1. The data agrees with the reported literature values.[2,3]

2-(Boc-amino)-*N*-(6-(6-(pyridine-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3yl)pyridine-3-yl)acetamide



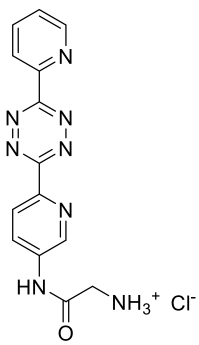
(S2). Boc-glycine (622 mg, 3.55 mmol, 2.0 equiv.) was dissolved in dry THF (9 mL) under nitrogen and cooled to 0 °C. *N*-Methylmorpholine (977 µL, 8.88 mmol, 5.0 equiv.) and isobutyl chloroformate (464 µL, 3.55 mmol, 2.0 equiv.) were added and the mixture was stirred for 5 min. Then amine **S1** (450 mg, 1.78 mmol, 1.0 equiv.) was added and the mixture was stirred overnight. Water and EtOAc were added, the layers were separated and the water layer was extracted with EtOAc (2 x). The combined organic layers were washed with sat. $NaHCO_3$ (aq.), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The product was purified using column chromatography (70-100% EtOAc in heptane) yielding amide **S2** as a yellow solid (575 mg, 79%). R_f = 0.37 (70% EtOAc in heptane). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H), 8.93 (s, 1H), 8.88 (s, 1H), 8.82 (d, *J* = 2.5 Hz, 1H), 8.67-8.57 (m, 1H), 8.15 (dd, *J* = 8.7, 2.5 Hz, 1H), 8.00-7.87 (m, 3H), 7.53 (ddd, *J* = 7.2, 4.9, 1.5 Hz, 1H), 7.13 (t, *J* = 6.1 Hz, 1H), 3.78 (d, *J* = 6.1 Hz, 2H), 1.40 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.2, 155.9, 148.6, 147.3, 146.3, 146.1, 141.3, 138.9, 137.4, 137.0, 126.8, 125.3, 121.4, 121.0, 78.2, 43.8, 28.2. LRMS (ESI+) *m/z* calcd for $C_{19}H_{23}N_8O_3^+$ $[M+H]^+$ 411.2, found: 411.1. The data agrees with the reported literature values.[2,3]

2-(Boc-amino)-*N*-(6-(6-(pyridine-2-yl)-1,2,4,5-tetrazin-3-yl)pyridine-3-yl)acetamide (S3).



Dihydrotetrazine **S2** (300 mg, 0.73 mmol, 1.0 equiv.) was dissolved in acetic acid (15 mL). Sodium nitrite (93 mg, 1.10 mmol, 1.1 equiv.) was added and the solution was stirred for 10 min. The mixture was diluted with DCM, and washed 3 times with sat. $NaHCO_3$ (aq.). The organic layer was dried with Na_2SO_4 and the volatiles were removed under reduced pressure. The product was purified using column chromatography (0-8% MeOH in DCM) yielding tetrazine **S3** as a pink solid (151 mg, 51%). R_f = 0.45 (10% MeOH in DCM). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.05 (d, *J* = 2.5 Hz, 1H), 8.97-8.90 (m, 1H), 8.64 (d, *J* = 8.7 Hz, 1H), 8.59 (td, *J* = 8.0, 1.1 Hz, 1H), 8.43 (dd, *J* = 8.7, 2.5 Hz, 1H), 8.16 (dt, *J* = 7.7, 1.7 Hz, 1H), 7.73 (ddd, *J* = 7.7, 4.7, 1.2 Hz, 1H), 7.18 (t, *J* = 6.1 Hz, 1H), 3.84 (d, *J* = 6.1, 2H), 1.41 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.5, 163.0, 162.8, 156.0, 150.6, 150.2, 144.0, 141.3, 138.2, 137.8, 126.6, 126.3, 124.9, 124.2, 78.2, 43.9, 28.2. LRMS (ESI+) *m/z* calcd for $C_{19}H_{21}N_8O_3^+$ $[M+H]^+$ 409.2, found: 409.1. The data agrees with the reported literature values.[2,3]

2-Amino-*N*-(6-(6-(pyridine-2-yl)-1,2,4,5-tetrazin-3-yl)pyridine-3-yl)acetamide hydrochloride (12).



Boc-protected amine **S3** (40 mg, 98 µmol, 1.0 equiv.) was dissolved in dry DCM (2.2 mL) under nitrogen. 4M HCl in dioxane (735 µL, 2.94 mmol, 30 equiv.) was slowly added and the mixture was stirred for 30 min. The solvent was removed under reduced pressure, whereupon the pink solid was lyophilized yielding amine **17** (34 mg, quant.). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.57 (s, 1H), 9.16 (d, J = 2.4 Hz, 1H), 8.98-8.91 (m, 1H), 8.68 (d, J = 8.7 Hz, 1H), 8.61 (dt, J = 8.0, 1.1 Hz, 1H), 8.44 (dd, J = 8.7, 2.5 Hz, 1H), 8.35 (br. t, J = 5.6 Hz, 2H), 8.18 (dt, J = 7.7, 1.8 Hz, 1H), 7.75 (ddd, J = 7.7, 4.7, 1.2 Hz, 1H), 3.99-3.91 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.1, 163.0, 162.7, 150.5, 150.0, 144.6, 141.3, 138.0, 137.6, 126.7, 126.6, 125.1, 124.3, 41.3. LRMS (ESI+) *m/z* calcd for $C_{14}H_{13}N_8O^+$ $[M+H]^+$ 309.1, found: 309.1. The data agrees with the reported literature values.[2,3]

Experimental procedures

^{1}H NMR study of the click-to-release reaction. The reactions between dipyridyl tetrazine **10** and the alkenes **6** and **9** in 3:1 CD_3OD/deuterated PBS were followed using ^{1}H NMR (500 MHz). Prior to the start of the tetrazine ligation, pinacol ester **6** (5 mM) was incubated in 3:1 MeOD-*d*$_4$/deuterated PBS. The hydrolysis of the pinacol ester was followed over time and showed full conversion to the free boronic acid within 2 h (Figure SI-1). Next, tetrazine **10** (5.0 mM) and the deprotected VBA **6** or the vinyl ether **9** (5.0 mM) were mixed 1:1 for a final concentration of 2.5 mM and the reactions were followed over time up to 14 days at room temperature.

Second order rate constant experiment. The reactions between the alkenes **6** and **9** and dipyridyl tetrazine derivative **10** in 75% MeOH/PBS were followed on a plate reader (Spark M10 microplate reader (Tecan)) at a controlled temperature of 20 °C, by measuring the absorbance of the tetrazine at 540 nm. The tetrazine and alkene **6** or **9** were both dissolved in methanol, and then diluted with PBS. After addition of the tetrazine to the alkene solution, the measurement was started directly. The final concentration of the tetrazine was 500 µM and of the excess of alkene was 10-20 equiv. (5, 6.25, 7.50, 8.75 or 10 mM). The time between the addition of the tetrazine and the start of the measurement was ± 20s. All reactions were performed in quadruplo. The kinetics were normalized and plotted in Figure SI-2A and 2B. The observed reactions were normalized by setting the absorbance at t = 0 s as 100%. It was taken into account that the measurement was started after a certain time. Since the reactions did not end in a plateau after the set time, the plateau was set equal to the background absorbance of pyridazine **11** absorption at the given wavelength.
Pseudo-first-order rate constant determination. The pseudo first-order rate constants k_{obs} for the tetrazine reactions with an excess of alkene was determined. The decay of the absorbance of the tetrazine was plotted against time (min) for the 5 different concentrations of the alkene. The k_{obs} was determined by fitting an exponential 'one phase decay' (nonlinear regression) using PrismGraphPad Software whereby Y = (Y0 -plateau)*exp(-k_{obs}*time(s)) + plateau.
Second-order rate constant determination. To determine the second-order rate constant, the k_{obs} of the reactions was plotted against the concentration of the alkenes. The line was fitted using a linear regression and the slope gave k_2. The goodness of the fit is shown by the coefficient of determination (R^2). The data is shown in Figure SI-2A and 2B.

Click-to-release LCMS measurements. The reaction between tetrazine **12** and VBA-doxorubicin **14** in 1% DMSO/PBS was followed using LCMS. The reaction was followed over 24 hours at 37 °C. The amount of doxorubicin was determined and was quantified by the area under the curve. The graph shows the relative amount compared to the amount of starting material.

Cell culture. Hela cells were maintained in DMEM supplemented with 10% heat-inactivated donor bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin (all purchased at Life Technologies). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Cells were passaged every 3-4 days.

Cell viability assay. The toxicity of several compounds was tested with a cell proliferation and cytotoxicity assay using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories). Hela cells were seeded in a 96-well plate (10 000 cells/well in 200 µL of medium). After one day of cell growth, the cells were washed once with 1 x PBS followed by the addition of 100 µL of growth medium containing 1% DMSO and different concentrations of doxorubicin, VBA-doxorubicin **14**, tetrazine **12**, and the tetrazine ligation of **12** with VBA-doxorubicin **14**. After 72 h of incubation, the medium was removed, the cells were washed 3 x with growth medium and 100 µL of medium containing 10% of CCK-8 was added. After incubation for 3 h, the absorbance of 450 nm using a microplate reader (Sunrise™, Tecan) was measured. The background absorbance of cell medium containing 10% CCK-8 was abstracted from the measured values. The viability of the cells was determined to be 100% by measuring the absorbance of cells with CCK-8 that were first incubated with 1% DMSO only. All conditions were measured in six-fold, mean values with SD are shown.

Supporting Information Figure 1

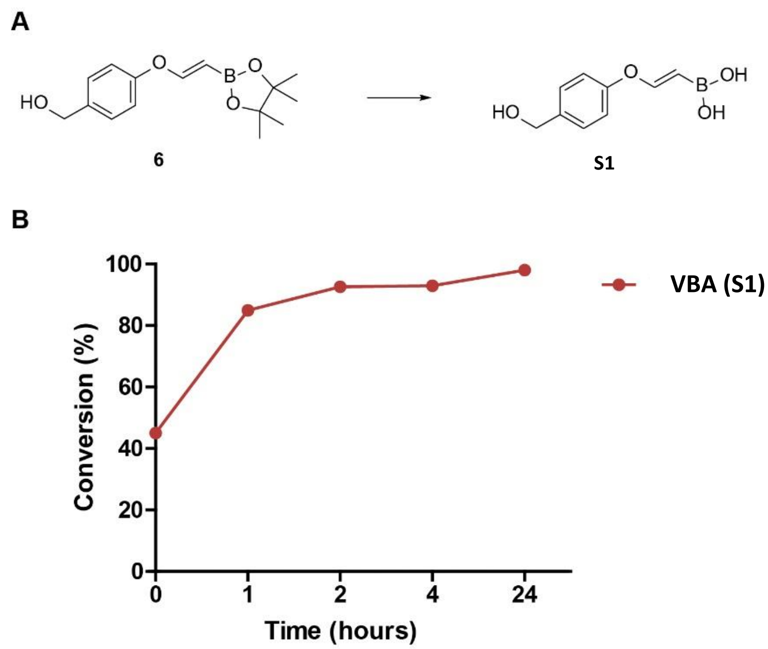


Figure SI-1. Pinacol hydrolysis in PBS. A) Schematic representation of the pinacol hydrolysis of VBA **6**; B) Studies were performed with 5mM VBA **6** dissolved in 75% MeOD/deuteurated PBS at room temperature and analyzed at different time points by ^{1}H NMR.

Supporting Information Figure 2

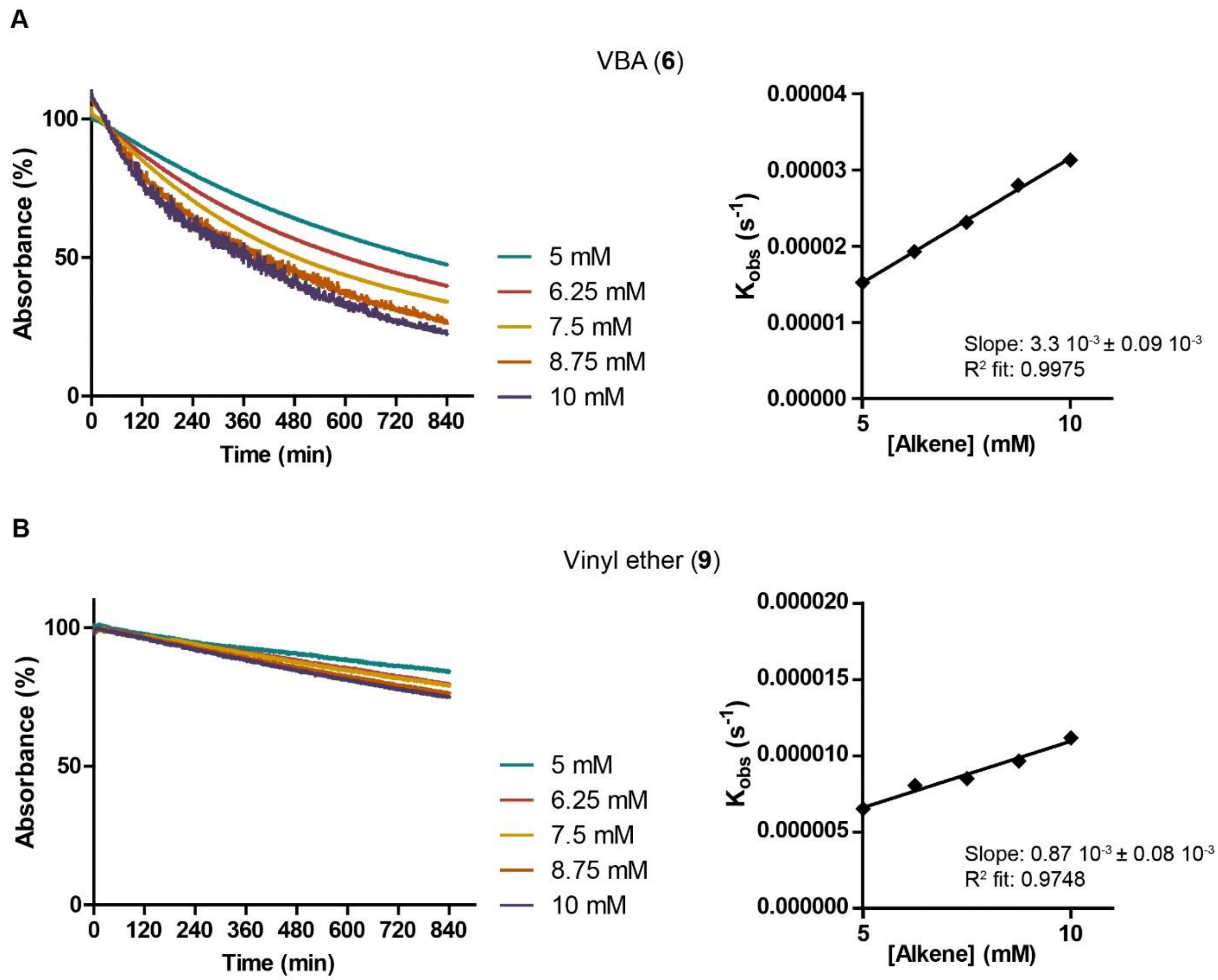
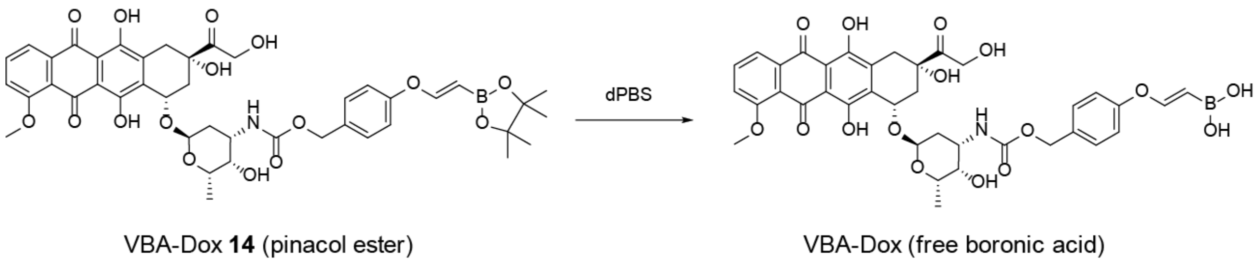


Figure SI-2. Kinetics of the click-to-release reaction with tetrazine **10** (500 µM) with 10 - 20 equiv. of the alkene in 75% MeOH/PBS. The left graph is the normalized absorbance at 540 nm of the reaction between tetrazine **10** and the alkene (A: VBA **6** and B: vinyl ether **9**) at different concentrations against time (min). The right graph shows the plot of the k_{obs} values against the alkene (A: VBA **6** and B: vinyl ether **9**) concentration. The slope of the linear fit is the second order rate constant, the goodness of the fit is shown by R^2.

Supporting Information Figure 3



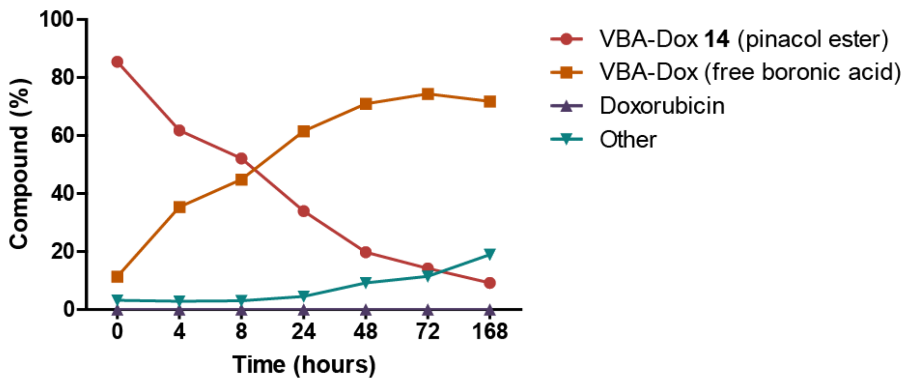


Figure SI-3. Stability of VBA-Dox **14** in deuterated PBS. Studies were performed with 0.1 mM of pinacol protected VBA-doxorubicin dissolved in deuterated PBS at 37 °C and analyzed at different time points by ^{1}H NMR. Slow hydrolysis of the pinacol was observed yielding the free boronic acid.

Supporting Information Figure 4

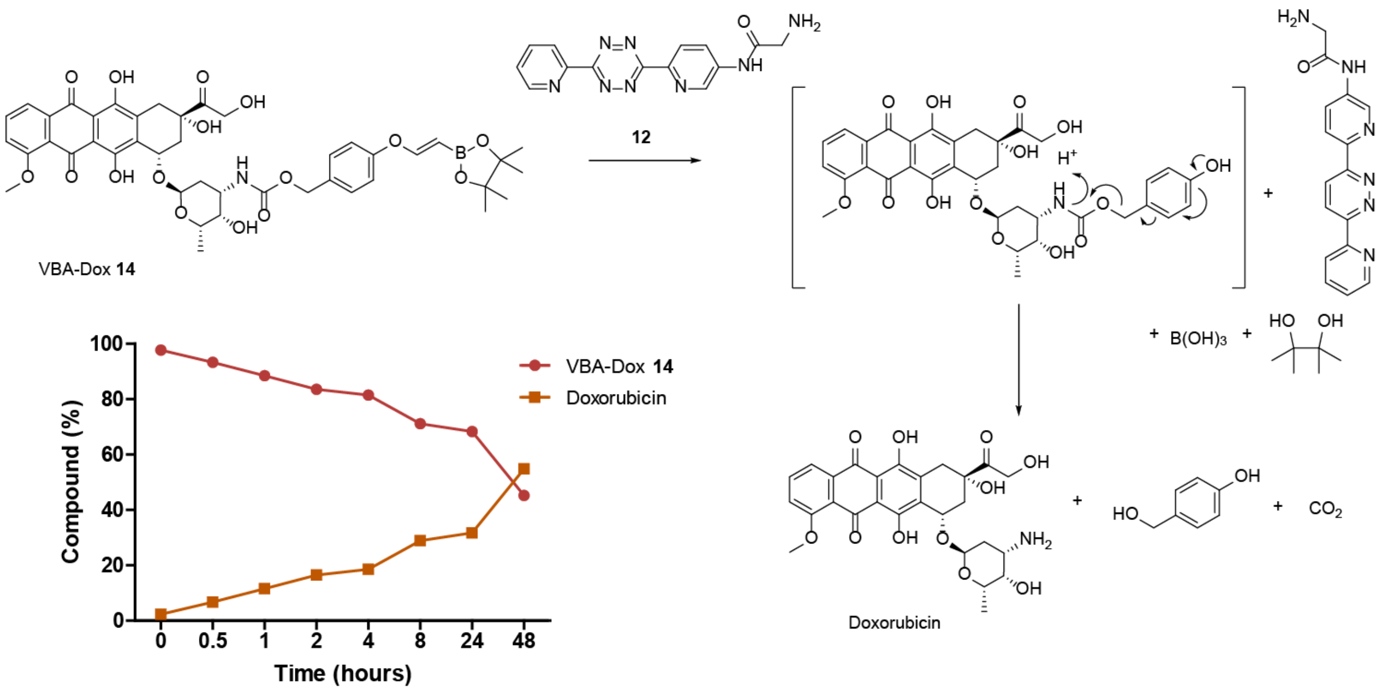


Figure SI-4. Analysis of click-to-release with 0.1 mM VBA-Dox **14** with tetrazine **12** at 37 °C in PBS and analyzed at different time points by LCMS.

Supporting Information Figure 5

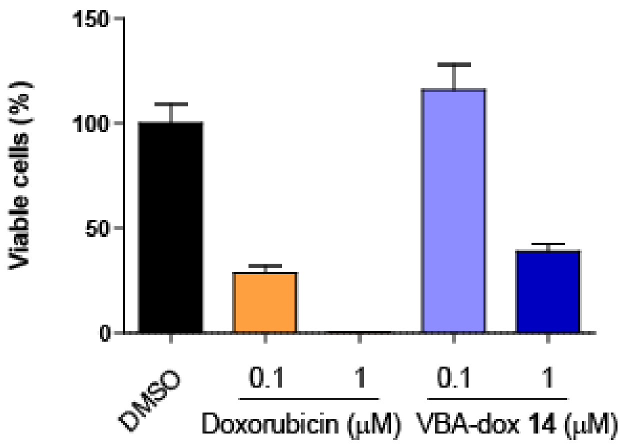
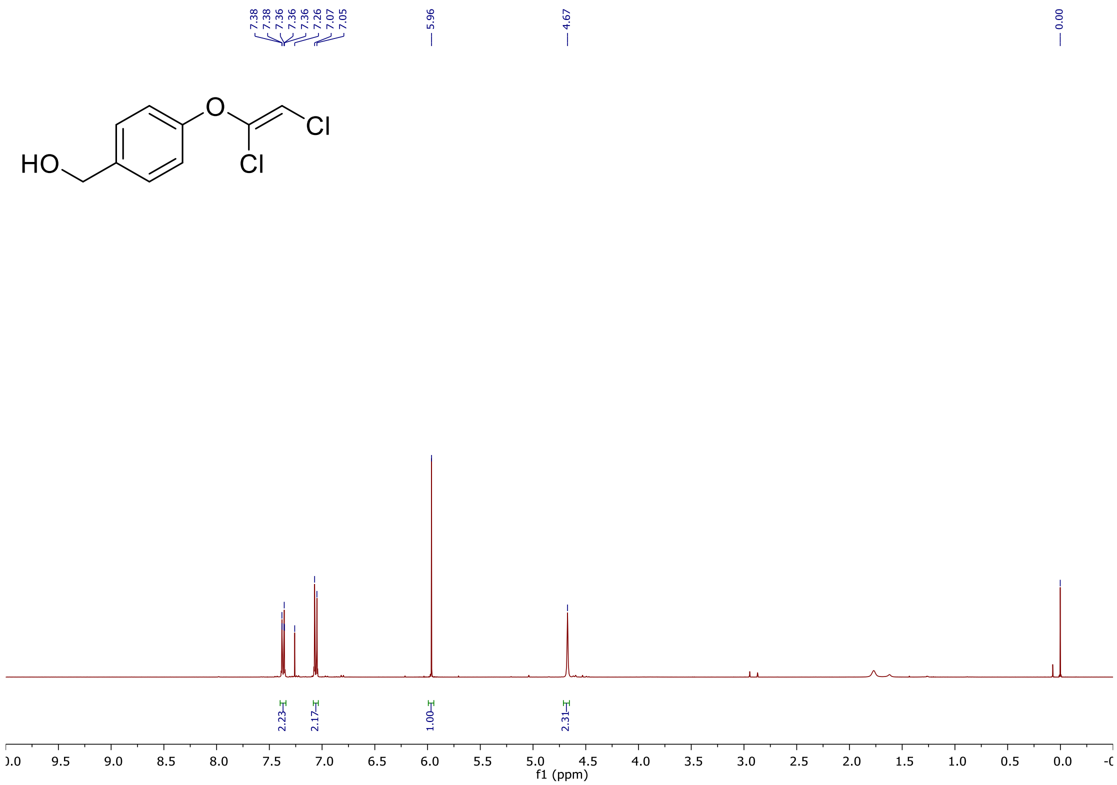


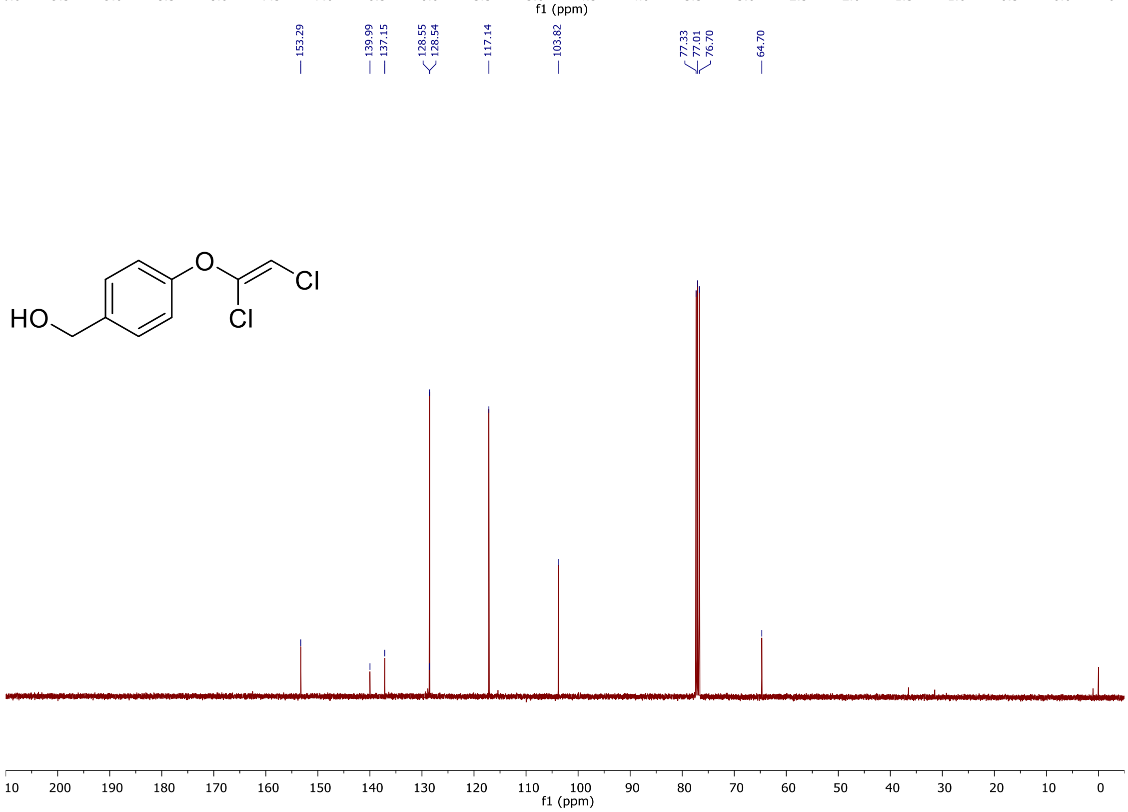
Figure SI-5. A) Cell viability of 0.1 and 1 μM of doxorubicin and VBA-doxorubicin **14** after 3 days at 37 °C. B) Cell viability of 1, 10 and 100 μM of water soluble tetrazine **12** after 3 days at 37 °C. The highest concentrations of VBA-Dox **14** and tetrazine **12** showed significant toxicity and were not used in our toxicity studies to uncage VBA-Dox **14**.

References

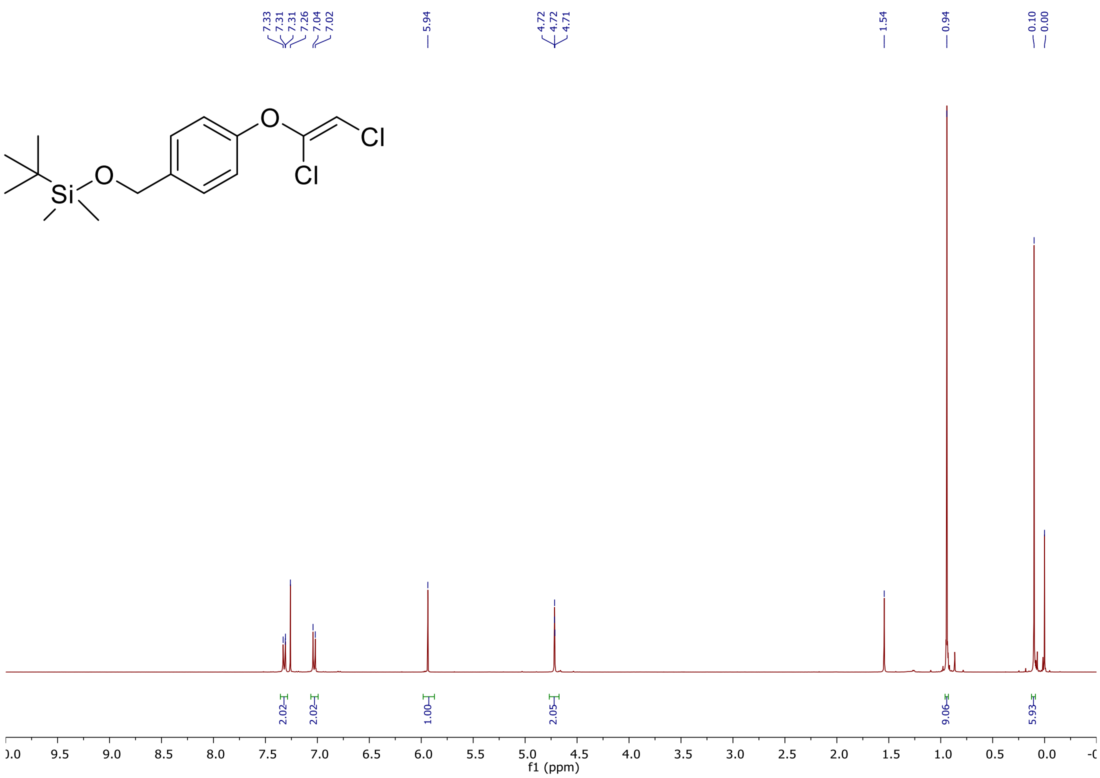
1. J. Jiang, W. Liu, J. Cheng, L. Yang, H. Jiang, D. Baib, W. Liu, A sensitive colorimetric and ratiometric fluorescent probe for mercury species in aqueous solution and living cells. *Chem. Commun.,* 2012, **48**, 8371–8373.
2. K. Lang, L. Davis, L.; J. Torres-Kolbus, C. Chou, A. Deiters, J. W. Chin, Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. *Nat. Chem.* 2012, **4**, 298-304.
3. S. Eising, F. Lelivelt, K. M. Bonger, Vinylboronic Acids as Fast Reacting, Synthetically Accessible, and Stable Bioorthogonal Reactants in the Carboni–Lindsey Reaction. *Angew. Chem. Int. Ed.* 2016, **55**, 12243-12247.

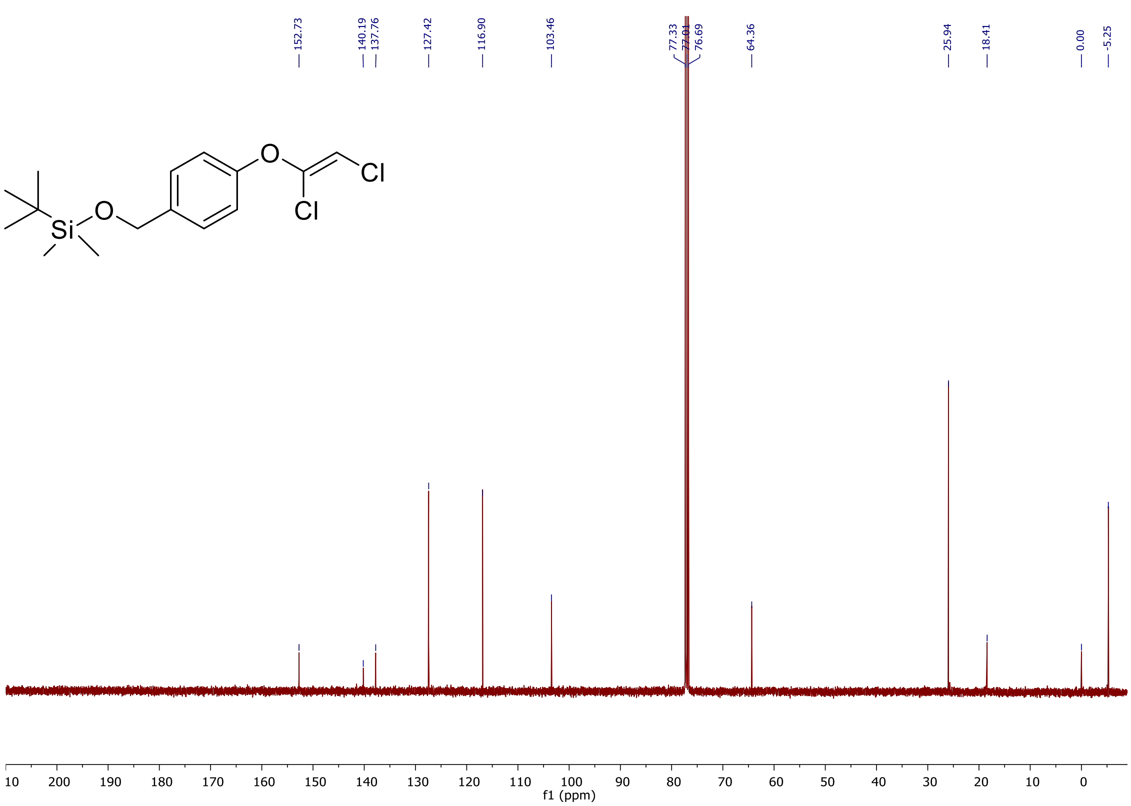
Copies of ^{1}H and ^{13}C NMR spectra of (Z)-(4-((1,2-Dichlorovinyl)oxy)phenyl)methanol (**2**).



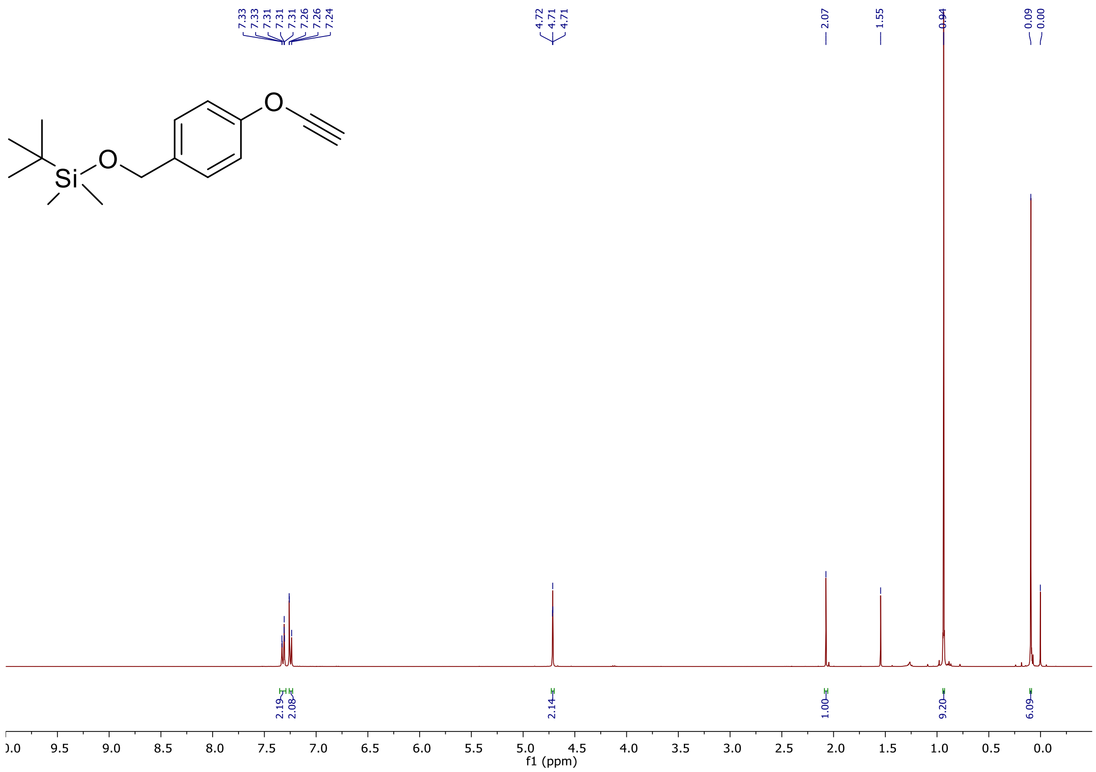


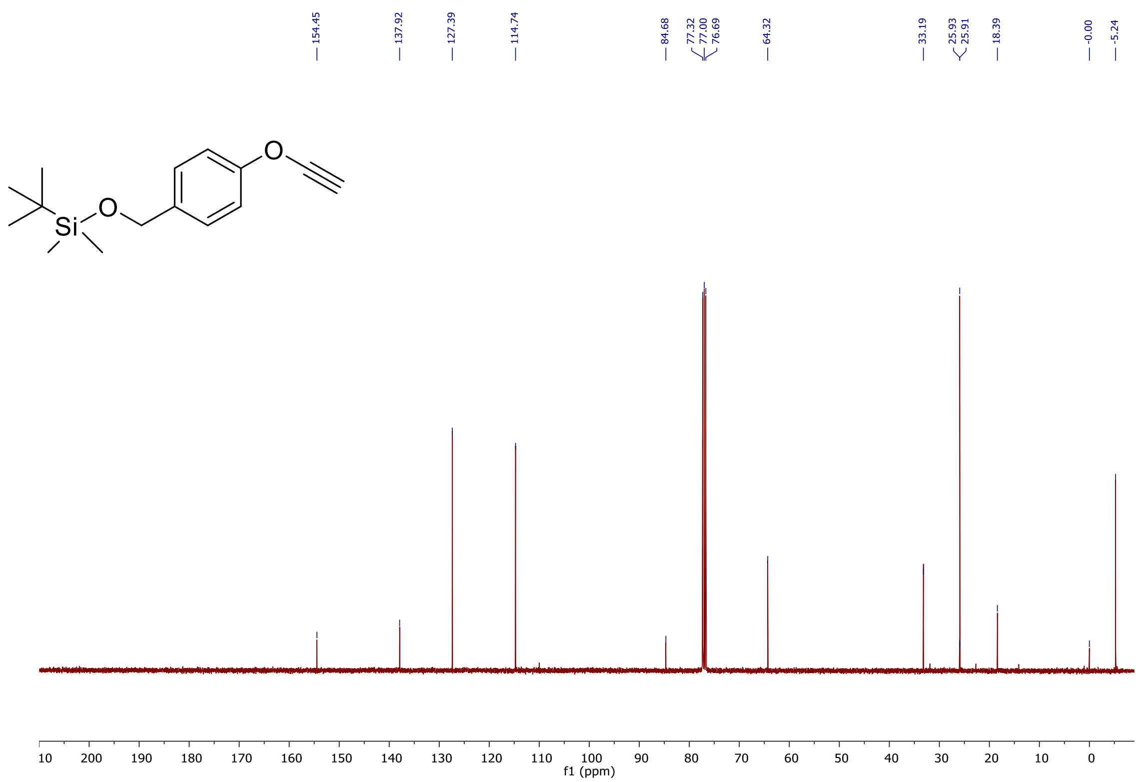
Copies of ^{1}H and ^{13}C NMR spectra of (Z)¬-tert-Butyl((4-((1,2-dichlorovinyl)oxy)benzyl)oxy)dimethylsilane (**3**).



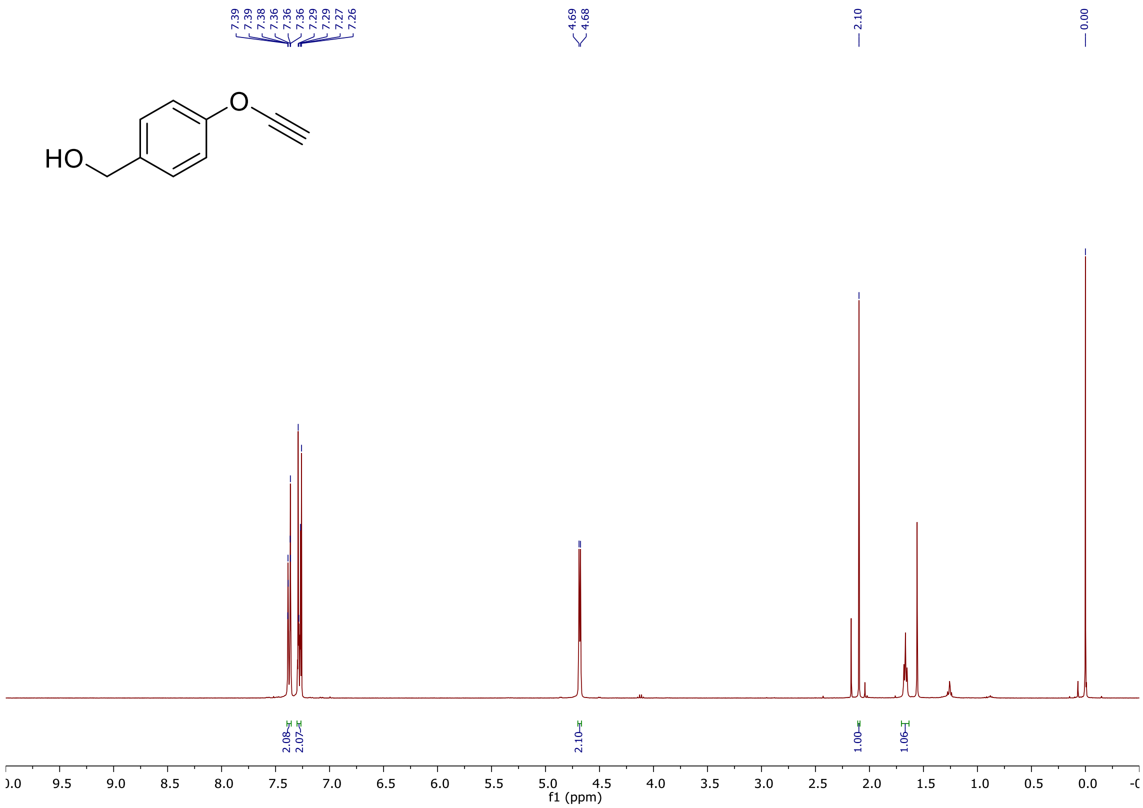


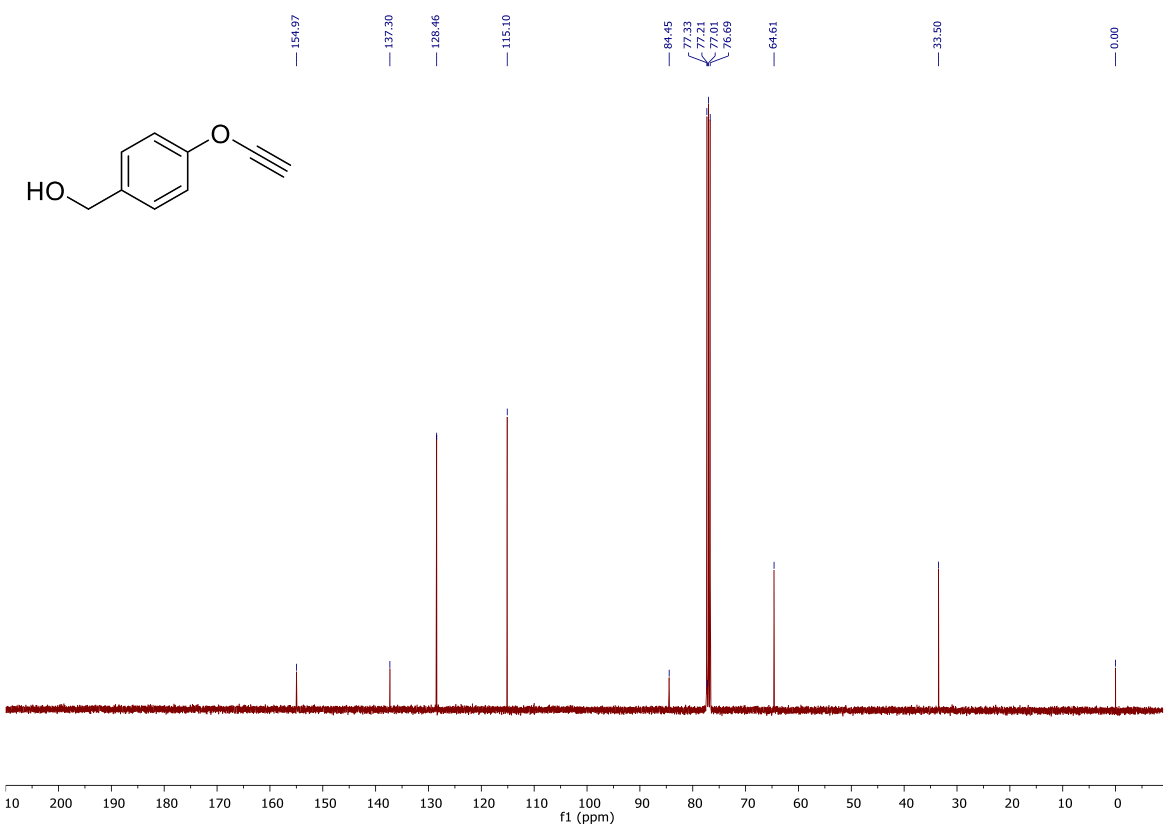
Copies of ^{1}H and ^{13}C NMR spectra of tert-Butyl((4-(ethynyloxy)benzyl)oxy)dimethylsilane (**4**).



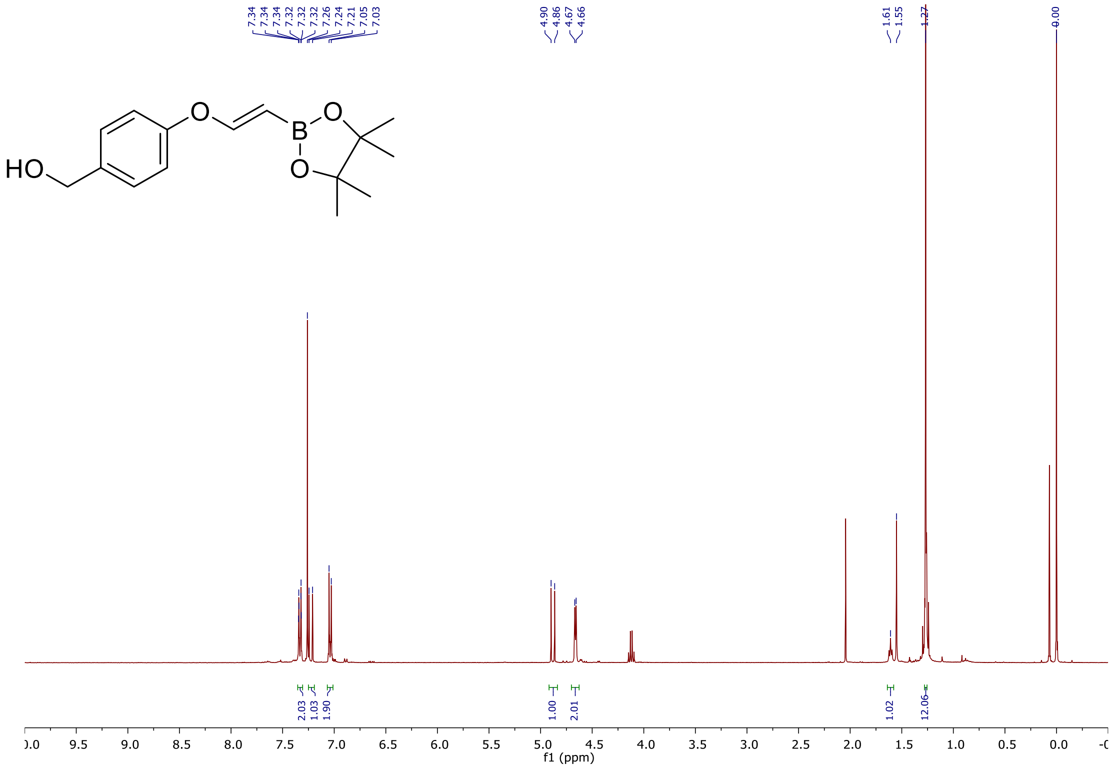


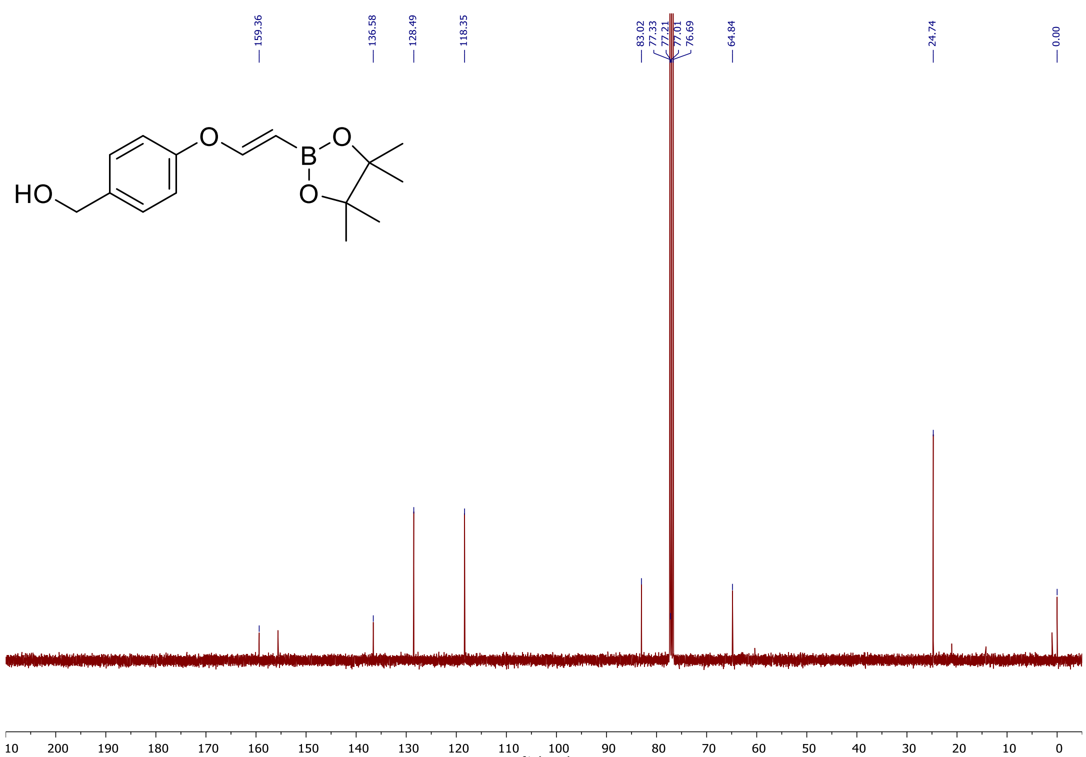
Copies of ^{1}H and ^{13}C NMR spectra of (4-(Ethynyloxy)phenyl)methanol (**5**).

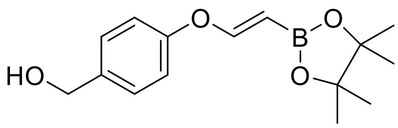


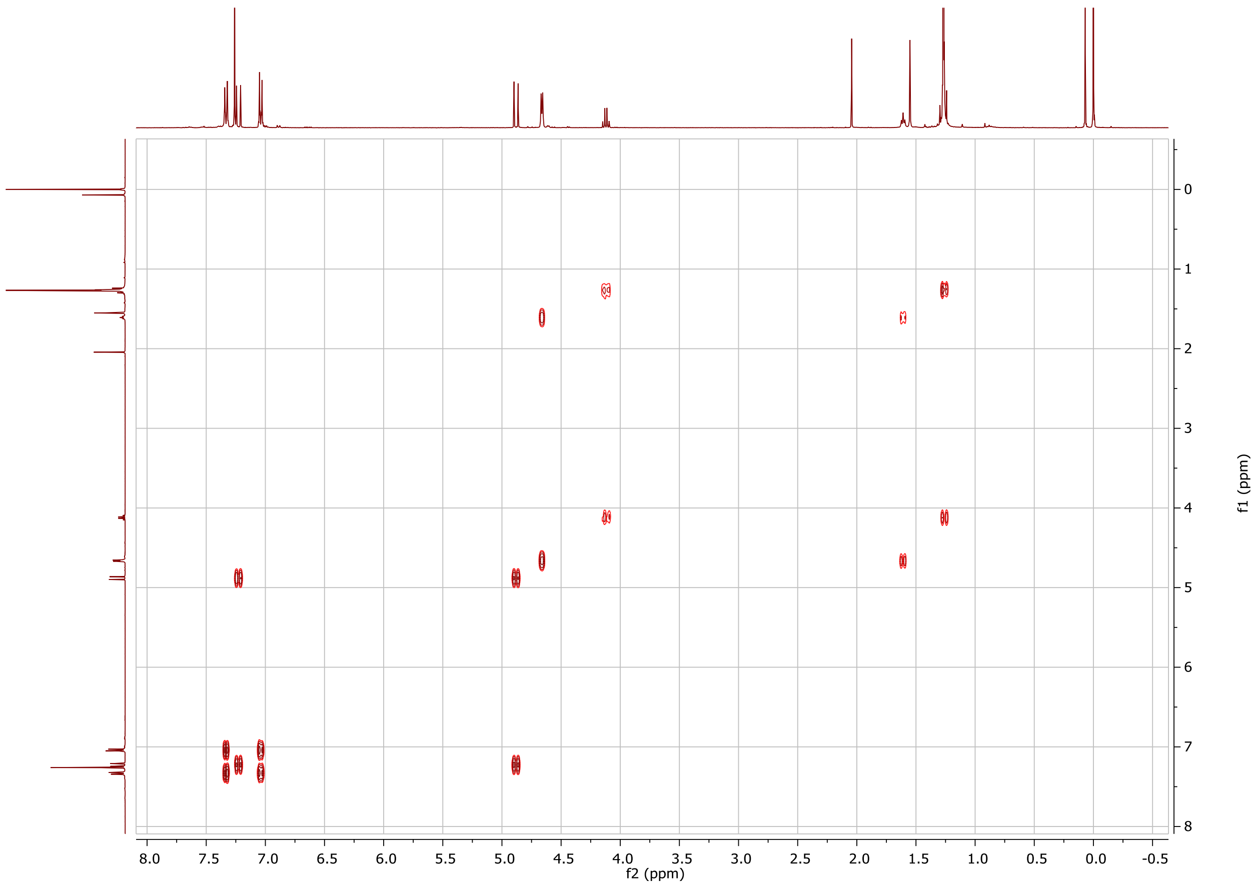


Copies of ^{1}H, ^{13}C and ^{1}H COZY NMR spectra of (E)-(2-(4-(Hydroxymethyl)phenoxy)vinyl)boronic acid pinacol ester (**6**).

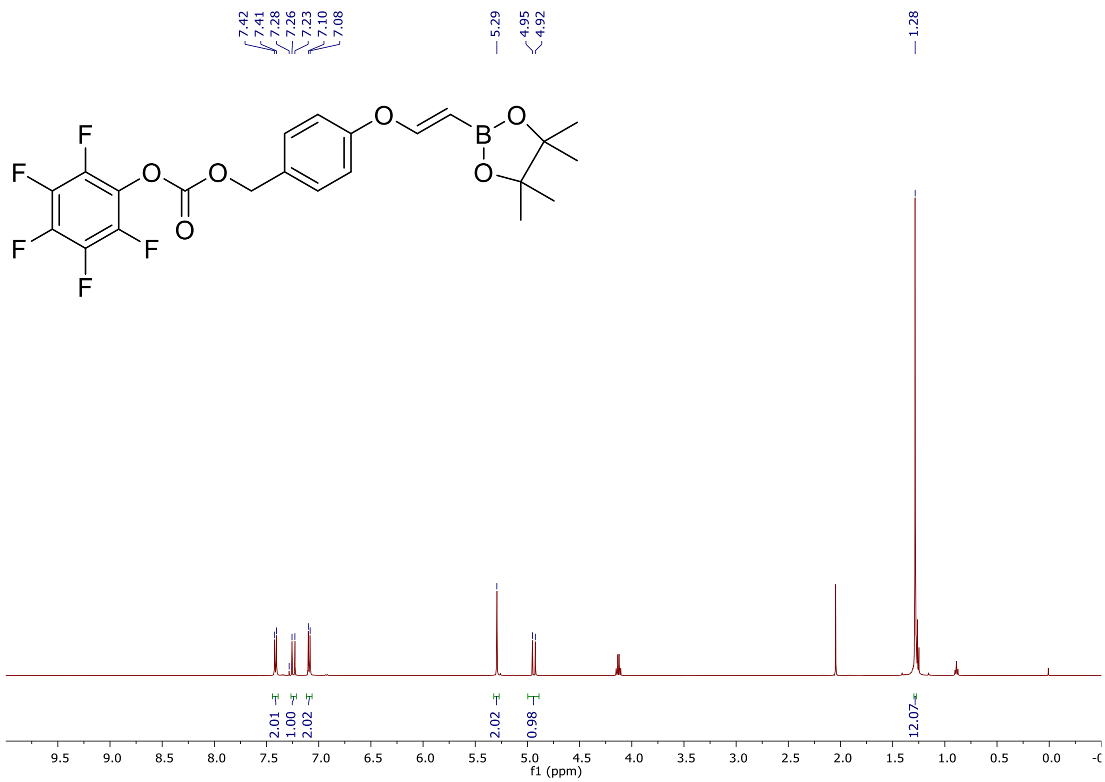


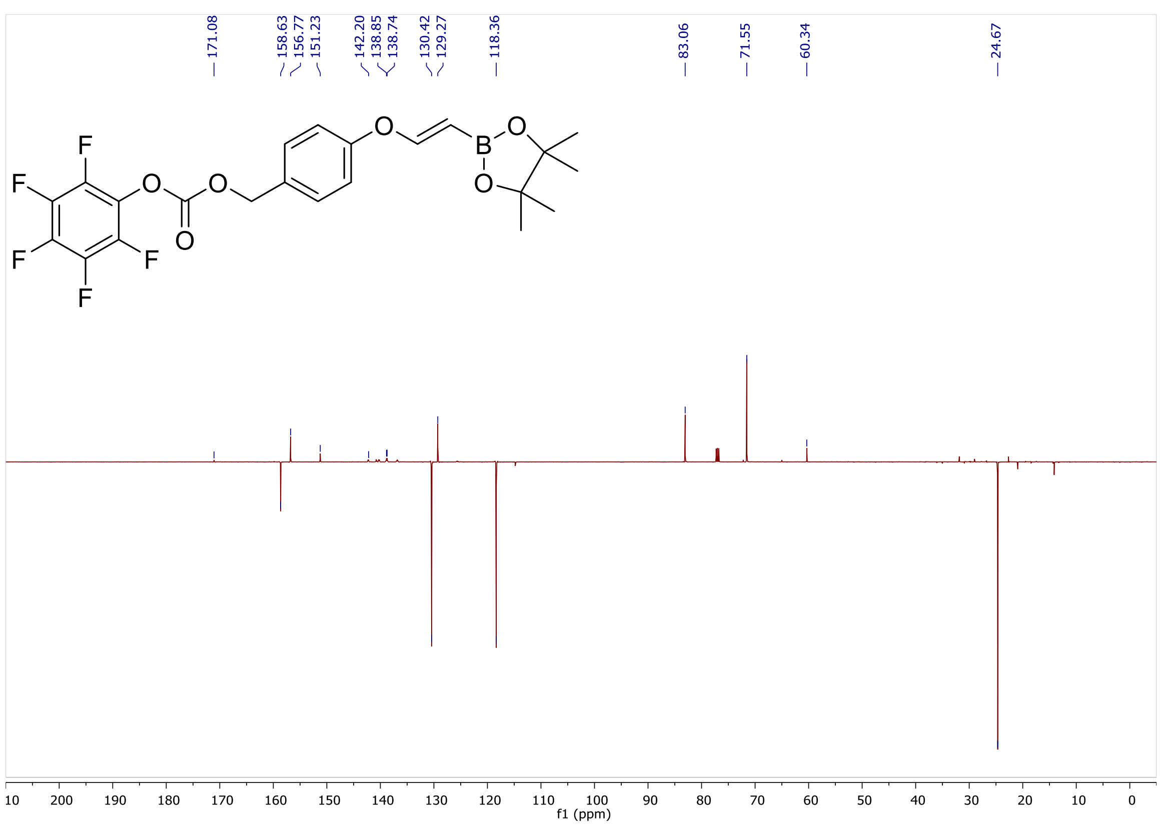



HO
O
B
O
O

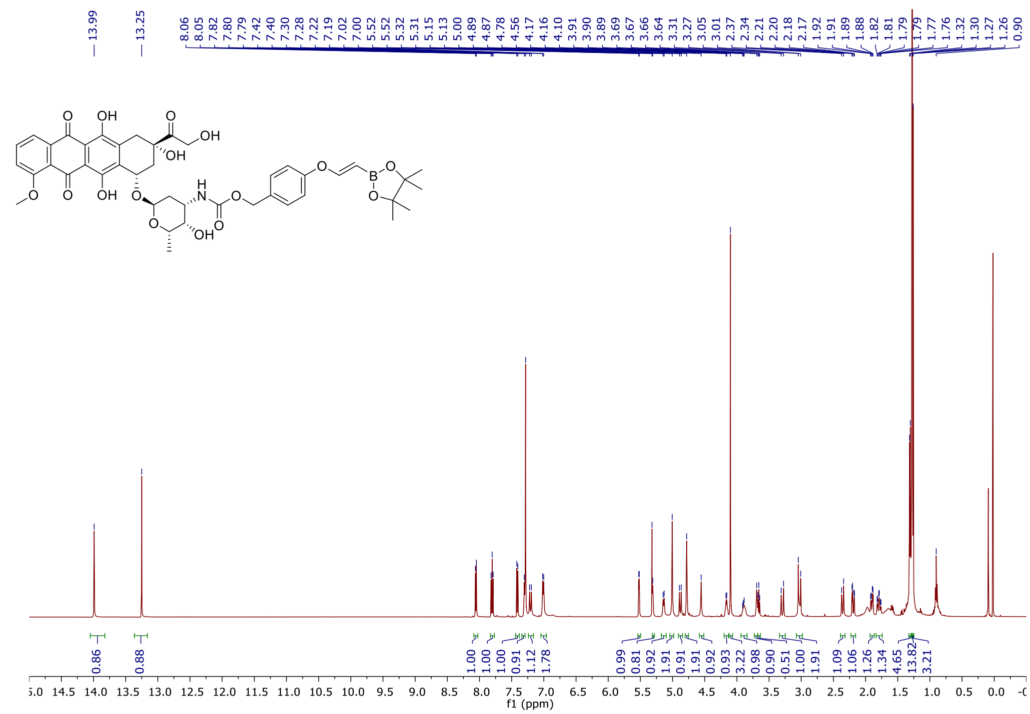

8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f2 (ppm)
0
1
2
3
4
5
6
7
8
f1 (ppm)

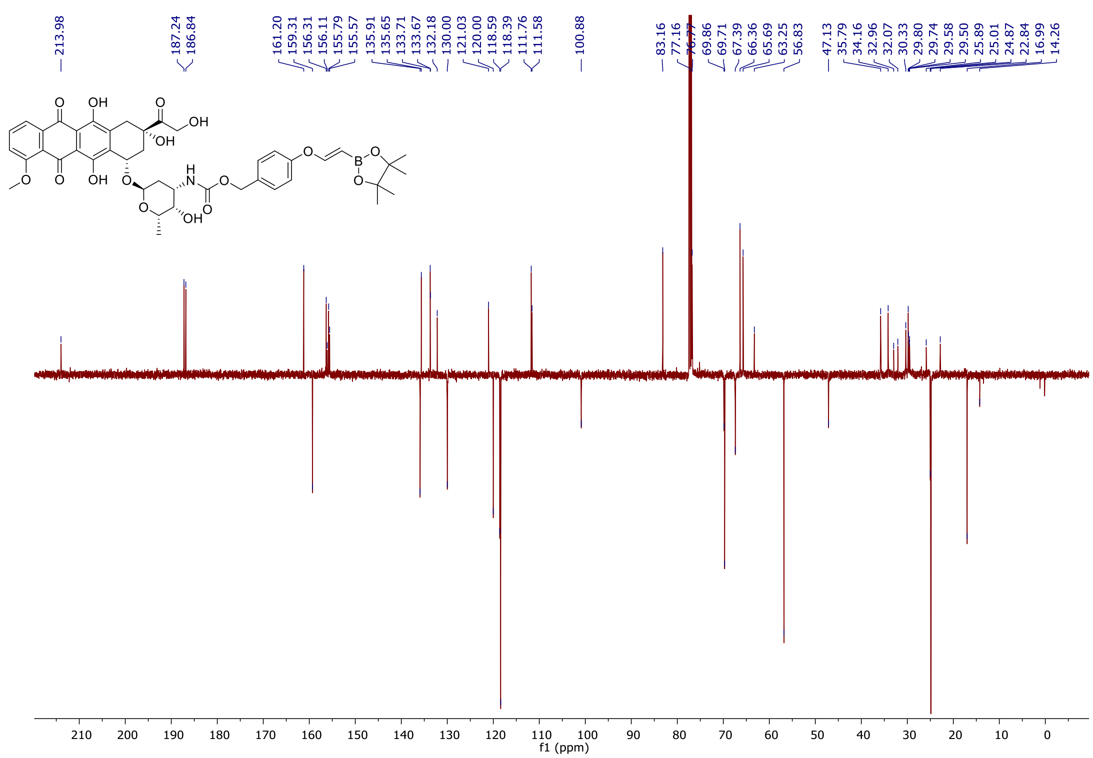
Copies of ^{1}H and ^{13}C NMR spectra of (E)-(2-(4-(Perfluorophenyl carbonate)phenoxy)vinyl)boronic acid pinacol ester (**13**).

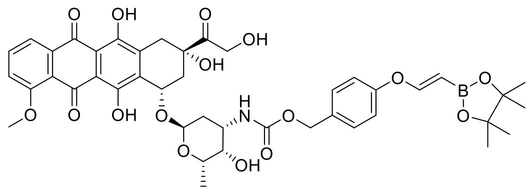


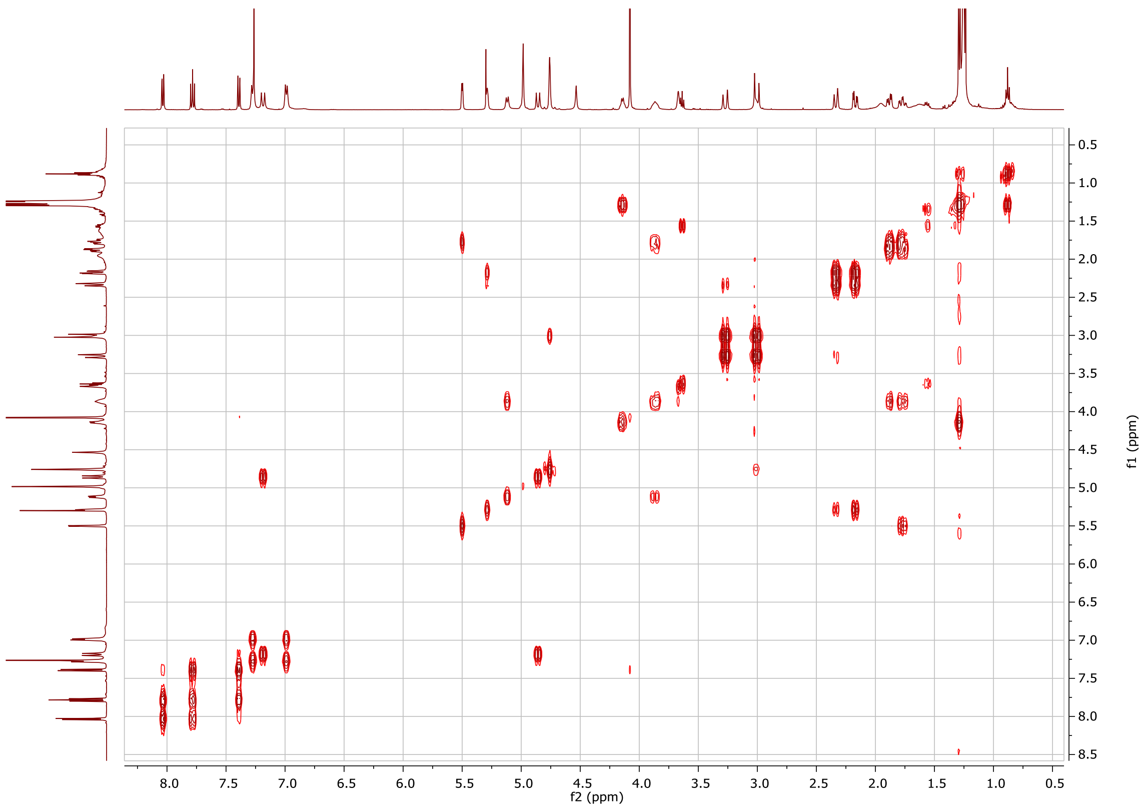


Copies of ^{1}H, ^{13}C APT and ^{1}H COZY NMR spectra of (E)-(2-(4-(Doxorubicin carbamate)phenoxy)vinyl)boronic acid pinacol ester (**14**).









f2 (ppm)
f1 (ppm)