Supporting information for

Dichloromethanol but not difluoromethanol as a viable surrogate of carbon monoxide for prodrug design

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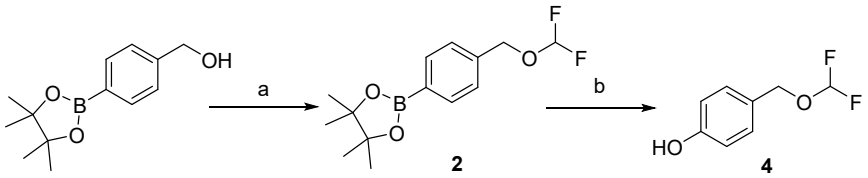
1. Chemistry

General information: All reagents and solvents were of reagent grade. Column chromatography was carried out using flash silica gel (Sorbent 200-300 mesh). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded on 300, 400 or 600 MHz spectrometers. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively, using the respective solvent (^{1}H NMR, ^{13}C NMR) as the internal reference. Compound **1** was purchased from Tokyo Chemical Industry. CO probe **1-AC** was prepared according to literature procedures [1].



Scheme S1. The synthesis of the compound **2** and **4**. Reagents and conditions: a) (Bromodifluoro methyl)trim-ethylsilane, KOAc, DCM/H_2O, rt, 24 h. b) H_2O_2, CH_3CN/PBS, 37 ^{o}C.

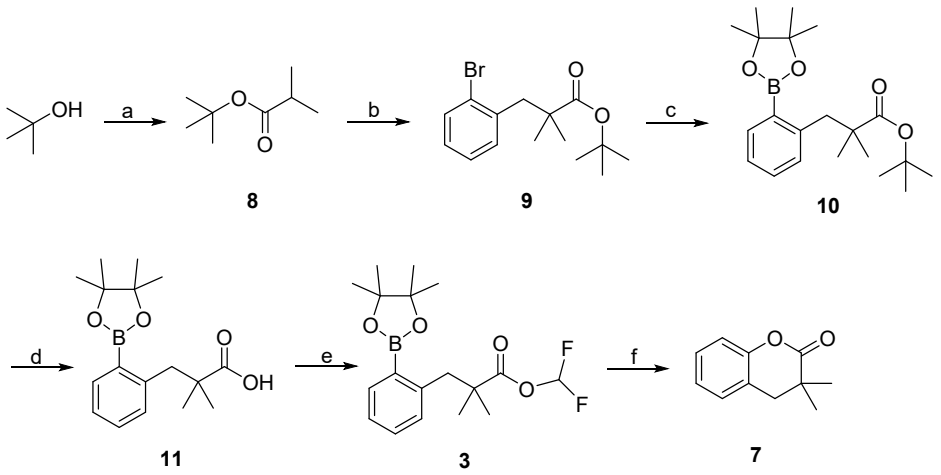
The synthesis of compound 2

To a solution of 4-(Hydroxymethyl)phenylboronic acid pinacol ester (100 mg, 0.427 mmol, 1 eq.) in DCM (0.5 mL) was added a solution of difluorobromomethyltrimethylsilane (173 mg, 0.854 mmol, 2 eq.)) dropwisely at room temperature. Then KOAc (167 mg, 1.708 mmol, 4 eq.) dissolved in deionized water (0.5 mL) was added to the reaction mixture. The obtained reaction mixture was vigorously stirred at room temperature for 24 h, extracted with dichloromethane (10 mL × 3), washed with saturated brine (10 mL × 3), the obtained organic phase was dried over anhydrous sodium sulfate. After filtration and concentration, the crude product was purified by column chromatography (petroleum ether: ethyl acetate = 20:1, v/v) to yield the title compound as a white solid (85 mg, yield: 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, J = 8.1 Hz, 2H), 7.36 (d, J = 8.1 Hz, 2H), 6.30 (t, J = 74.4 Hz, 1H), 4.91 (s, 2H), 1.35 (s, 12H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.5, 135.2, 127.1, 116.0 (t, J = 261.1 Hz), 84.0, 65.3 (t, J = 6.1 Hz), 25.0. ^{19}F NMR (563 MHz, $CDCl_3$) δ -84.34 (d, J = 74.4 Hz). HRMS (EI) m/z calculated for $C_{14}H_{19}BF_2O_3$ [M]: 284.1395; found: 284.1394.

The synthesis of compound 4

To a solution of Compound **2** (50 mg, 0.176 mmol, 1 eq.) in CH_3CN/PBS (v/v = 2:1, 3 mL) was added H_2O_2 (200 μL, wt% = 30%) at 37 ^{o}C and stirred for 30 min. The reaction solution was extracted with EA (10 mL × 3), the organic phases were combined, dried over anhydrous sodium sulfate, filtered and concentrated, and column chromatography (PE: EA = 10:1) gave the product **4** as a colorless oil (28 mg, yield: 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (d, J = 8.5 Hz, 2H), 6.88 –

6.78 (m, 2H), 6.27 (t, J = 74.6 Hz, 1H), 5.04 (s, 1H), 4.81 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.9, 130.2, 127.7, 115.6, 116.0 (t, J = 261.1 Hz), 65.3 (t, J = 6.1 Hz). ^{19}F NMR (376 MHz, $CDCl_3$) δ -84.03 (d, J = 74.7 Hz). HRMS (EI) m/z calculated for $C_8H_8F_2O_2$ [M]: 174.0492; found: 174.0490.



Scheme S2. The synthesis of the compound **3**. Reagents and conditions: a) Isobutyryl chloride, TEA, 4-DMAP, DCM, 0 °C - rt, 12 h; b) LDA, 1-bromo-2-(bromomethyl)benzene, dry THF, -78 °C - rt, 2 h; c) Pinacol biborate, $Pd(dppf)Cl_2$, AcOK, dioxane, 80 °C, 3 h; d) CF_3COOH, 0 °C - rt, DCM, 1 h; e) (Bromodifluoromethyl)trimethylsilane, KOAc, DCM/H_2O, rt, 24 h. f) H_2O_2, THF/PBS, 0 °C - rt, 30 min.

The synthesis of compound 8

A solution of isobutyryl chloride (12 g, 103.24 mmol, 1 eq.) in anhydrous dichloromethane (85 mL) was treated consecutively with a solution of tert-butanol (12 mL) at 0 °C, followed by triethylamine (20.89 g, 206.48 mmol, 2 eq.), and 4-DMAP (63 mg, 0.516 mmol, 0.5% eq.). Then, the ice bath was removed and the mixture was stirred at room temperature overnight. The resulting reaction mixture was then treated with aq. HCl solution (2 M, 50 mL) and extracted with dichloromethane (50 mL × 3). The obtained organic phase was washed with saturated $NaHCO_3$ solution and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified by distillation under reduced pressure to yield the desired product **8** as a colorless liquid (8.43 g, yield: 57%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.49 – 2.30 (m, 1H), 1.42 (s, 9H), 1.10 (d, J = 6.9 Hz, 6H).

The synthesis of compound 9

A flame-dried round-bottom flask was charged with compound **8** (500 mg, 3.47 mmol, 1 eq.) and anhydrous THF (12 mL) under a nitrogen atmosphere. The reaction was cooled to -78 °C and then treated dropwise with 2 M *n*-butyllithium in hexanes (2.08 mL, 4.16 mmol, 1.2 eq.). After stirring for an additional hour at -78 °C, a solution of 2-bromobenzyl bromide (954 mg, 3.81 mmol, 1.1 eq.) in anhydrous tetrahydrofuran (3 mL) was added dropwise to the reaction mixture. After that, the reaction was warmed to room temperature and stirred for one hour, and then was quenched with sat. $NaHCO_3$ (50 mL). The mixture was extracted with ethyl acetate (50 mL × 3), and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified via chromatography on a silica column (PE: EA = 100: 0 ~ 98: 2) to obtain a colorless oil (761 mg,

yield: 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (d, *J* = 8.0 Hz, 1H), 7.24 (d, *J* = 1.3 Hz, 1H), 7.19 (t, *J* = 7.4 Hz, 1H), 7.09 – 7.01 (m, 1H), 3.10 (s, 2H), 1.45 (s, 9H), 1.18 (s, 6H).

The synthesis of compound 10

A solution of compound **9** (1 g, 3.19 mmol, 1 eq.), pinacol biborate (980 mg, 3.86 mmol, 1.21 eq.), Pd(dppf)Cl_2 (233 mg, 0.319 mmol, 0.1 eq.) and KOAc (783 mg, 7.98 mmol, 2.5 eq.) in dry 1,4-dioxane (15 mL) was heated at 135 ºC for 3 h under nitrogen atmosphere. After the reaction reached completion, the reaction was cooled to room temperature and concentrated under reduced pressure. The obtained residue was then dissolved in PE: EA (v/v = 85:15), and filtered on a silica gel plate. The filtrate was concentrated, and the residue was purified via chromatography on a silica column (PE: EA = 100: 1) to obtain the title compound a colorless oil **10** (543 mg, 47%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 – 7.72 (m, 1H), 7.34 – 7.28 (m, 1H), 7.24 – 7.14 (m, 2H), 3.26 (s, 2H), 1.44 (s, 9H), 1.34 (s, 12H), 1.07 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.6, 145, 135.8, 130.6, 130.4, 125.5, 83.6, 79.9, 44.2, 43.3, 28.2, 25.1, 24.9. HRMS (ESI): calculated for $C_{21}H_{33}BO_4Na$ $[M + Na]^+$: 383.2370; found: 383.2372.

The synthesis of compound 11

To a solution of compound **10** (500 mg, 1.39 mmol, 1 eq.) in DCM (10 mL) was added dropwise trifluoroacetic acid (1.58 mL) at 0 ºC. The resulting mixture was stirred at room temperature for one hour. The solvent was then evaporated under reduced pressure, and product **11** was obtained as a colorless oil (375 mg, yield: 89%), which was directly used for the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 7.5 Hz, 1H), 7.36 – 7.30 (m, 1H), 7.24 – 7.18 (m, 2H), 3.33 (s, 2H), 1.35 (s, 12H), 1.17 (s, 6H).

The synthesis of compound 3

Compound **11** (500 mg, 1.39 mmol, 1 eq.) was dissolved in a mixed solution of dichloromethane/water (v/v = 1:1, 1.5 mL/1.5 mL, 3 mL), followed by the addition of potassium acetate (500 mg, 3.62 mmol, 2.2 eq.) and difluorobromomethyltrimethylsilane (511 μL, 3.29 mmol, 2 eq.). The obtained mixture was stirred vigorously at room temperature for 24 h. The reaction solution was extracted with DCM (10 mL × 3), and the combined organic phase was dried over anhydrous sodium sulfate. After filtration and concentration, the crude product was purified by column chromatography (PE: EA = 100: 1) to yield **3** as a colorless oil (14 mg, yield: 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (dd, *J* = 7.5, 1.4 Hz, 1H), 7.34 (td, *J* = 7.5, 1.6 Hz, 1H), 7.23 (td, *J* = 7.4, 1.2 Hz, 1H), 7.12 (d, *J* = 7.7 Hz, 1H), 7.25 – 6.89 (t, *J* = 71.4 Hz, 1H), 3.35 (s, 2H), 1.35 (s, 12H), 1.19 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.1 (t, *J* = 2.4 Hz), 143.2, 136.2, 130.7, 130.2, 126.0, 112.8 (t, *J* = 257.0 Hz), 83.7, 44.0, 42.7, 24.9, 24.0. ^{19}F NMR (563 MHz, $CDCl_3$) δ -91.94 (d, *J* = 71.2 Hz). HRMS (ESI): calculated for $C_{18}H_{25}BF_2O_4Na$ $[M + Na]^+$: 377.1712; found: 377.1715.

The synthesis of compound 7

Compound **3** (50 mg, 0.141 mmol, 1 eq.) was dissolved in tetrahydrofuran (2 mL) and PBS buffer (2 mL, pH 7.4), and 30% hydrogen peroxide solution (79.6 μL, 1.41 mmol, 1 eq.) was added. The solution was then heated to 37 °C and stirred for 30 min. The mixture was then diluted and extracted with dichloromethane, and the organic phase was washed with deionized water, dried over

anhydrous sodium sulfate, and concentrated under reduced pressure. Column chromatography (PE: EA = 2: 1) gave product **7** as a white solid (21 mg, yield: 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 – 7.22 (m, 1H), 7.15 (d, *J* = 6.2 Hz, 1H), 7.09 (td, *J* = 7.4, 1.1 Hz, 1H), 7.02 (dd, *J* = 8.1, 0.7 Hz, 1H), 2.85 (s, 2H), 1.29 (s, 6H).

2. CO release quantification by the CO-meter

The compound **1/2/3** (50 μM) was dissolved in 5% CH_3CN/PBS (50 mL) in a 70 mL flask equipped with a CO-meter. Then, H_2O_2 (1 mM) was added to the mixture and the CO released was recorded over 2 h at 37 °C. Each experiment was repeated three times independently.

3. CO-myoglobin assay

The two-compartment CO-myoglobin assay was employed [2]. The inner vial was for the solution of **1** (20 mM) in CH_3CN/PBS (v/v = 9: 1, 2 mL), and the bigger outer vial contained the deoxy-Mb solution. Specifically, a myoglobin solution in PBS (0.5 mg/mL) was degassed by bubbling with nitrogen for 30 min. To this degassed solution was added a freshly prepared solution of sodium dithionite (0.1%) to afford the deoxy-Mb solution. Then, H_2O_2 (8 μL, 40 mM, w% = 30%) was added to the inner vial mixture (the experimental group) and for the control group, an equal volume of PBS was added instead. Next, the solution was put into a 2-8 °C refrigerator overnight to increase the solubility of CO in the water. Subsequently, UV-Vis spectra of the deoxy-Mb solution were taken.

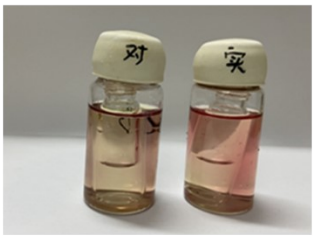


Figure **S1**: a) The "two-compartment" Mb-CO assay. Right: the experimental group; Left: the control group.

4. CO release quantification

The CO release from compound **1** was indirectly quantified by using a previous reported CO prodrug **BW-CO-103**[2] as standard, which was reported to release one equivalent of CO in a mixed aqueous solution. Specifically, H_2O_2 (1 mM) triggered CO release from compound **1** (50 μM) was recorded in 30% CH_3CN/PBS (v/v = 3:7, 50 mL) in a 70 mL flask equipped with a CO-meter, and CO release from **BW-CO-103** (50 μM) under the same conditions (without H_2O_2) was also

recorded. CO release efficiency from compound **1** was then roughly calculated by comparing the peak CO concentration of compound **1** with that of **BW-CO-103**. Each experiment was repeated three times independently.

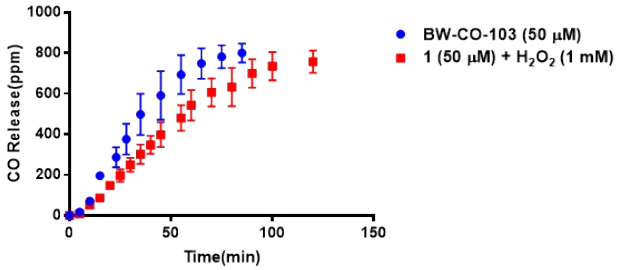


Figure S2. The CO-released quantity of compound **1** (50 μM) in the presence of H_2O_2 (1mM), as compared with **BW-CO-103** (50 μM) in 30% CH_3CN/PBS.

5. CO release specificity of 1 in response to various ROS and endogenously found species

Various ROS or endogenously found species (1 mM) and **1** (50 μM) was dissolved in 5% CH_3CN/PBS (50 mL) in a 70 mL flask equipped with a CO-meter. The resulting mixture was incubated at 37 °C for 2 h, and the CO release was quantified by the CO-meter. Each experiment was triplicated independently.

H_2O_2, tert butyl hydrogen peroxide (TBHP), hypochlorite (ClO^-), hydroxyl radical ($HO\cdot$), tert-butyloxy radical ($tBuO\cdot$), oxidized glutathione (GSSG), reduced glutathione (GSH), cysteine (Cys), nitrite (NO_2^-), peroxynitrite ($ONOO^-$) was prepared according to literature procedures [3]. The protocol for the preparation of other ROS was detailed below.

Hydroxyl radical ($HO\cdot$) solution (1 mM): $FeSO_4$ (37.89 mg) and H_2O_2 (5 μL, wt% = 30%) were dissolved in PBS (47.5 mL), and the resulting mixture was incubated at 37 °C for 30 min for later use [4].

tert-Butoxyl free radical ($t\text{-}BuO\cdot$) solution (1 mM): $FeSO_4$ (37.89 mg) and t-BuOOH (6.9 μL, wt%= 70%) were dissolved in PBS (47.5 mL), and the resulting mixture was incubated 37 °C for 1 h for later use [4].

Singlet oxygen ($^1O_2$) (200 mM): NaOH (40 mg), H_2O_2 (200 μL, wt% = 30%) and $Na_2MoO_4\cdot 7H_2O$ (24.2 mg) were dissolved in PBS (10 mL), and the resulting mixture was incubated at 37 °C for one hour for later use [5].

Superoxide ($O_2^{\bullet-}$) (5 mM): Allopurinol (27.2 mg) and xanthine oxidase (0.1 mg, 8.6 U/mg) were dissolved in PBS (10 mL), and the resulting mixture was incubated at 37 °C for 30 min for later use [6].

6. The decomposition kinetics of 3 and 4

A solution of compound **4** (500 μM) in 50% of CH_3CN in PBS (pH = 7.4) was incubated at 37 °C. An aliquot of 50 μL reaction mixture was taken out and analyzed by HPLC at intervals to monitor the formation of compound **5**. The results were summarized in Figure **S3**.

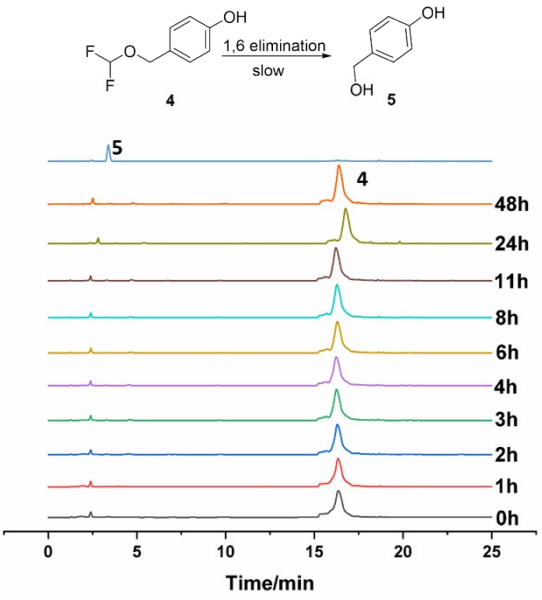


Figure S3. The HPLC trace at different time points. The analysis was performed using the Shimadzu Prominence UFLC with a reversed-phase analytical column (Waters C18 5 μm, 4.6 x 250 mm) at 25 °C. The flow rate was set at 1 mL/min. Gradient elution with acetonitrile and deionized water (0 -10 min, 40% acetonitrile; 10 - 25 min, 100% acetonitrile) was used. Detection wavelength: 254 nm. Injection sample volume: 50 μL.

A solution of compound **3** (500 μM) with H_2O_2 (10 mM) in 50% CH_3CN/PBS (pH = 7.4) was incubated at 37 °C. An aliquot of 50 μL reaction mixture was taken out and analyzed by HPLC at intervals to monitor the formation of the lactone compound **7**. The results were summarized in Figure **S4**. The peak areas of compound **7** was plotted against time, and the formed curve was fitted using GraphPad Prism to give the first order reaction kinetics of the lactonization step (Figure **S4**).

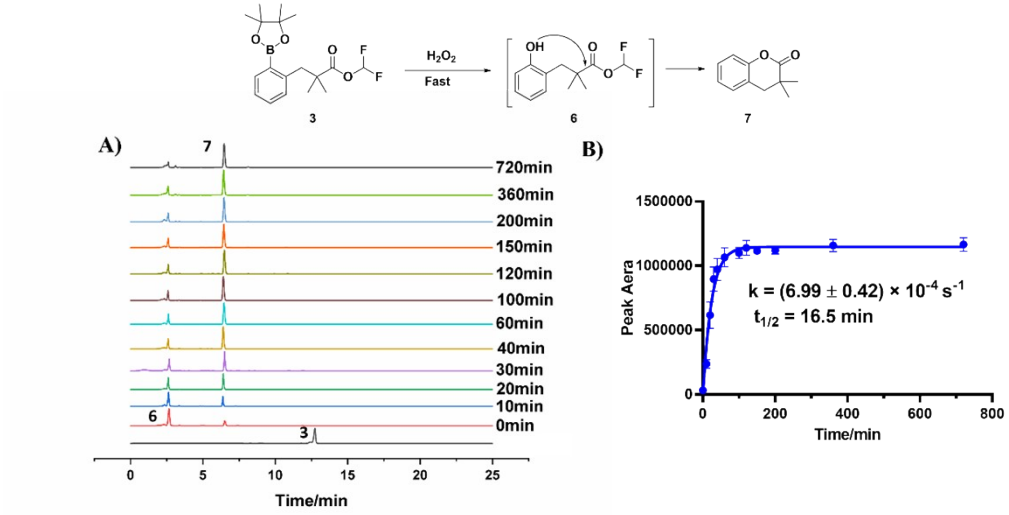


Figure **S4.** The decomposition kinetics of compound **3** in the presence of H_2O_2. A) The HPLC trace at different time points at 37 °C. B) The first-order reaction kinetics of the lactonization step. The analysis was performed using the Shimadzu Prominence UFLC with a reversed-phase analytical column (Waters C18 5 µm, 4.6 x 100 mm) at 25 °C. The flow rate was set at 1 mL/min. Gradient elution with acetonitrile and deionized water (0 - 25 min, 70% acetonitrile) was used. Detection wavelength: 254 nm. Injection sample volume: 50 µL.

7. Cell imaging studies

7.1. Imaging studies of ROS levels in 4T1 cells

4T1 cells were cultured in 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37 °C. The cells are seeded into a 6-well plate for 24 h, after which the cells were treated with either beta-lapachone (2 µM) or vehicle control for 6 h. Then, a ROS fluorescent probe 2 ', 7' - dichlorofluorescein - 3 ', 6' - diacetate (5 µM) was added and the cells were incubated at 37 °C with 5% CO_2 for another 2 h. Next, the live cells were imaged under the green channel using a Nikon fluorescent microscope, and the results were summarized in Figure **S5**.

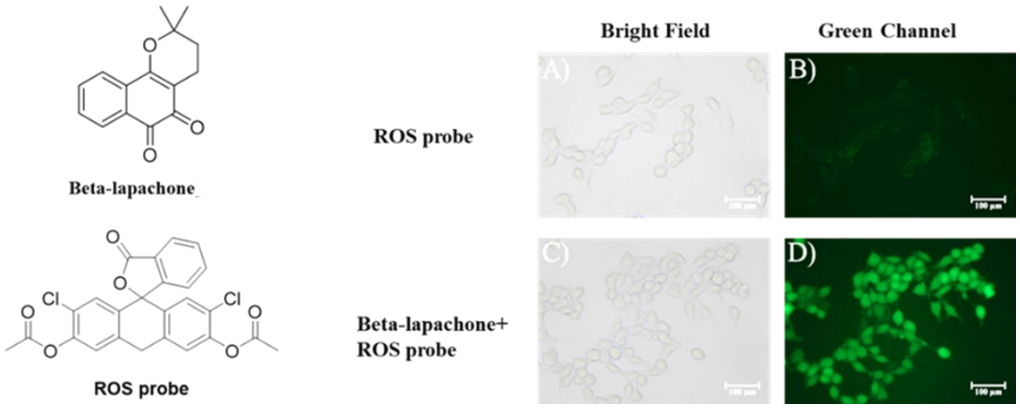


Figure **S5**. The cell imaging studies in 4T1 cells. The cells were treated with ROS probe (5 µM, A-B), beta-lapachone (2 µM) + ROS probe (5 µM, C-D). The live cells were imaged under the FITC channel. Scale bar: 100 µm.

7.2. Imaging of intracellular CO release in 4T1 cells

4T1 cells were seeded on the 6-well plates (1×10^5 cells $well^{-1}$) and incubated overnight in RPMI 1640 containing 10% FBS. Four groups were set. The cells in the first and the second group were cultured for 6 hours without any other treatment. the cells in the third and fourth groups were treated with beta-lapachone (2 µM) for 6 h. After that, CO probe **1-Ac** (2 µM) was added to all groups, and the cells in the second and fourth groups were treated with **1** (100 µM) for 2 h. Next, the cells were washed twice with PBS, and the fluorescence images of the cells were acquired under the red channel using a Nikon laser confocal microscope.

8. Cytotoxicity assay

4T1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C with 5% CO_2. The cells were seeded in a 96-well plate and incubated for 24 h. Then, the tested compounds were added to the 96-well plate and the cells were cultured in the incubator for another 24 h. Then, the culture medium was replaced with 10% CCK8 medium, and the incubation was continued for 1-2 h. The OD value at 450 nm of each well was recorded with a microplate reader. The data were processed and the cell survival rate (%) was calculated. The results were summarized in Figure **S6**.

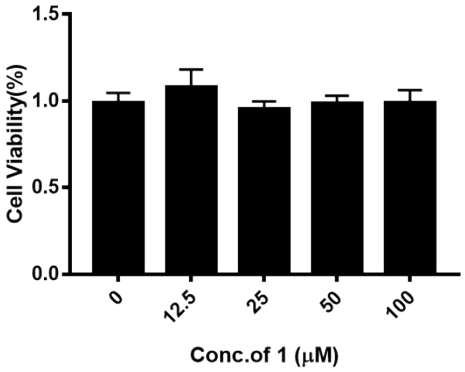
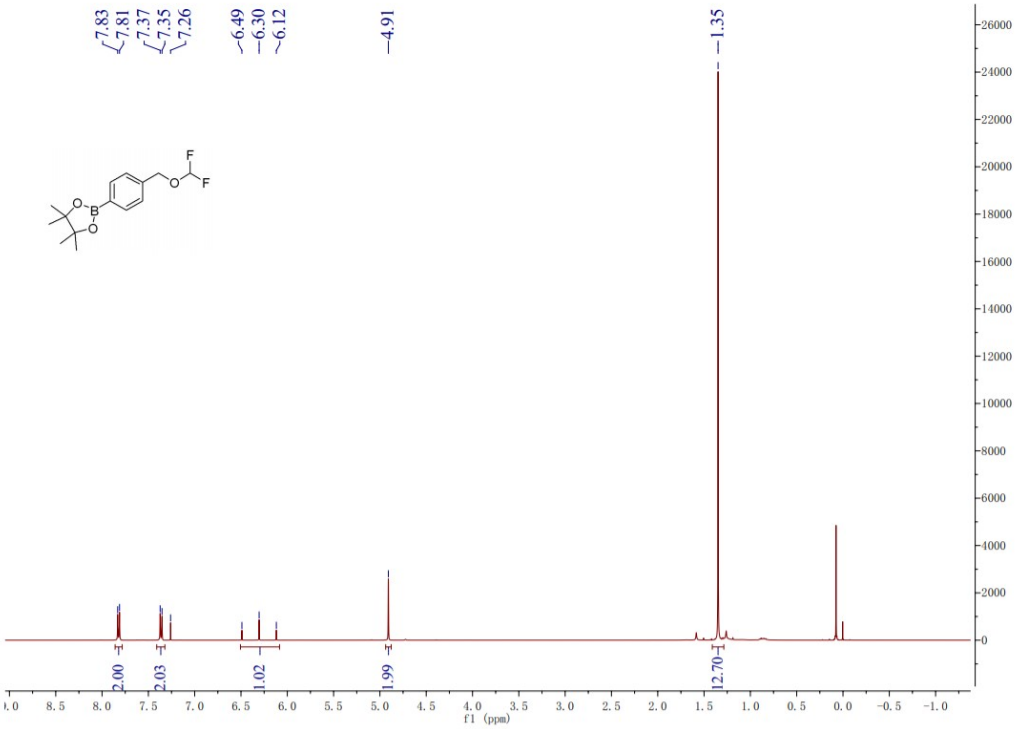
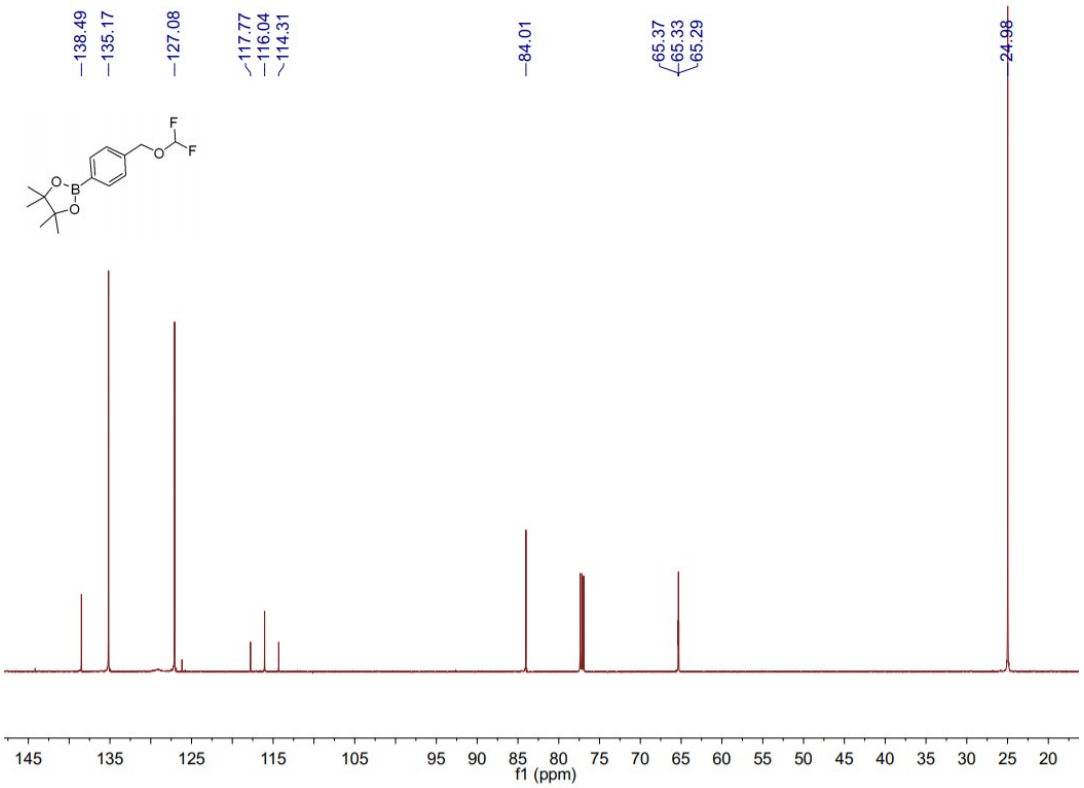


Figure **S6**. The cytotoxicity of **1** in 4T1 cells.

9. NMR spectra

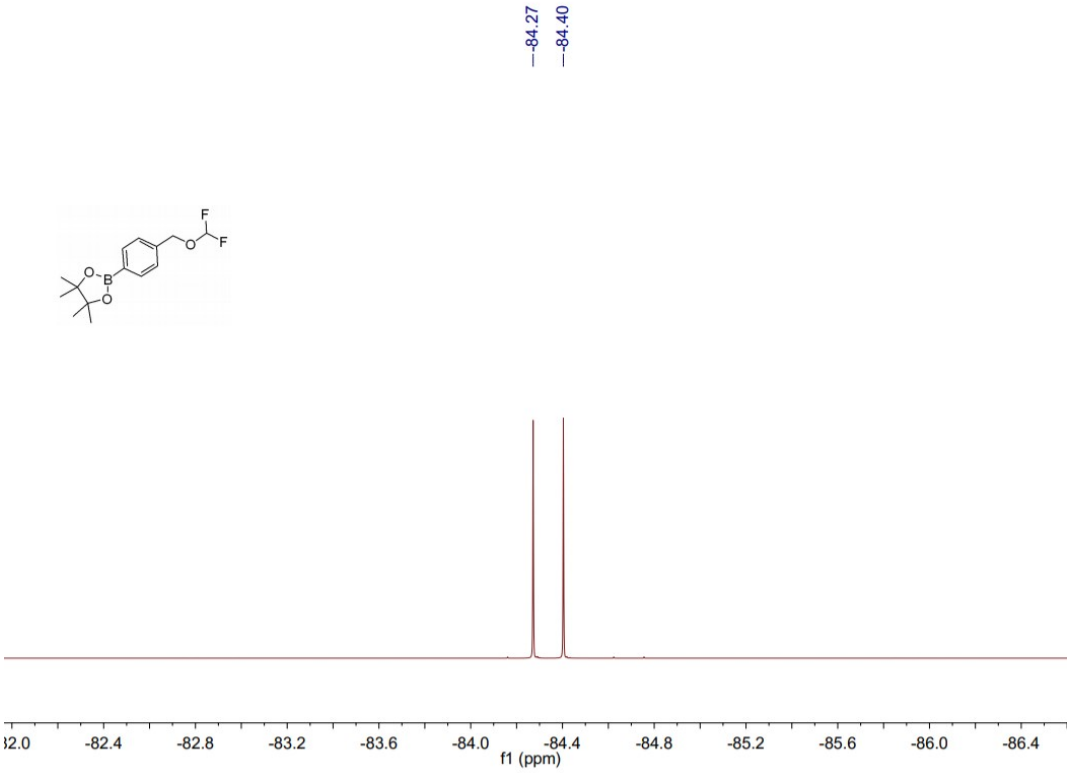


^{1}H NMR (400 MHz, $CDCl_3$) of 2-(4-((difluoromethoxy)methyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**2**).

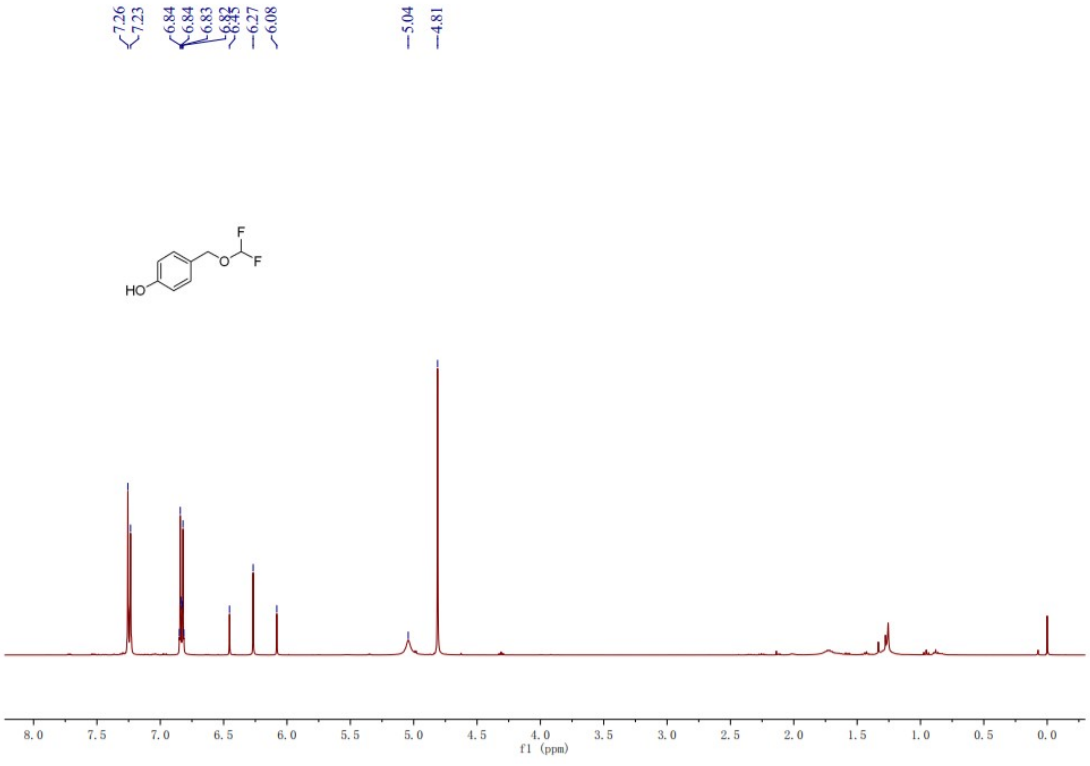


^{13}C NMR (150 MHz, $CDCl_3$) of 2-(4-((difluoromethoxy)methyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-

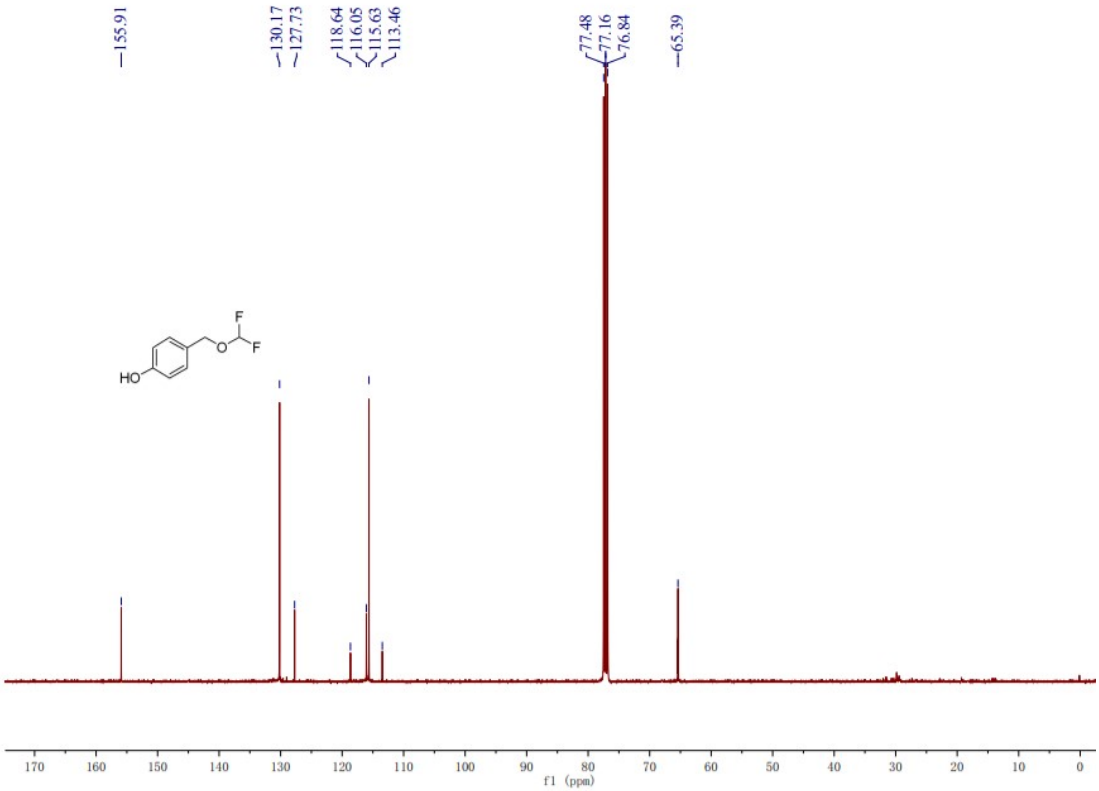
dioxaborolane (**2**).



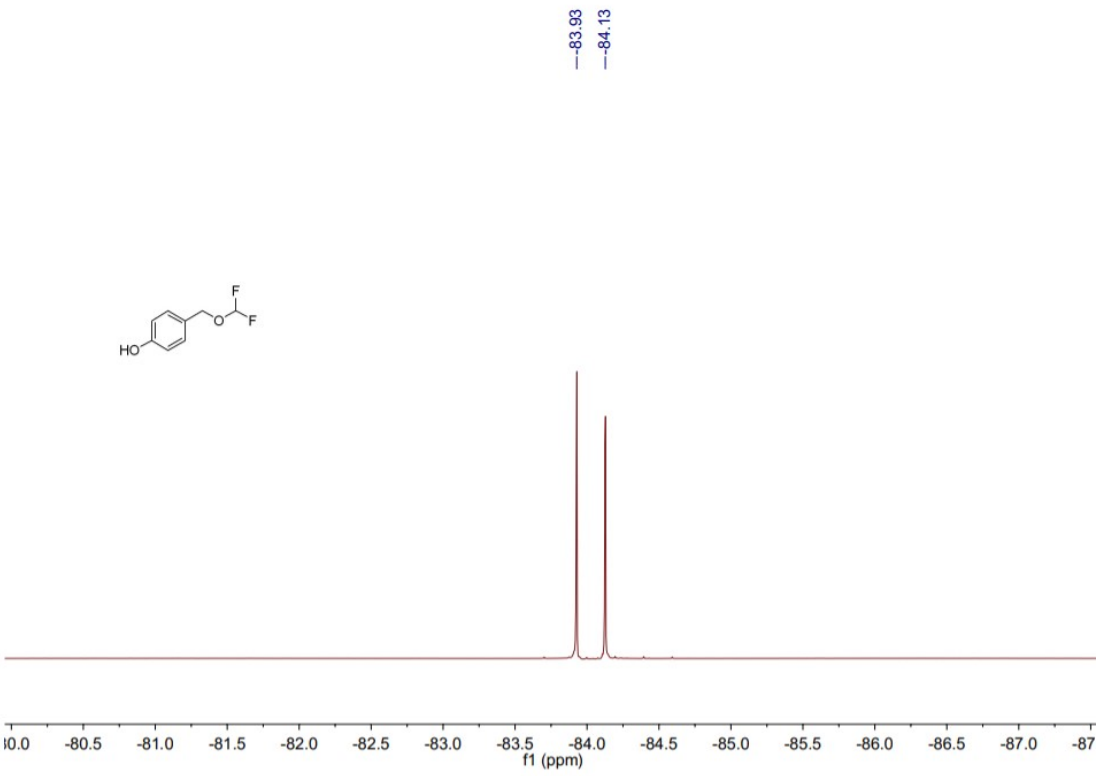
^{19}F NMR (563 MHz, $CDCl_3$) of 2-(4-((difluoromethoxy)methyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**2**).



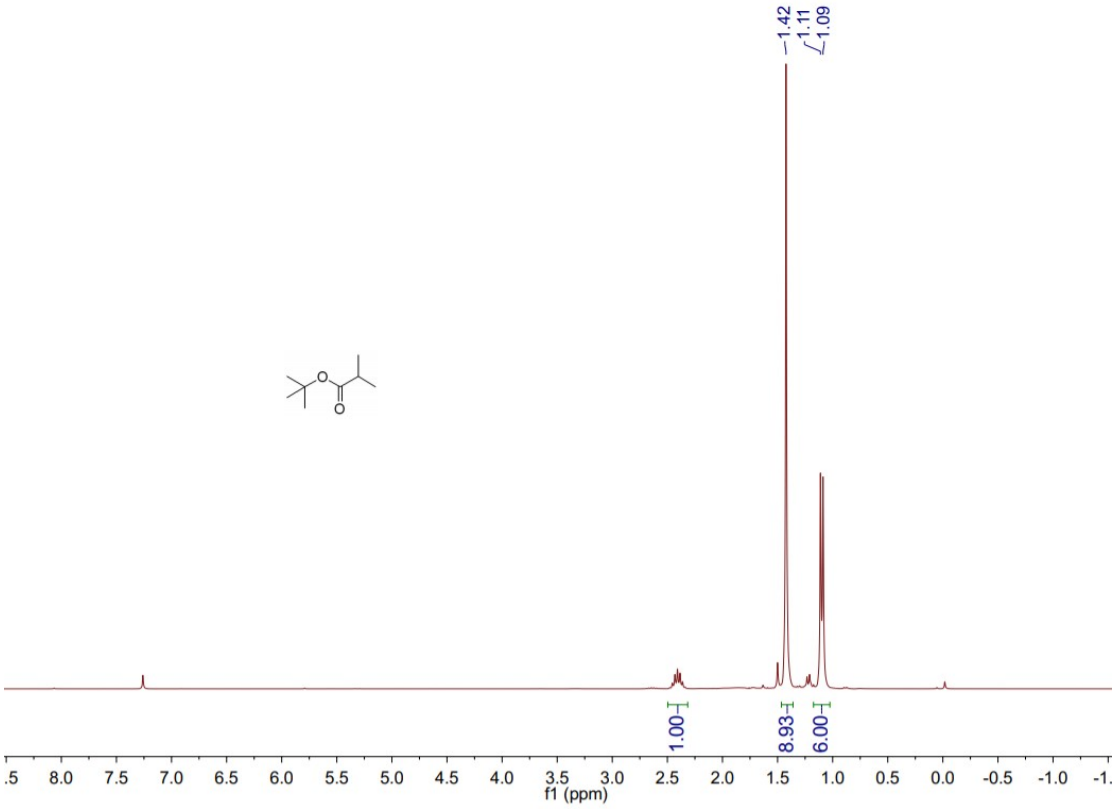
^{1}H NMR (400 MHz, $CDCl_3$) of 4-((difluoromethoxy)methyl)phenol (**4**).



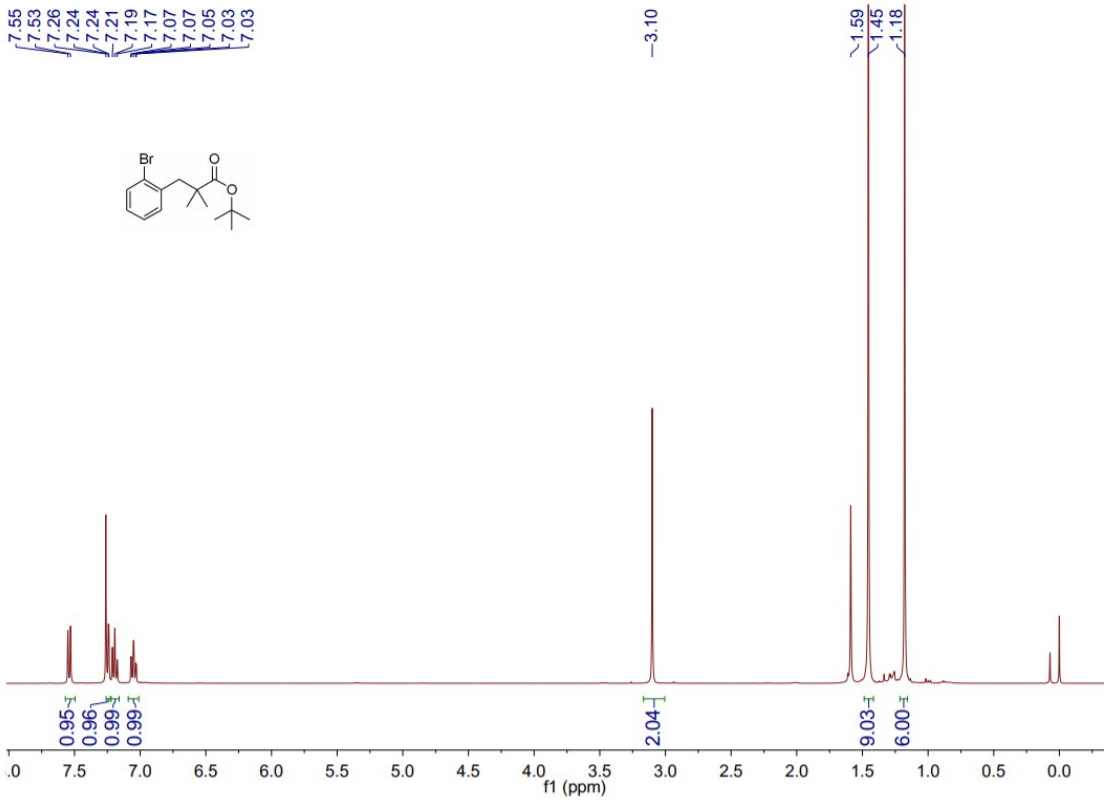
^{13}C NMR (100 MHz, $CDCl_3$) of 4-((difluoromethoxy)methyl)phenol (**4**).



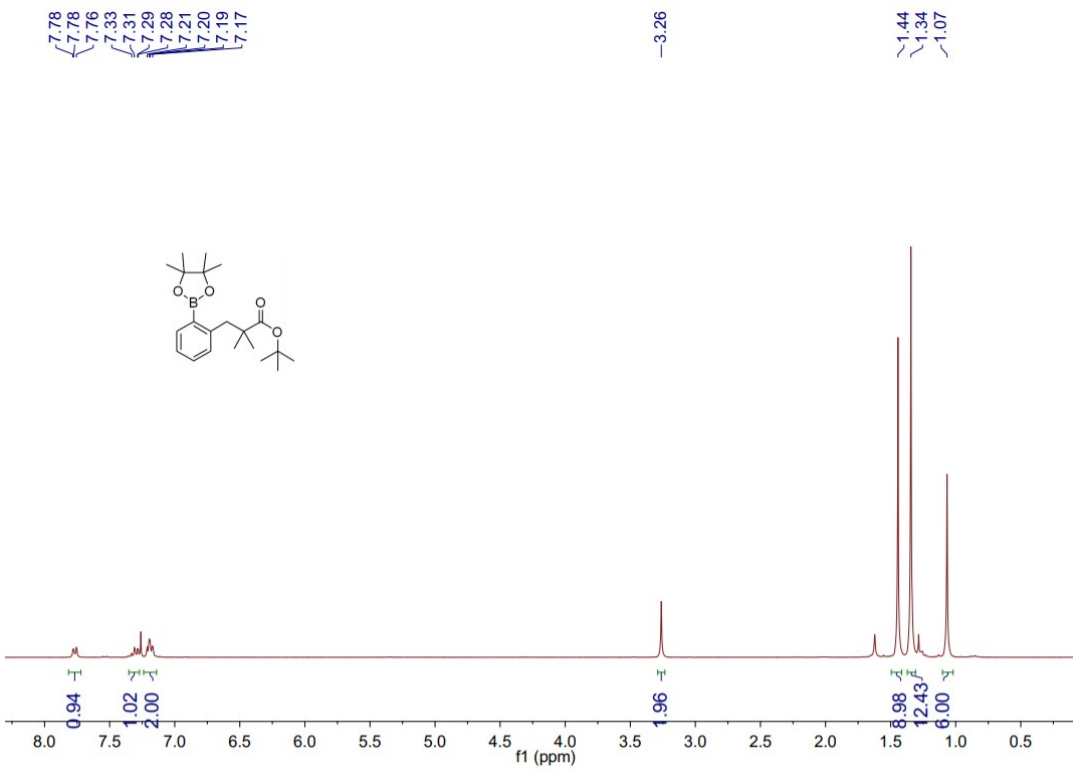
^{19}F NMR (376 MHz, $CDCl_3$) of 4-((difluoromethoxy)methyl)phenol (**4**).



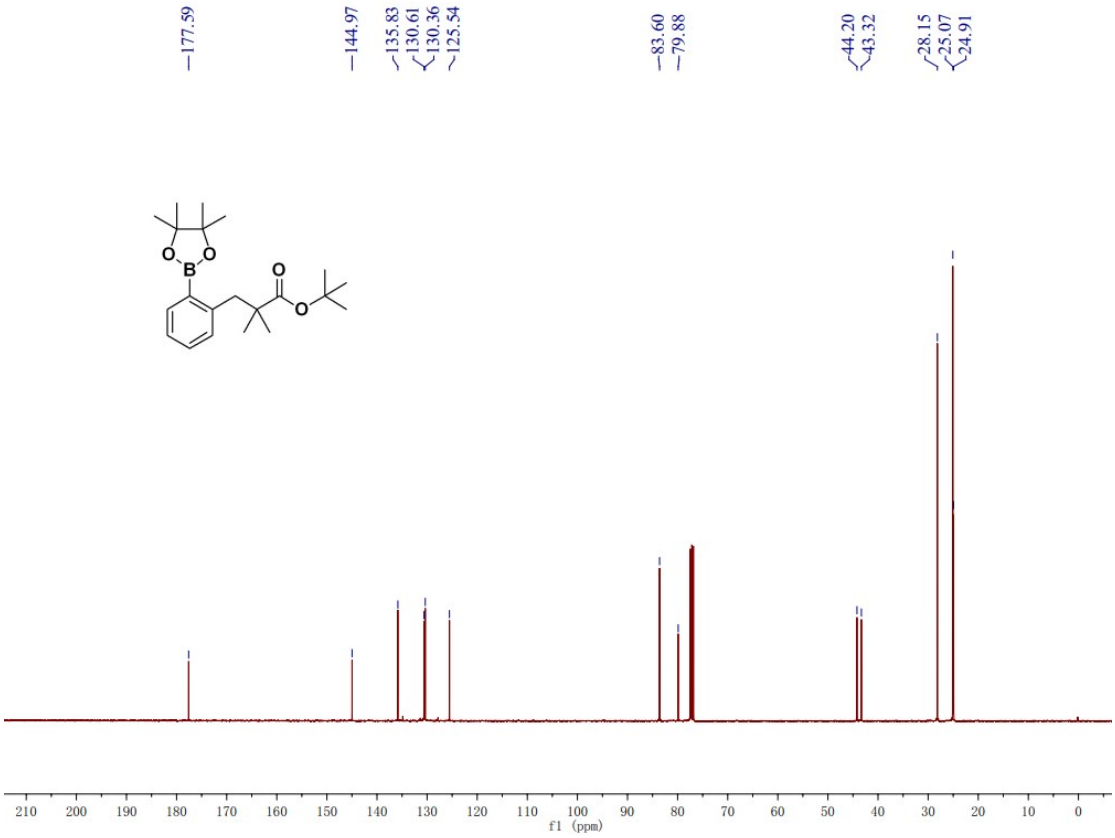
^{1}H NMR (300 MHz, $CDCl_3$) of tert-butyl isobutyrate (**8**).



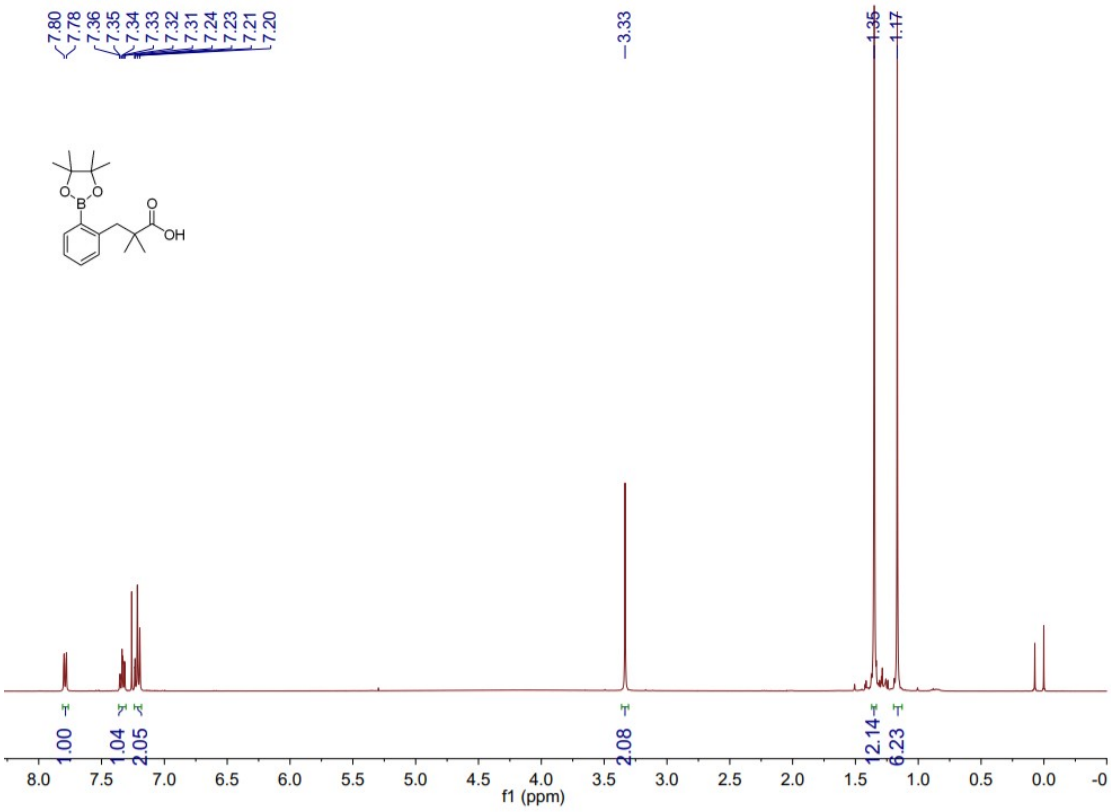
^{1}H NMR (400 MHz, $CDCl_3$) of tert-butyl 3-(2-bromophenyl)-2,2-dimethylpropanoate(**9**).



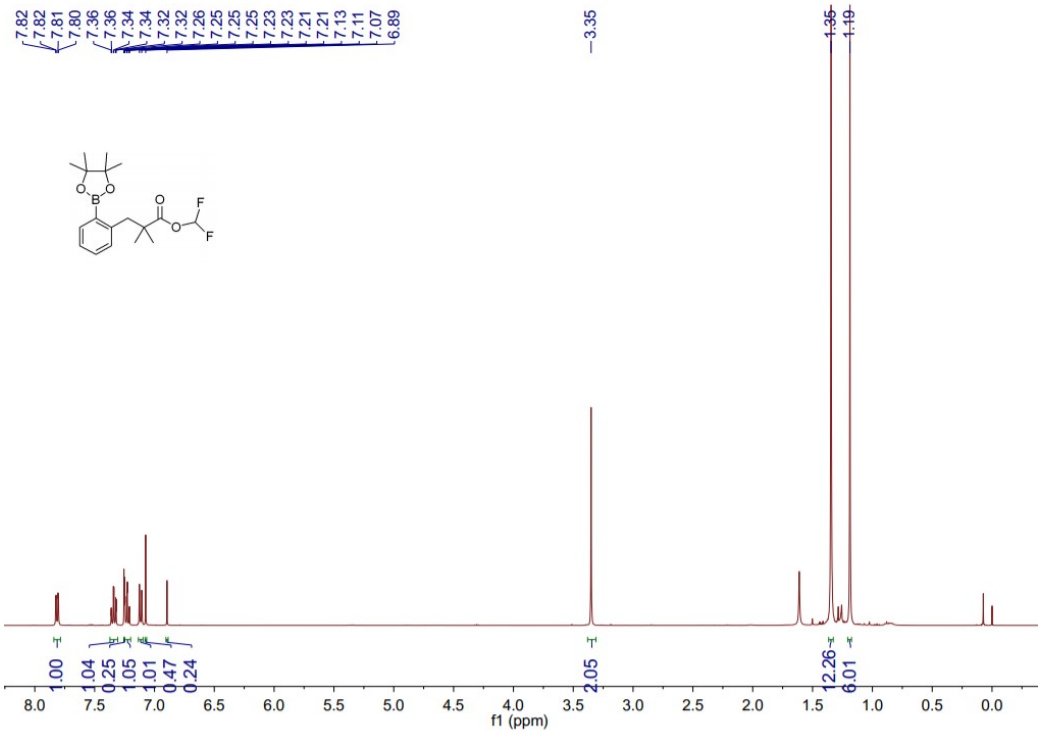
^{1}H NMR (400 MHz, $CDCl_3$) of tert-butyl 2,2-dimethyl-3-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (**10**).



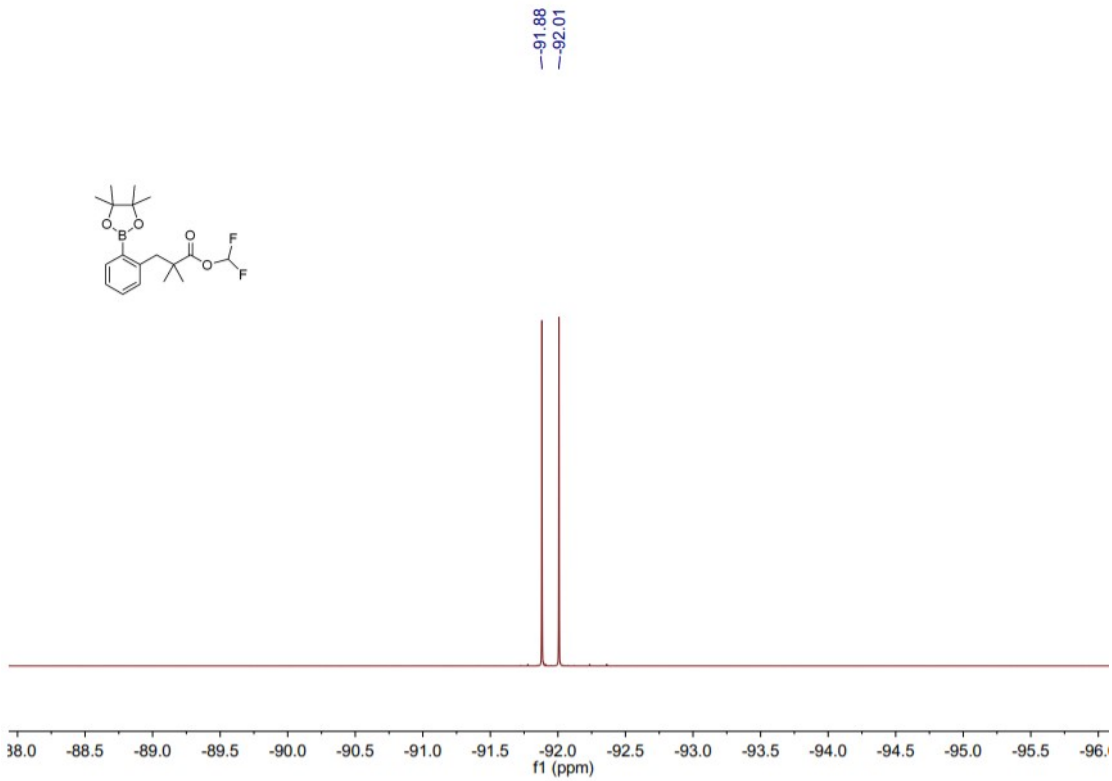
^{13}C NMR (100 MHz, $CDCl_3$) of tert-butyl 2,2-dimethyl-3-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (**10**).



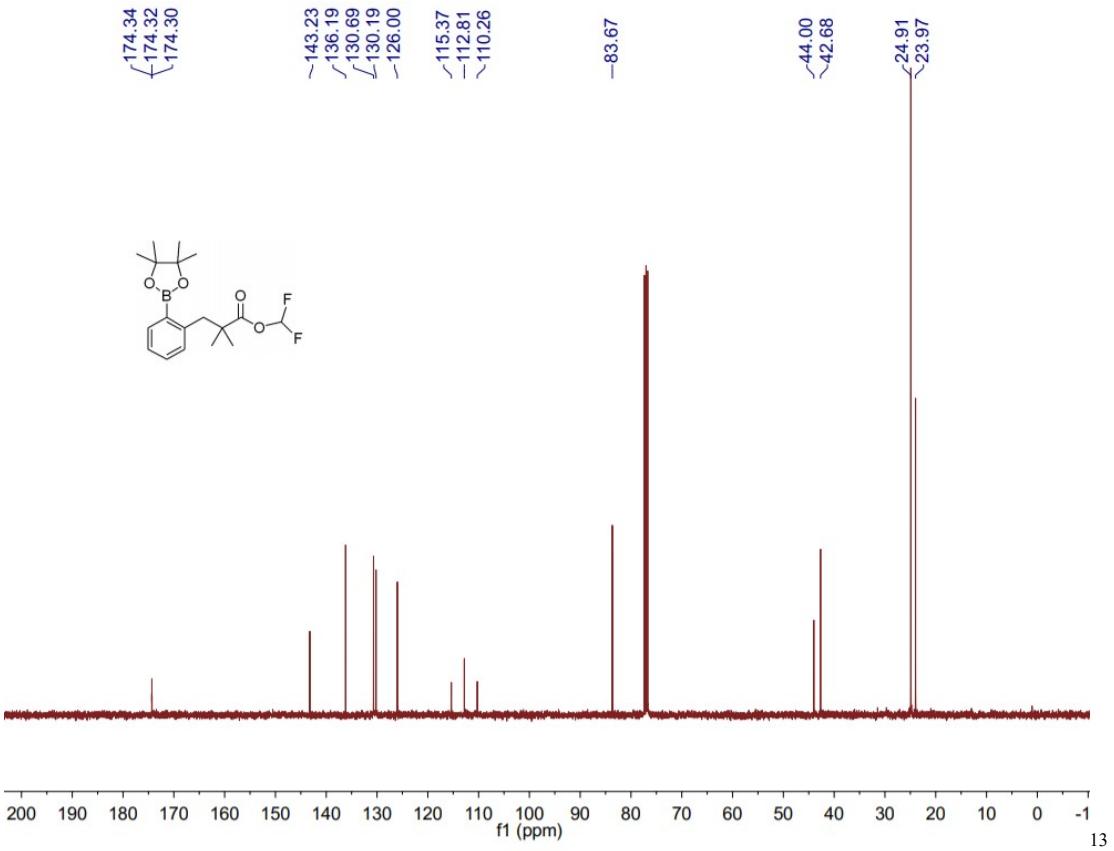
^{1}H NMR (400 MHz, $CDCl_3$) of 2,2-dimethyl-3-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoic acid (**11**).



^{1}H NMR (400 MHz, $CDCl_3$) of difluoromethyl 2,2-dimethyl-3-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (**3**).

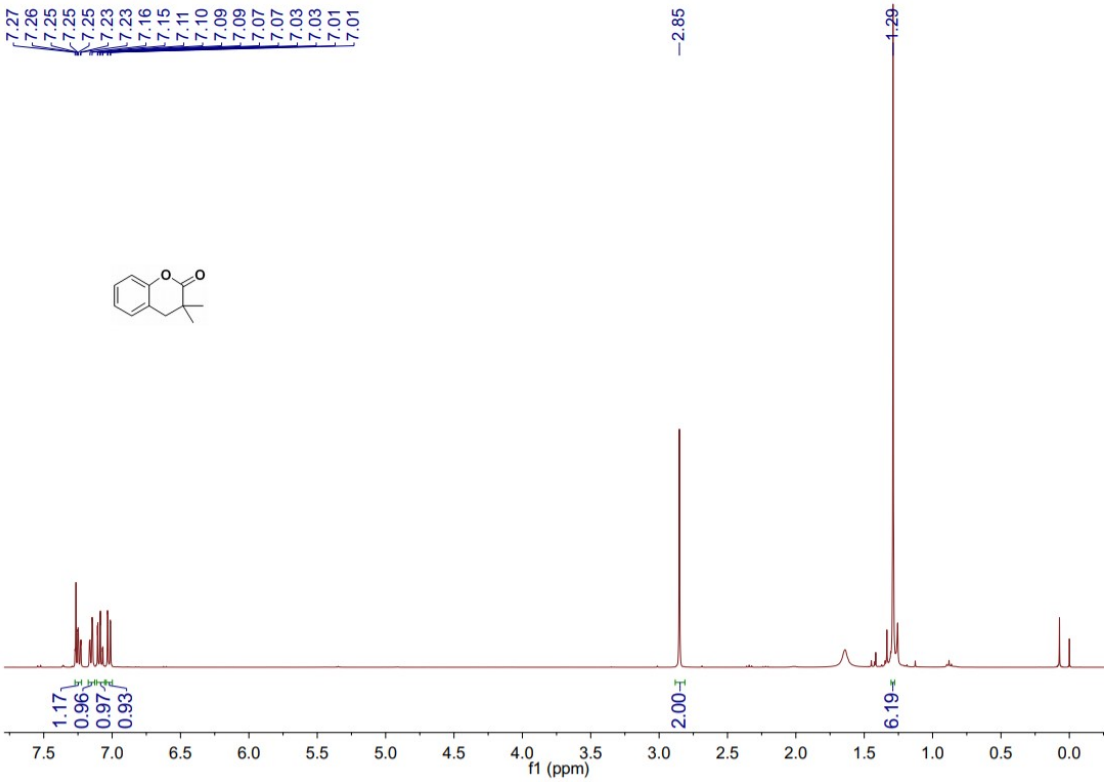


^{19}F NMR (376 MHz, $CDCl_3$) of difluoromethyl 2,2-dimethyl-3-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (**3**).



C NMR (100 MHz, $CDCl_3$) of difluoromethyl 2,2-dimethyl-3-(2-(4,4,5,5-tetramethyl-1,3,2-

dioxaborolan-2-yl)phenyl)propanoate (**3**).



^{1}H NMR (400 MHz, $CDCl_3$) of 3,3-dimethylchroman-2-one (**7**).

10. Reference

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