Supporting Information

Biocompatible Boron-Containing Prodrugs of Belinostat for the Potential Treatment of Solid Tumor

Shilong Zheng, Shanchun Guo, Qiu Zhong, Changde Zhang, Jiawang Liu, Lin Yang, Qiang Zhang, Guangdi Wang*

RCMI Cancer Research Center and Department of Chemistry, Xavier University of Louisiana, New Orleans, Louisiana 70125, United States

General chemistry. All reagents and solvents were purchased from Asta Tech, Combi-Blocks, AK Scientific, ACROS and Pharmaco-AAPER and were used as received. **1** and **2** were purchased from Apexbio through Fisher Scientific. ^{1}H and ^{13}C NMR spectra were obtained on a Bruke-300 NMR and Agilent 400-MR NMR spectrometer. Chemical shifts are reported as parts per million (ppm) relative to TMS. HRMS spectra data were collected on a Thermo LTQ Orbitrap-XL mass spectrometer in positive ion modes. The tested compounds were confirmed to be >95% pure by HPLC.

***O*-(4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)hydroxylamine.** It was prepared from *N*-hydroxy phthalimide in two steps following the reference procedure.[1] ^{1}H-NMR (300 MHz, $CDCl_3$): 7.81 (d, J = 7.8 Hz, 2H), 7.23 (d, J = 7.8 Hz, 2H), 5.40 (s, 2H), 4.71 (s, 2H), 1.35 (s, 12H). ^{13}C-NMR (75 MHz, $CDCl_3$): 140.6, 135.0, 127.5, 83.8, 77.9, 24.9.

***O*-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)hydroxylamine.** It was prepared from *N*-hydroxy phthalimide through the same procedure for the synthesis of *O*-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)hydroxylamine. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.81 (s, 1H), 7.76 (d, J = 6.9 Hz, 1H), 7.23 (d, J = 7.5 Hz, 1H), 7.38 (m, 1H), 5.39 (s, 2H), 4.70 (s, 2H), 1.35 (s, 12H). ^{13}C-NMR (75 MHz, $CDCl_3$): 136.6, 134.8, 134.5, 131.3, 127.9, 83.7, 78.0, 24.9.

Procedure for the synthesis of 7, 8 and 9. The mixture of methyl (*E*)-3-(3-(N-phenyl sulfamoyl)phenyl)acrylate (0.32 g, 1 mmol), NaOH (0.04g, 1 mmol) in 5 ml water was refluxed for 2 h, acidified with 1M HCl, then extracted with dichloromethane and the combined organic layer was dried over MgSO4. After filtering, the filtrate was added *O*-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)hydroxylamine or *O*-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)hydroxylamine (1 mmol), DCC (0.21g, 1 mmol) and pyridine (0.5 mL) . The

resultant mixture was stirred at room temperature overnight. After the reaction, the reaction was quenched with an aqueous NH_4Cl solution and washed with brine. The organic layer was separated and dried over MgSO4. After the solvent was removed under vacuum, the residue was purified by flash chromatography to afford the corresponding product.

(*E*)-3-(3-(*N*-Phenylsulfamonyl)phenyl)-*N*-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benyl)oxy)acrylamide (**7**) Yield: 28% (two steps). ^{1}H-NMR (300 MHz, DMSO-d6): 11.34 (s, 1H), 10.32 (s, 1H), 7.91 (s, 1H), 7.79 (d, J = 6.9 Hz, 1H), 7.70-7.69 (m, 3H), 7.60-7.48 (m, 2H), 7.43 (d, J = 7.5 Hz, 2H), 7.22-7.20 (m, 2H), 7.09-7.03 (m, 3H), 6.46 (d, J = 15.9 Hz, 1H), 4.90 (s, 2H), 1.29 (s, 12H). ^{13}C-NMR (75 MHz, DMSO-d6): 162.8, 140.7, 139.7, 138.3, 137.9, 136.0, 134.9, 132.6, 130.5, 129.7, 128.6, 127.8, 125.5, 124.8, 121.2, 120.8, 84.2, 77.3, 25.1. HRMS (ESI) for $C_{28}H_{32}BN_2O_6S$ (M+H): Cacld. 535.2074; Found 535.2070.

(*E*)-3-(3-(*N*-Phenylsulfamonyl)phenyl)-N-((3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benyl) oxy) acrylamide (**8**): Yield: 15% (two steps). ^{1}H-NMR (400 MHz, DMSO-d6):11.33 (s, 1H), 10.28 (s, 1H), 7.90 (s, 1H), 7.77-6.97 (m, 13H), 6.46 (d, J = 16.0 Hz, 1H), 4.87 (s, 2H), 1.25 (s, 12H). ^{13}C-NMR (100 MHz, DMSO-d6): 162.8, 140.7, 138.3, 137.9, 136.0, 135.5, 134.8, 134.3, 132.4, 131.4, 130.4, 129.6, 128.4, 128.3, 125.5, 124.8, 121.2, 120.8, 84.2, 74.7, 25.4. HRMS (ESI) for $C_{28}H_{32}BN_2O_6S$ (M+H): Cacld. 535.2074; Found 535.2078.

(*E*)-(3-(((3-(3-(*N*-Phenylsulfamoyl)phenyl)acrylamido)oxy)methyl)phenyl)boronic acid (**9**): Yield: 9.0% (two steps). ^{1}H-NMR (400 MHz, DMSO-d6):11.30 (s, 1H), 10.29 (s, 1H), 8.07 (s, 1H), 7.89 (s, 1H), 7.81-7.76 (m, 2H), 7.69 (d, J = 8.0 Hz, 1H), 7.56-7.34 (m, 6H), 7.22-7.19 (m, 2H), 7.08-7.01 (m, 3H), 6.46 (d, J = 16.0 Hz, 1H), 4.85 (s, 2H). ^{13}C-NMR (100 MHz, DMSO-d6): 162.7, 140.7, 138.2, 137.9, 136.1, 135.2, 134.6, 134.4, 132.5, 131.1, 130.5, 129.6, 127.9,

127.7, 125.4, 124.8, 121.3, 120.8, 77.8. HRMS (ESI) for $C_{22}H_{22}BN_2O_6S$ (M+H): Cacld. 453.1292; Found 453.1296.

Antiproliferative assays. Human breast adenocarcinoma (MDA-MB-231), human non-small cell lung carcinoma (A549), human cervix carcinoma (HeLa), human breast adenocarcinoma (MCF-7), human melanoma (SK-MEL-28), human large cell lung carcinoma (NCI-H460), and non-cancer breast epithelial (MCF-10A) cell lines were grown in DMEM medium supplemented with 115 units/mL of penicillin G, 115 μg/mL of streptomycin, and 10% fetal bovine serum (all from Life Technologies, Grand Island, NY). Cells were seeded in 96-well plates (5×10^3 cells/well) containing 50 μL growth medium for 24 hrs. After medium removal, 100 μL fresh medium containing individual prodrugs and both **1** and **2** controls at different concentrations was added to each well and incubated at 37 °C for 72 h. After 24 hrs of culture, the cells were supplemented with 50 μL of prodrugs, **1** and **2** dissolved in DMSO (less than 0.25% in each preparation). After 72 h of incubation, 20 μL of resazurin was added for 2 h before recording fluorescence at 560 nm (excitation) and 590 nm (emission) using a Victor microtiter plate fluorometer (Perkin-Elmer, USA). The IC_{50} was defined as the compound concentration required inhibiting cell proliferation by 50%, in comparison with cells treated with the maximum amount of DMSO (0.25%) and considered as 100% viability. MCF-10A cell line was treated with two concentrations of **7** and **2** (Figure S1).

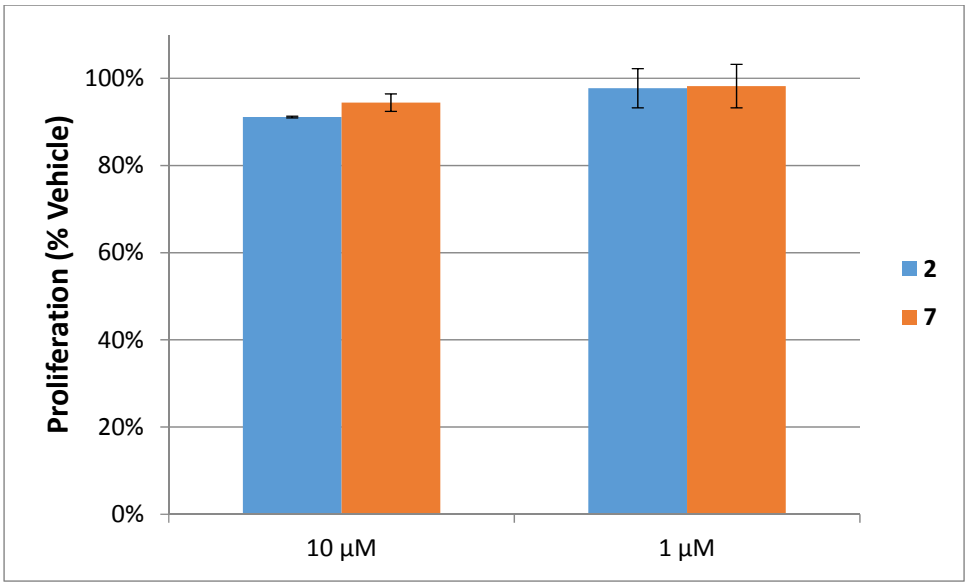


Figure S1 Antiproliferation activity of prodrug **7** and **2** against MCF-10A

HPLC-MS analysis of prodrugs and active belinostat in the *in vitro* assays. Reaction products and cell culture media samples were analyzed on a TSQ Advantage UHPLC-MS/MS system (Thermo-Fisher Scientific, West Palm Beach, FL). Chromatographic separations were achieved by using a Hypersil gold column (50 × 2.1 mm). Samples were eluted from the analytical column with mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid) at a flow-rate of 0.2 mL/min over 22 min using a linear gradient of 0–100% B for 13 min; holding at 100% B for 5 min before returning to 0% B. For analysis of the relative concentrations of prodrugs and belinostat, selected ion monitoring (SIM) method was used where m/z 453 was monitored for the boronic acid prodrugs **9** and **10**, m/z 535 for prodrugs **7** and **8**, and m/z 319 for belinostat **2**. The peak areas of the above-mentioned ions were recorded and used to calculate the relative concentrations of their corresponding molecules.

HDAC assay for prodrug 7. The histone deacetylase activity assay kit (Fluorometric) ab156064 (Abcam, Cambridge, UK) was used to detect the HDAC activity in lysates and measure the HDAC inhibition of prodrug **7**. In duplicate, ddH2O, HDAC assay buffer, and fluoro-substrate

peptide or fluoro-deacetylated peptide were added to microtiter plate wells. Then, **1**, **2**, **7**, trichostatin A, or DMSO (control) in which compound was dissolved was added to the assay wells and mixed. Next, developer was added to each well of the microtiter plate and mixed well. Reactions were initiated by adding 5 μL of HDACs to each well and mixed thoroughly at RT and incubated for 20 min at RT. 20 μL of stop solution and 5 μL of developer were added to each well of the microtiter plate and mixed thoroughly. The reaction mixture was incubated for 20 min at RT and the fluorescence intensity was read for 30~60 minutes at 1~2-minute intervals using a microplate fluorescence reader at Ex/Em = 355 nm/460 nm. The average fluorescence of each data point based on the duplicates was determined. The efficacy of inhibition of **7** on the HDAC activity was calculated as follows: Inhibition effect = [Prodrug **7** Assay – DMSO Control Assay]. The EC_{50} was defined as the compound concentration required to inhibit HDAC activity by 50%. The rate of reaction was measured and calculated while the reaction velocity remained constant.

The *in vitro* assay of prodrug **7** against HDAC isoforms 1-11 has been performed by Reaction Biology Corporation. Prodrug 7 was tested in singlet 10-dose IC_{50} mode with 3-fold serial dilution starting at 100 μM against HDACs 1-11. HDAC reference compounds Trichostatin A (TSA) and TMP269 were tested in a 10-dose IC_{50} with 3-fold serial dilution starting at 10 μM. Substrate for HDAC 1, 2, 3, 6, and 10: Fluorogenic peptide from p53 residues 379-382 (RHKK(Ac)AMC). Substrate for HDAC 4, 5, 7, 9, and 11: Fluorogenic HDAC Class2a Substrate (Trifluoroacetyl Lysine). Substrate for HDAC 8: Fluorogenic peptide from p53 residues 379-382 (RHK(Ac)K(Ac)AMC). IC_{50} values were calculated using the Graph Pad Prism 4 program based on a sigmoidal dose-response equation. The blank (DMSO) value was entered as 1.00×10^{-12} of concentration for curve fitting. All data for the curve fitting and parameter

setting can be viewed by clicking the graph in the tabs.

Table S1 Inhibitory activity of prodrug 7 against HDAC isoforms

	EC_{50}(μM)										
Compounds	HDAC1	HDAC2	HDAC3	HDAC4	HDAC5	HDAC6	HDAC7	HDAC8	HDAC9	HDAC10	HDAC11
7	*	*	*	*	*	20.2	*	341	*	*	*
Trichostatin A	0.0133	0.0365	0.0254	ND	ND	0.00243	ND	0.498	ND	0.0489	4.39
TMP 269	ND	ND	ND	0.301	0.281	ND	0.0863	ND	0.0254	ND	ND

*: Indicates no inhibition or compound activity that could not be fit to an EC_{50} curve. ND: Indicates compound not tested against this enzyme isoform. EC_{50} value higher than 10 μM is firstly estimated based on the best curve fitting available and is retested with an adjusted compound concentration range in order to obtain a more definitive result.

In vivo efficacy assay in mice and PK study

Four to six weeks old female ovariectomized Nu/Nu mice were purchased from Charles River Laboratories (Wilmington, MA). MCF-7 cells were cultured and harvested in the exponential growth phase using a PBS/EDTA solution. The animals were injected with 5×10^6 viable cells suspended in 50 μL sterile PBS mixed with 100 μL Matrigel (reduced factor; BD Biosciences, Bed- ford, MA) bilaterally into the mammary fat pad (MFP). 17β-Estradiol pellets (0.72 mg, 60 day release; Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the lateral area of the neck using a precision trochar (10 gages) at the time of cell injection. Tumors were allowed to form and at day 15 post cell injection mice were randomized into three groups and treated with vehicle, belinostat **2** and prodrug **7** at 10 mg/kg/day by subcutaneous injection. Tumor volumes were monitored and recorded every other day for three weeks of treatment duration. At the last day of the study, the tumor tissues were collected, weighed and snap frozen for further PK analysis.

Table S2. Tumor weights of each mouse in 7- and 2-treated groups after the treatment

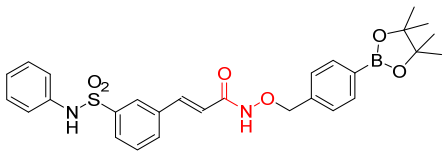
Tumor weights of **2**-treated group (mg, 4 mice)				Tumor weights of **7**-treated group (mg, 4 mice)			
191.2	144.5	149.6	183.9	117.8	95.4	119.7	101.5
Average 167.3 ± 23.7				Average 108.6 ± 12.0			
T-test: *p*-value 0.00445							

All procedures involving the animals were conducted in compliance with State and Federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by Xavier University Animal Care and Use Committee. The facilities and laboratory animals program of Xavier University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

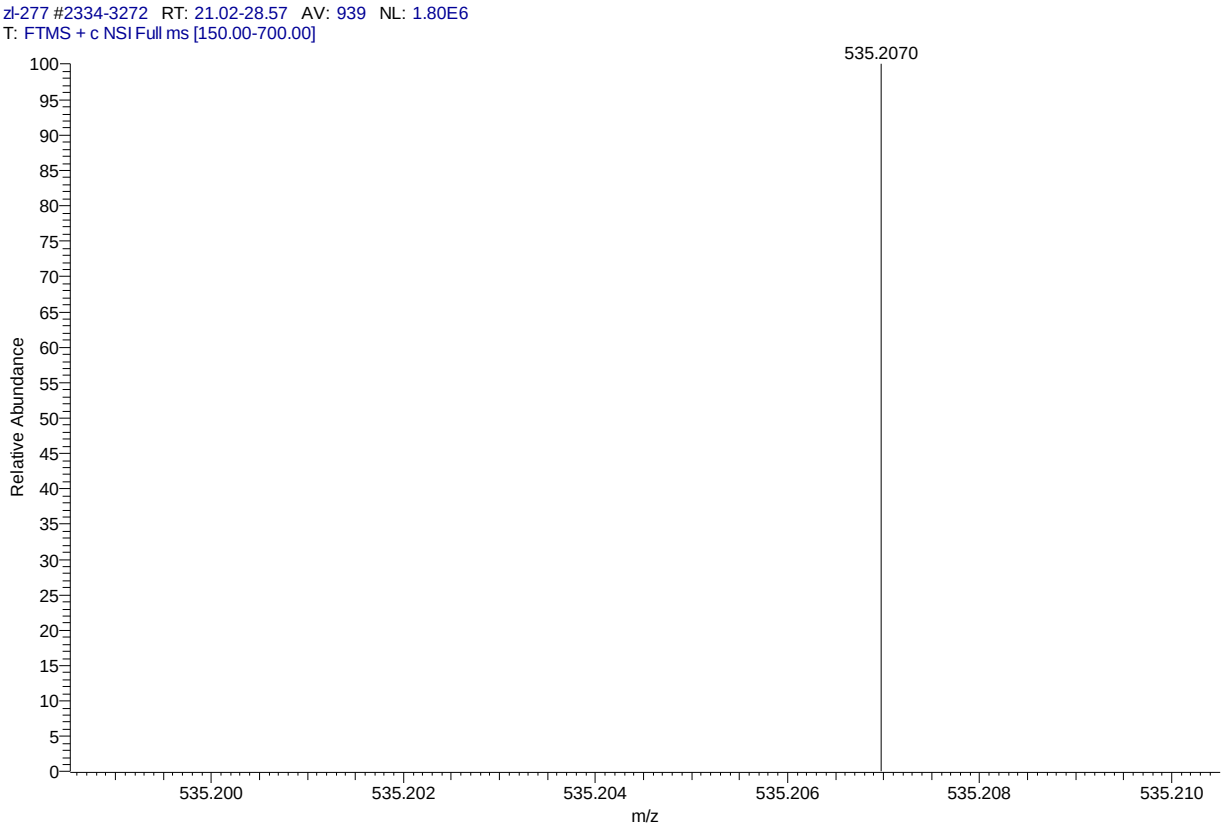
References

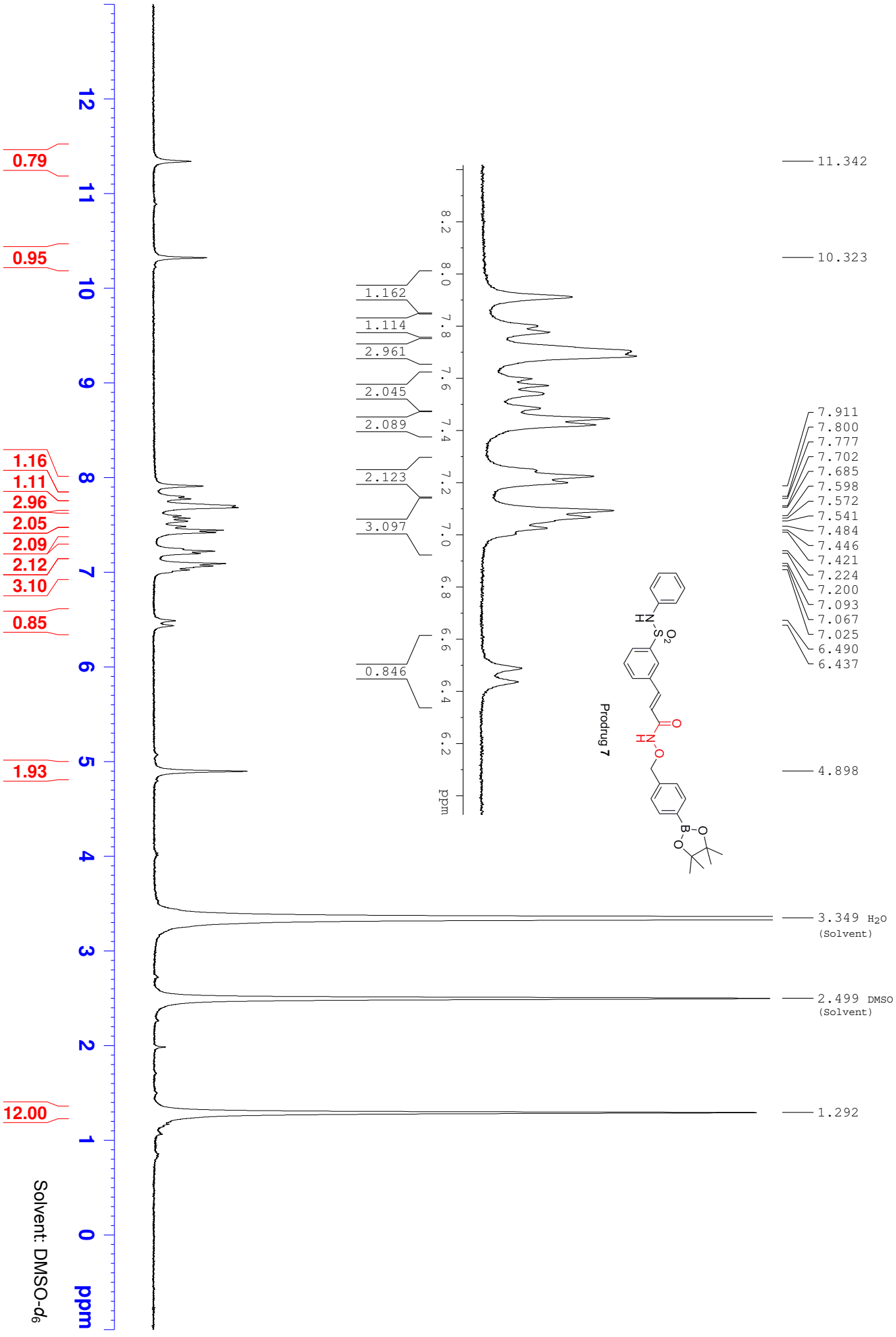
1. Wang, M.-Z.; Xu, H.; Liu, T.-W.; Feng, Q.; Yu, S.-J.; Wang, S.-H.; Li, Z.-M. Design, synthesis and antifungal activities of novel pyrrole alkaloid analogs. *Eur. J Med. Chem.* **2011**, *46*, 1463-1472.

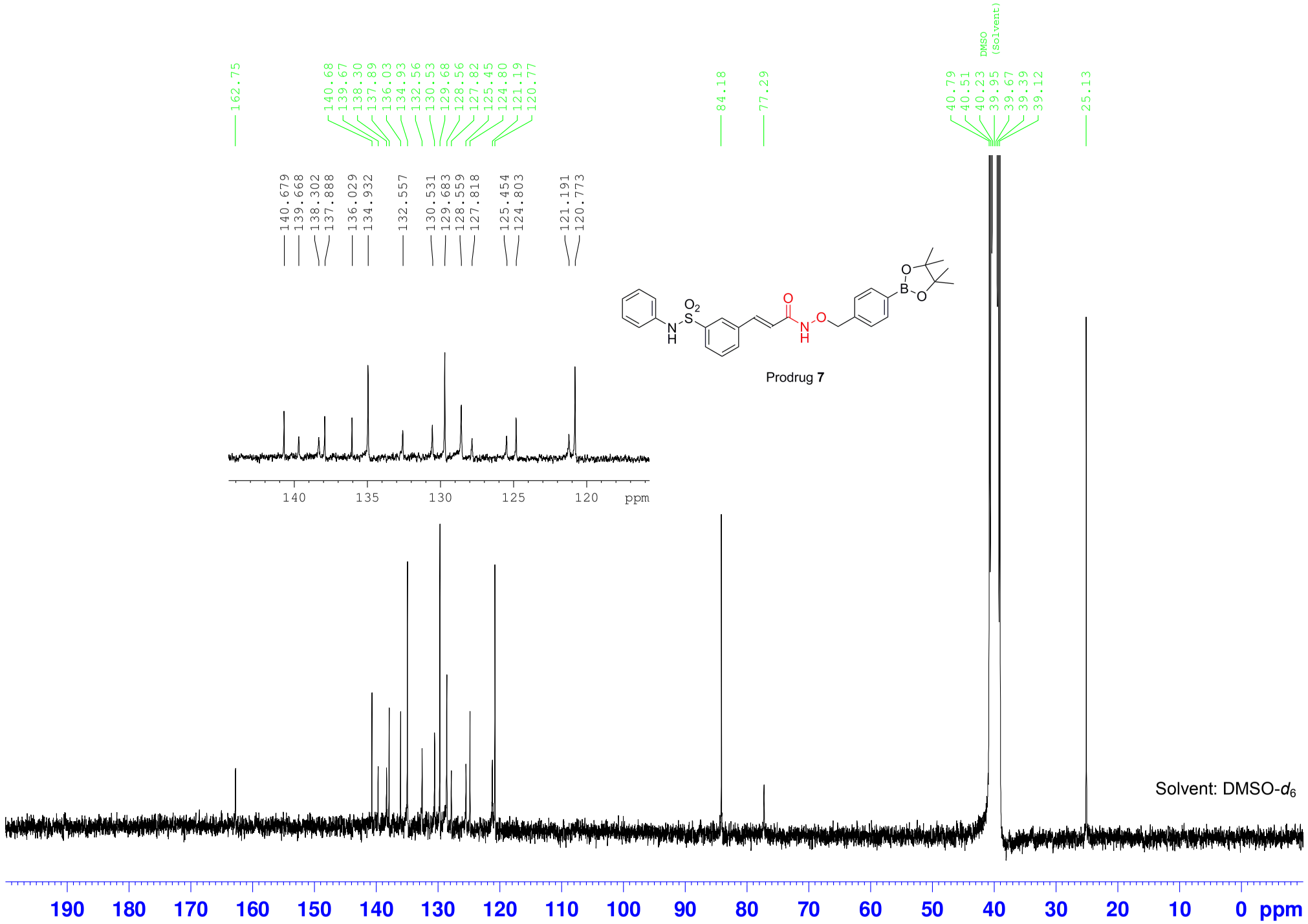
Prodrug **7** Calc. $(M+H)^+$ m/z 535.2074 *Found* *535.2070*



Prodrug **7**




0.79
0.95
1.16
1.11
2.96
2.05
2.09
2.12
3.10
0.85
1.93
12.00
12
11
10
9
8
7
6
5
4
3
2
1
0
ppm
Solvent: DMSO-d_6
11.342
10.323
7.911
7.800
7.777
7.702
7.685
7.598
7.572
7.541
7.484
7.446
7.421
7.224
7.200
7.093
7.067
7.025
6.490
6.437
4.898
3.349 H_2O
(Solvent)
2.499 DMSO
(Solvent)
1.292
1.162
1.114
2.961
2.045
2.089
2.123
3.097
0.846
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
ppm
Prodrug 7


162.75
140.68
139.67
138.30
137.89
136.03
134.93
132.56
130.53
129.68
128.56
127.82
125.45
124.80
121.19
120.77
84.18
77.29
DMSO (Solvent)
40.79
40.51
40.23
39.95
39.67
39.39
39.12
25.13
140.679
139.668
138.302
137.888
136.029
134.932
132.557
130.531
129.683
128.559
127.818
125.454
124.803
121.191
120.773
Prodrug 7
140
135
130
125
120
ppm
Solvent: DMSO-d_6
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm