Electronic Supplementary Information

Elucidation of working principle of a gene-directed caged HDAC inhibitor with cell-type selectivity

Kotoko Sakamoto, Ayumi Hirano, Rika Hidaka, Akinobu Z. Suzuki, Taro Ueno, and Toshiaki Furuta*
Faculty of Science, Department of Biomolecular Science, Toho University

Table of contents

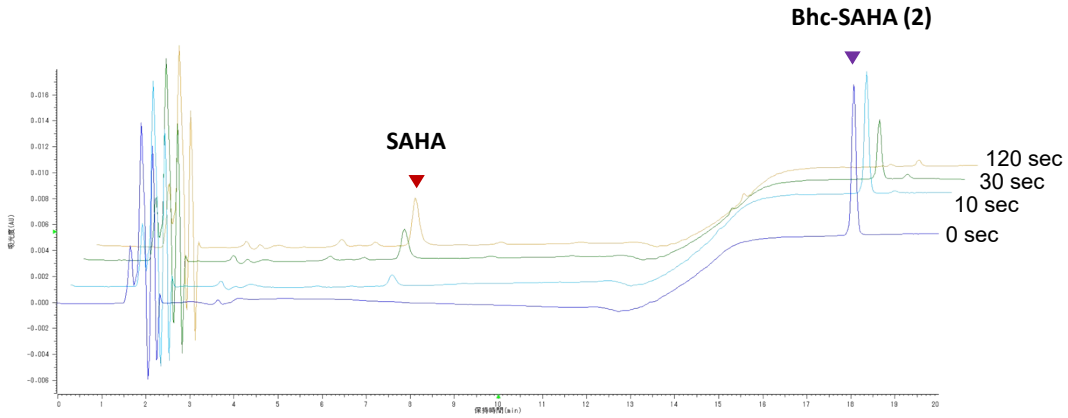


Fig. S1 Typical HPLC traces of **Bhc-SAHA (2)** upon 405 nm irradiation. Samples (10 μM in 10 mM HEPES pH 7.5 containing 0.1% DMSO) were irradiated and analyzed by reverse phase HPLC after the irradiation time shown in the figure. Purple triangle: **2** (retention time 18.1 min), red triangle: **SAHA** (retention time 7.3 min).

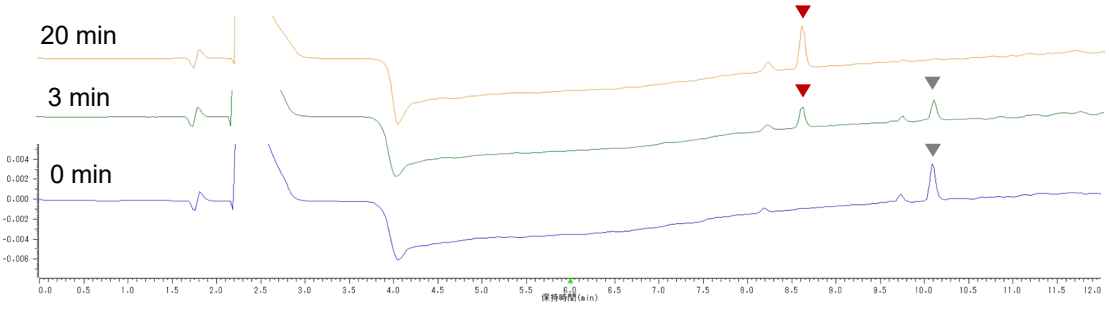


Fig. S2 Typical HPLC traces of **CM-Bhc-SAHA (3)** in the presence of PLE. 5.4 μM of **3** was treated with 0.1 μg/mL of PLE (from the porcine liver). Samples were analyzed after the incubation time shown in the figure. Grey triangle: **3** (retention time 10.1 min), red triangle: **2** (retention time 8.6 min).

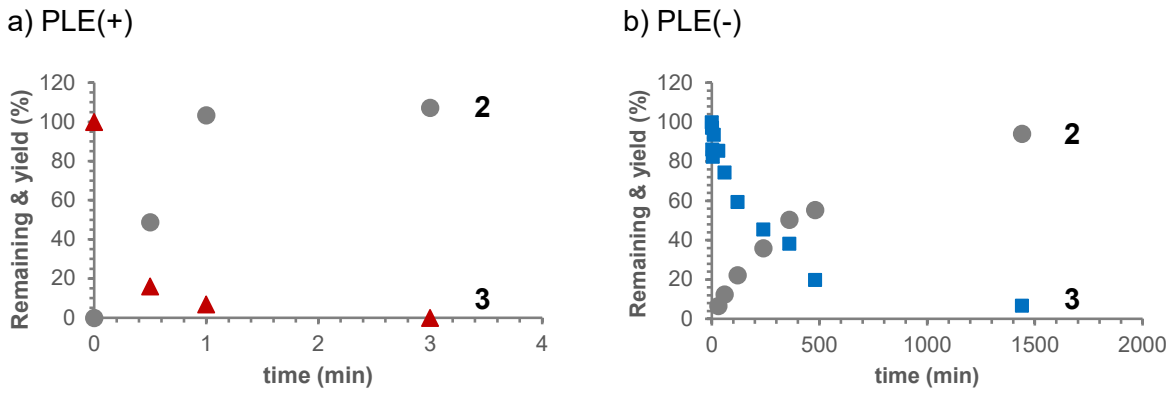


Fig. S3 Enzymatic transformation of **3** in the presence of HEK293T cell lysates. (a) 5.4 μM of **3** was treated with HEK293T/PLE (PLE(+)) lysates. **3**: red triangle, released **2**: gray circle, (b) with HEK293T (PLE(-)) lysates. **3**: blue square, released **2**: gray circle.

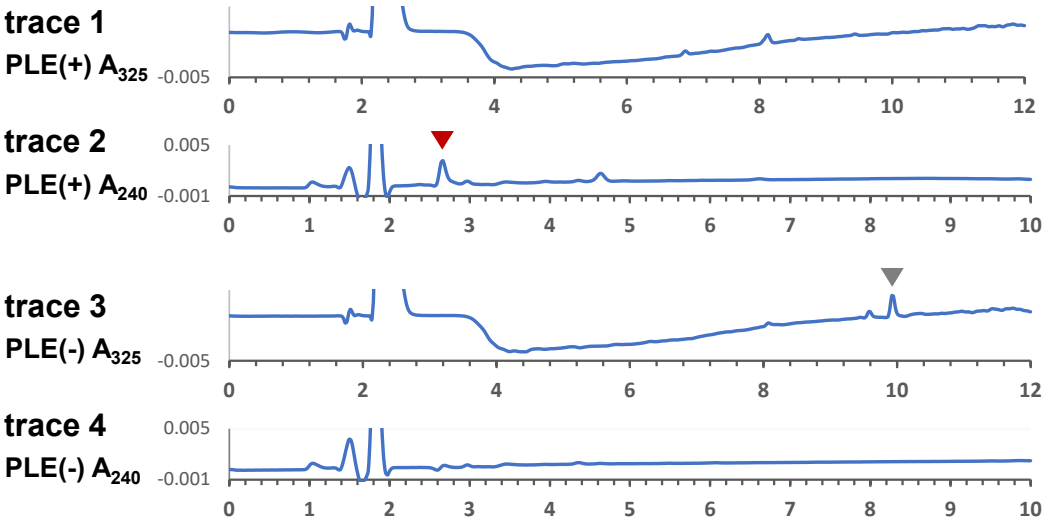


Fig. S4 HPLC traces of 3 after exposure to 405 nm light.

CM-Bhc-SAHA (3) was exposed to 405 nm light (408 mJ cm^{-2}) in the presence of HEK293T/PLE lysates (traces1 & 2) or HEK293T lysates (traces 3 & 4). The x-axis represents retention time (min). A_{325}: Traces 1 and 3 were monitored at 325 nm to detect **2** and **3**. A_{240}: Traces 2 and 4 were monitored at 240 nm to detect **SAHA**. **3** was not detected in trace 1 (gray triangle in trace 3, retention time 9.9 min). **SAHA** was detected in trace 2 (red triangle, retention time 2.6 min) and not in trace 4. **2** (retention time 8.6 min) was not detected both in traces 1 and 3.

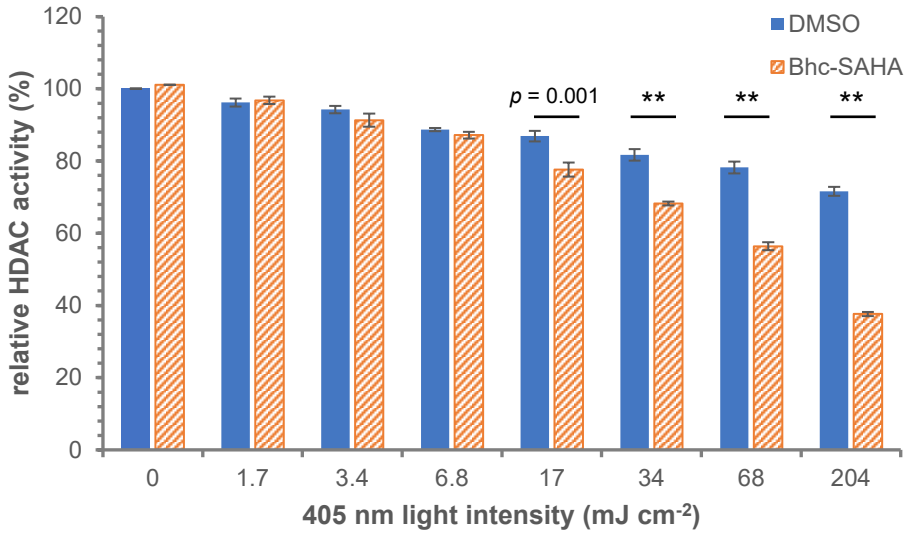


Fig. S5 Photo-mediated inhibition of HDAC activity in the presence of **Bhc-SAHA (2)**. HeLa nuclear extracts were exposed to a 405-nm light at the indicated integrated light intensity in the presence of 500 nM of **2** (orange, shaded). HDAC activity from control extracts that were not treated with **2** was quantified (blue, solid). Percentages of HDAC activity were normalized to these groups. The data points represent the averages of quadruplicate experiments ± the standard deviation (SD) of the mean. ** $P < 0.0001$

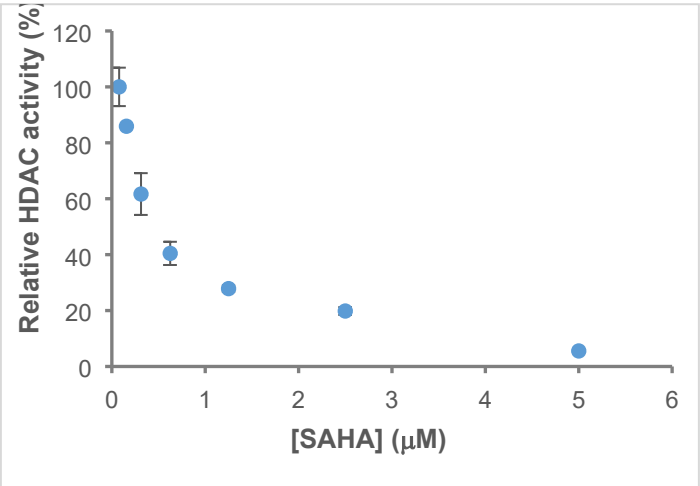


Fig. S6 HDACi activity of **SAHA** against endogenous HDAC in HEK293T cells. Cells were treated with the indicated concentration of **SAHA** for 1 h, and were washed with fresh culture media to remove excess **SAHA** outside the cells. The enzymatic activity of HDACs from control cells treated with DMSO (0 μM) was quantified. Percentages of the HDAC activity were normalized to this group. The values represent the averages of triplicate experiments ± the standard deviation (SD) of the mean.

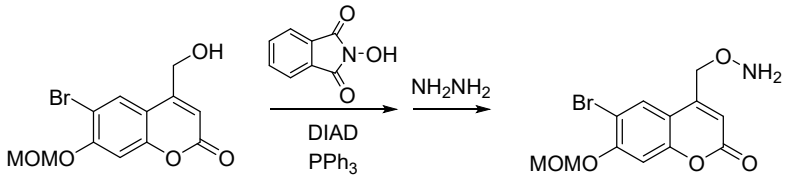
Synthetic Procedures

General Synthesis Methods

All reagents and solvents were purchased from commercial sources and used without further purification. Flash column chromatography was carried out on a YAMAZEN EPCLC-AI-580S system using universal column premium (30 μm silica gel). NMR spectra were recorded on a Brucker Biospin Avance 300M at 300 MHz for ^{1}H and 75 MHz for ^{13}C and Brucker Ascend 400 at 400 MHz for ^{1}H with a deuterated solvent and TMS as an internal standard. IR spectra were recorded on a Thermo Nicolet Avatar 320 in ATR mode. Analytical HPLC was run on a Hitachi Chromaster system with DAD detection. HRMS spectra were recorded on a JEOL JMS-700 using *m*-nitrobenzyl alcohol as a matrix to facilitate sample ionization.

Synthesis of Bhc-SAHA (2)

Synthesis of 4-((aminooxy)methyl)-6-bromo-7-(methoxymethoxy)-2H-chromen-2-one (1):



MOMBhc-CH_2OH **MOMBhc-CH_2ONH_2 (1)**

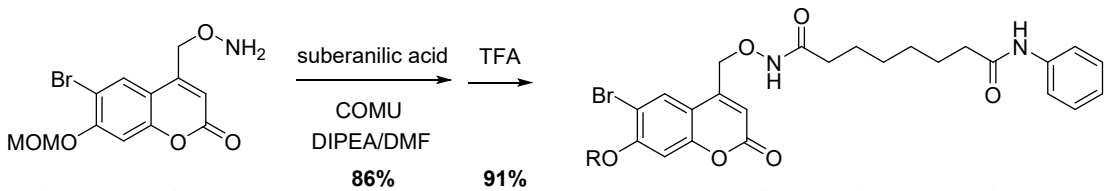
To a stirred solution of MOM-Bhc-CH_2OH[1] (344.1 mg, 1.092 mmol), N-hydroxyphthalimide (214.5 mg, 1.315 mmol), and triphenylphosphine (510.1 mg, 1.945 mmol) in anhydrous THF (5 mL) was added dropwisely 258 μL (1.311 mmol) of DIAD at 0 °C. After 2 h of stirring at 0 °C, hydrazine monohydrate (127.4 μL, 2.621 mmol) was added to the mixture, and the stirring was continued at rt for 24 h. The reaction mixture was diluted with ethyl acetate and washed with water. The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification using flash column chromatography (Yamazen Universal Column premium Silica gel, linear gradient of 35% to 56% ethyl acetate in hexane as eluents) yielded 227.0 mg (0.688 mmol, 66 % yield) **1**.

MOM-Bhc-CH_2ONH_2 ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δH 3.52 (3H, s, CH_3O), 4.81 (2H, d, *J* 1.2, C(4)-CH_2O), 5.32 (2H, s, CH_3CH_2O), 5.69 (2H, br s, NH_2), 6.37 (1H, t, *J* 1.2, H-3), 7.16 (1H, s, H-8), 7.80 (1H, s, H-5).

^{13}C NMR (75 MHz, $CDCl_3$) δC 56.81, 73.62, 95.31, 104.15, 108.54, 112.58, 113.42, 128.35, 159.22, 154.48, 156.31, 160.51.

IR (neat) ν_{max}/cm^{-1} 1731vs (C=O), 1603s, 1151s, 1083s, 1067s, 1026s

Synthesis of N^1-((6-bromo-7-hydroxy-2-oxo-2H-chromen-4-yl)methoxy)-N^8-phenyloctanediamide (2, Bhc-SAHA):



A mixture of Suberanilic acid (52.9 mg, 0.212 mmol), DIPEA (75.6 µL, 0.434 mmol) and COMU (110.7 mg, 0.2585 mmol) in DMF (1.5 mL) was stirred at an ambient temperature for 1.5 h under an Ar atmosphere. To this was added DIPEA (37.8 µL, 0.217 mmol) and **1** (64.9 mg, 0.197 mmol) in DMF (2 mL) and the stirring was continued for 2 h. The reaction was quenched by adding sat. NH_4Cl solution and diluted with ethyl acetate. The organic layer was separated, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was suspended in methanol. The precipitate was collected by vacuum filtration and dried under a vacuum to yield **MOM-Bhc-SAHA** (94.4 mg, 0.168 mmol, 86% yield) as a white solid.

MOM-Bhc-SAHA ^{1}H NMR (300 MHz, DMSO-d_6, Me_4Si) δH 1.26-1.29 (4H, m), 1.49-1.59 (4H, m), 1.98 (2H, t, *J* 7.5), 2.28 (2H, t, *J* 7.5), 3.42 (3H, s, OCH_3), 5.00 (2H, br s, C(4)-CH_2-O), 5.43 (2H, s, CH_3OCH_2O), 6.49 (1H, s, 3-H), 7.00 (1H, m), 7.24-7.30 (2H, m), 7,27 (1H, s, 8-H), 7.56-7.59 (2H, m), 8.30 (1H, s, 5-H), 9.84 (1H, br s, NH), 11.27 (1H, br s, NH)

^{13}C NMR (75 MHz, DMSO-d_6) δC 24.54, 24.71, 24.93, 28.28, 32.09, 36.33, 56.21, 72.77, 94.80, 103.45, 107.62, 113.07, 113.20, 119.00, 122.87, 128.59, 129.59, 139.31, 149.24, 153.80, 155.28, 159.50, 169.94, 171.16.

IR (neat) ν_{max}/cm^{-1} 1718, 1690, 1667, 1152, 1029, 958

HRMS (ESI) Calcd for $C_{26}H_{30}{}^{79}BrN_2O_7$ $[M+H]^+$: 561.1231; Found: 561.1221.

To a stirred solution **MOM-Bhc-SAHA** (28.9 mg, 0.0515 mmol) in anhydrous CH_2CH_2 (1 mL) was added dropwise TFA (1 mL) at 0 °C. After 2 h of stirring at rt, the solvents were removed by a rotary evaporator. The trace amount of TFA was azeotropically removed by repeated treatment of toluene and evaporation under a high vacuum to yield **Bhc-SAHA (2)** (26.4 mg, 0.0500 mmol, 91% yield).

Bhc-SAHA (2) ^{1}H NMR (400 MHz, $CDCl_3$, Me_4Si) δH 1.37-1.45 (4H, m), 1.68-1.78 (4H, m), 2.36 (2H, t, *J* 7.2), 2.47 (2H, t, *J* 7.2), 5.23 (2H, s, CH_3CH_2O), 6.35 (1H, s, 3-H), 7.03 (1H, s, 8-H), 7.10 (1H, m), 7.30-7.34 (2H, m), 7.36 (1H, m, NH), 7.52-7.54 (2H, m), 7.65 (1H, s, 5-H).

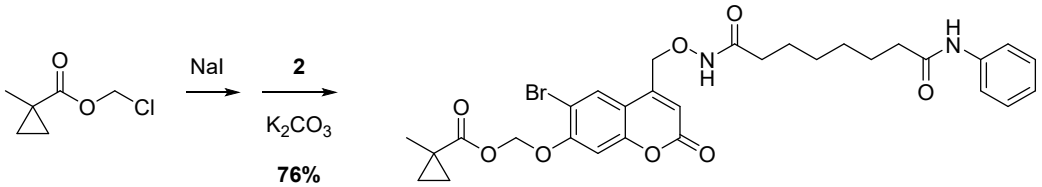
^{13}C NMR (75 MHz, CD_3OD, Me_4Si) δC 26.32, 26.65, 29.76, 29.84, 33.57, 37.82, 74.56, 104.26, 108.21, 112.83, 113.07, 121.27, 125.10, 129.74, 130.54, 139.87, 150.71, 155.88, 159.44, 162.60, 173.33, 174.58.

IR (neat) ν_{max}/cm^{-1} 1710vs (C=O), 1651s, 1164s, 1077s, 1041s

UV-vis: λ_{max} (10 mM HEPES (pH 7.5)) 374 nm (ε/dm^3 mol^{-1} cm^{-1} 11,800)

HRMS (ESI) Calcd for $C_{24}H_{26}{}^{79}BrN_2O_6$ $[M+H]^+$: 517.0969; Found: 517.0958.

Synthesis of ((6-bromo-2-oxo-4-(((8-oxo-8-(phenylamino)octanamido)oxy)methyl)-2H-chromen-7-yl)oxy)methyl 1-methylcyclopropane-1-carboxylate (3, CM-Bhc-SAHA)



CM-Bhc-SAHA (3)

A mixture of chloromethyl 1-methylcyclopropane-1-carboxylate (15.6 mg, 0.105 mmol) and NaI (15.3 mg, 0.111 mmol) in anhydrous acetone (1 mL) was stirred at an ambient temperature for 2 h. A mixture of Bhc-SAHA (18.4 mg, 0.0356 mmol) and K_2CO_3 (10.1 mg, 0.0731 mmol) in acetone (1 mL) was stirred at an ambient temperature for 2 h. To this was added the acetone solution of iodomethyl 1-methylcyclopropane-1-carboxylate prepared above and stirring was continued for 1.5 h at 65 °C. The solvent was removed under a vacuum. The residue was directly subjected to a flash column chromatography (Universal column premium Silica gel, YAMAZEN, linear gradient of 1% to 9% methanol in dichloromethane as eluents) to yield 17.1 mg (0.0272 mmol, 76% yield) of **3** as a white solid.

CM-Bhc-SAHA (3) ^{1}H NMR (400 MHz, DMSO-d_6, Me_4Si) δH 0.81 (2H, dd, *J* 3.9 and 6.8), 1.14 (2H, dd, *J* 3.9 and 6.8), 1.23 (3H, s), 1.27-1.29 (4H, m), 1.49-1.59 (4H, m), 1.98 (2H, t, *J* 7.1), 2.28 (2H, t, *J* 7.4), 5.01 (2H, s, C(4)CH_2O), 5.92 (2H, s, OCH_2O), 6.52 (1H, s, 3-H), 7.01 (1H, m), 7.27 (2H, m), 7.36 (1H, s, 5-H), 7.57 (2H, m), 8.32 (1H, s, 8-H), 9.82 (1H, s, NH), 11.21 (1H, br s, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δC 17.11, 18.75, 19.10, 25.19, 25.24, 28.77, 28.84, 32.59, 36.82, 73.26, 85.69, 104.07, 107.93, 114.24, 114.36, 119.50, 123.38, 129.08, 130.34, 139.80, 149.31, 154.25, 155.54, 159.92, 170.42, 171.68, 174.24.

IR (neat) ν_{max}/cm^{-1} 1745s (C=O), 1655s, 1230s (C-O), 1055 (C-O), 1026s, 991s cm^{-1}

UV-vis: λ_{max} (50% EtOH/HEPES (10 mM, pH 7.5)) 328 nm (ε/dm^3 mol^{-1} cm^{-1} 12,600)

HRMS (ESI) Calcd for $C_{30}H_{34}{}^{79}BrN_2O_8$ $[M+H]^+$: 629.1493; Found: 629.1489

Measurements of Photophysical and Chemical Parameters

The absorption spectra shown in **Fig. 2a** were recorded by using a UV–vis spectrophotometer (Shimadzu UV-2600). Stock solutions of the samples in DMSO (10 mM) were diluted with 10 mM HEPES buffer (pH 7.5) or ethanol. The final concentrations of samples were 10 μM; **2** in HEPES containing 0.1% DMSO, and **3** in 50% ethanol in HEPES.

The hydrolytic stability of **3** under dark conditions was measured in both HEPES buffer (pH 7.4) at an ambient temperature and Dulbecco's modified Eagle's media (DMEM) containing 10% FBS at 37 °C.

Photolysis time courses and stability in the dark were monitored with an HPLC (Chromaster 5110 Pump with 5430 Diode Array Detector). A 300 W xenon lamp (Asahi Spectra MAX-301) with a 405-nm band-pass filter (HQBP405-VIS, Asahi Spectra) and short wavelength UV-cut filter (385 nm, Asahi Spectra) were used for photolysis experiments. Aliquots of 10 μL were removed periodically and analyzed by HPLC (column: Hitachi, L-column 2 ODS, 3 μm, 4.6 x 150 mm); eluent: 1.0 mL/min, linear gradient of 20–40% (9–12 min) acetonitrile in water containing 0.1% TFA; monitored at 240 nm). Peak areas of **Bhc-SAHA (2)** (retention time: 18.1 min) and **SAHA** (retention time: 7.3 min) were quantified and converted to the molar ratios of **2** and **SAHA** using the molar absorptivity of the compounds (**Fig. 2b** & **S1**). The light output for the quantum efficiencies measurement was performed using ferrioxalate actinometry.[2] Quantum yields of photolysis were calculated from the plot shown in **Fig. 2b** following the method described in our previous report.[3]

Enzymatic reactions

The PLE-dependent production of **Bhc-SAHA (2)** from **CM-Bhc-SAHA (3)** was monitored by reverse phase HPLC. The enzyme reactions were initiated by adding 1.5×10^{-2} U of PLE (Esterase from porcine liver, ammonium sulfate suspension, Sigma E2884-1KU) to the 1000 μL of reaction solution containing various concentrations of **3** in 10 mM HEPES buffer (pH 7.5). Enzymatic reactions were monitored by HPLC using the same conditions as described for the photolysis of **2** except for the following elution conditions: linear gradient of 1–99% (0–10 min) acetonitrile in water containing 0.1% TFA monitored at 325 nm (**Fig. 3a** & **S2**).

Kinetic parameters measurements: Kinetic parameters of **CM-Bhc-SAHA** toward PLE were determined by methods similar to those reported in our previous study.[4] Kinetic parameters K_m and V_{max} for the reaction of **CM-Bhc-SAHA** and PLE were determined directly from the Michaelis-Menten plots following the equation: $V_0 = (V_{max}*[S])/(K_m+[S])$ and least-squares method toward V_0 plots. The estimated kinetic parameters were K_m 3.2 μM and k_{cat}/K_m 2.7×10^{-5} M^{-1} s^{-1}.

Cell lysates preparation: HEK293T cells (PLE(-)) were provided by the RIKEN BRC through the National BioResource Project of the MEXT, Japan. PLE(+) cells were prepared by transducing HEK293T cells with

lentivirus carrying the *h*KO1 (humanized Kusabira Orange 1)-P2A-PLE gene. Preparation of cell lysates was done by following the methods reported in our previous study.[4] In brief, cells were washed once with PBS (-), and lysed with 1× lysis buffer (1 mL) by tapping the dish. Then the lysed cells were transferred into 1.5 mL microcentrifuge tubes, centrifuged at 13000 rpm at 4 °C for 10 min, and the supernatants were used as cell lysates.

Enzymatic reactions in the presence of PLE-expressing cell lysates: To a 10 μL of **CM-Bhc-SAHA** (5.4 mM) in 890 μL of HEPES buffer (10 mM, pH 7.5) was added a 100 μL of HEK293T cell lysates, and the mixture was incubated at 37 °C. Aliquots of the reaction mixture (100 μL) were monitored by HPLC. Photolysis was done by using a 300 W xenon lamp (Asahi Spectra MAX-301) with a band pass filter (405 nm) and a short wavelength UV-cut filter (385 nm). Samples in a quartz cuvette were irradiated and analyzed by reverse phase HPLC (**Fig. 3b, S3** & **S4**). Two elution conditions were used. Condition A_{325}: linear gradient of 1–99% (0-10 min) acetonitrile in water (0.1% TFA) monitored at 325 nm for **2** and **3**; Condition A_{240}: isocratic elution with 40% acetonitrile in water (0.1% TFA) monitored at 240 nm for **SAHA**.

Measurement of HDACi activity of caged SAHAs to estimate IC_{50} values

Inhibitory activity of caged SAHAs were measured using HDAC-Glo I/II Screening System (G6430, Promega). We followed the supplier's protocol. The HeLa nuclear extracts supplied with the kit were used as an HDAC enzyme source. HeLa nucleus mainly includes class I HDACs (HDAC 1, 2, 3, and 8). HDAC activity was quantified by measuring luminescence signals from the liberated luciferin-luciferase using a 2030 Multilabel Reader ARVO X4 (Perkin Elmer). Inhibitory profiles are shown in **Fig. 4a**, and IC_{50} of all the compounds was calculated from data from quadruplicated experiments.

Photo-mediated inhibition of HDAC activity in cuvettes

Inhibitory activity of **Bhc-SAHA** (**2**) was measured and quantified using HDAC-Glo I/II Screening System as described above. To the 300-fold diluted HeLa cell nuclear extracts, either an equal volume of DMSO or **2** (final concentration 500 nM) was added. The mixture was exposed to 405 nm light using the same light source as described in the photolysis experiments (**Fig. S5**).

As shown in **Fig. S5**, the difference in HDAC activity between UV(-) and (+) showed statistical significance even in the absence of caged SAHA **2** (**Fig. S5**, DMSO series, 0 mJ vs 204 mJ, for example). The reason for the decrease in HDAC activity can be accounted for by the increase in the amount of damaged protein, including HDACs, upon photoirradiation. Since we used the HeLa cell nuclear extracts (basically, mixtures of nuclear proteins) in the HDAC Glo I/II assay kit as an HDAC source instead of a purified enzyme, we speculated that some photon-absorbing ingredients cause non-selective damage to proteins upon 405 nm irradiation. In Nakagawa's paper[5], photoirradiation (400-430nm, 660 mJ/cm^2) showed no effect on HDAC activity (Fig. 5 in ref. [5]). In our live cell experiments shown in **Fig. 4**, the same intensity of 405 nm light (204 mJ/cm^2) as in **Fig. S5** showed almost no effect on intrinsic nuclear HDAC activity in HEK293T cells

(**Fig 4b**, DMSO, 405 nm - & +). These results infer that HDAC activity itself is not photosensitive upon 405 nm irradiation. Therefore, we believe that the decrease in HDAC activity observed in **Fig. S5** is specific to the nuclear extracts used in our experiments and cannot be generalized.

Live cell experiments

The PLE (-) and PLE (+) HEK293T cells described above were used. Their intrinsic HDAC activity was measured and quantified using HDAC Cell-Based Activity Assay Kit (Cayman Chemical, 600150). A stock solution of CM-Bhc-SAHA in DMSO (10 mM) was diluted by DMEM to the final concentration indicated in Fig. 4 and was added to cells plated on a 96-well black culture plate. The cells were incubated at 37 °C for 1 hr and were exposed to 405 nm light for 60 s using the same 405 nm light source as above. After the incubation and irradiation, the endogenous activity of HDACs was measured by following the manufacturer's standard protocol (**Fig. 4b-d** & **S6**).

Supplementary References

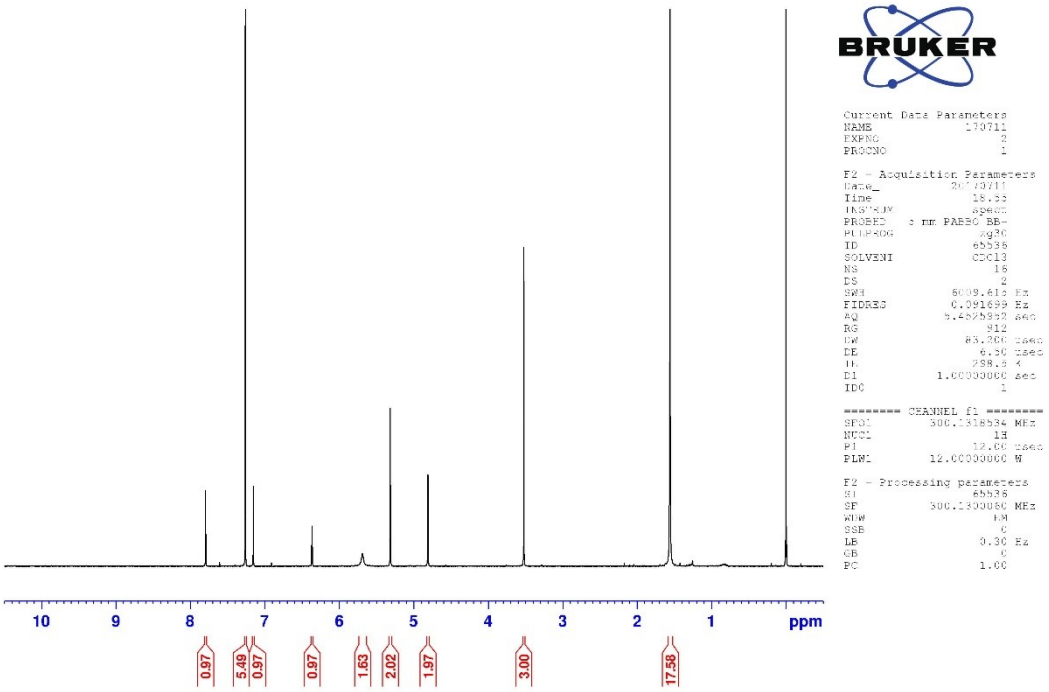
[1] A. Z. Suzuki, T. Watanabe, M. Kawamoto, K. Nishiyama, H. Yamashita, M. Ishii, M. Iwamura and T. Furuta, *Org. Lett.*, **2003**, *5*, 4867-4870.

[2] C. G. Hatchard and C. A. Parker, *Proc. R. Soc. London Ser. A*, **1956**, *235*, 518.

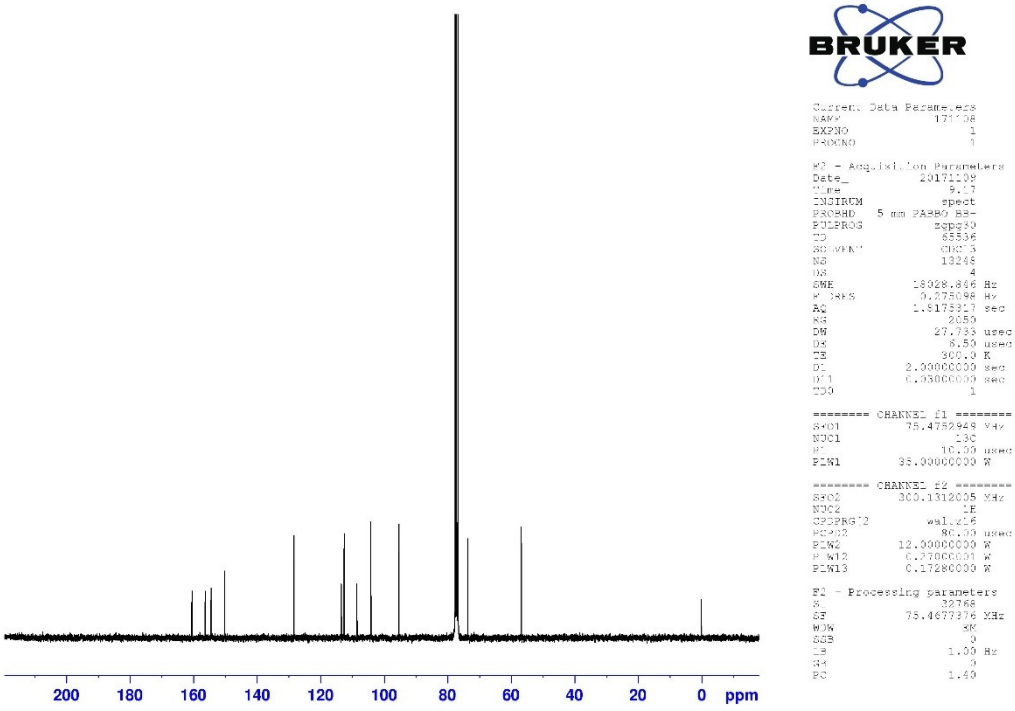
[3] T. Furuta, H. Takeuchi, M. Isozaki, Y. Takahashi, M. Kanehara, M. Sugimoto, T. Watanabe, K. Noguchi, T. M. Dore, T. Kurahashi, M. Iwamura and R. Y. Tsien, *ChemBioChem*, **2004**, *5*, 1119-1128.

[4] A. Z. Suzuki, T. Sakano, H. Sasaki, R. Watahiki, M. Sone, K. Horikawa, T. Furuta, *Chem. Commun.* **2021**, *57*, 5630-5633.

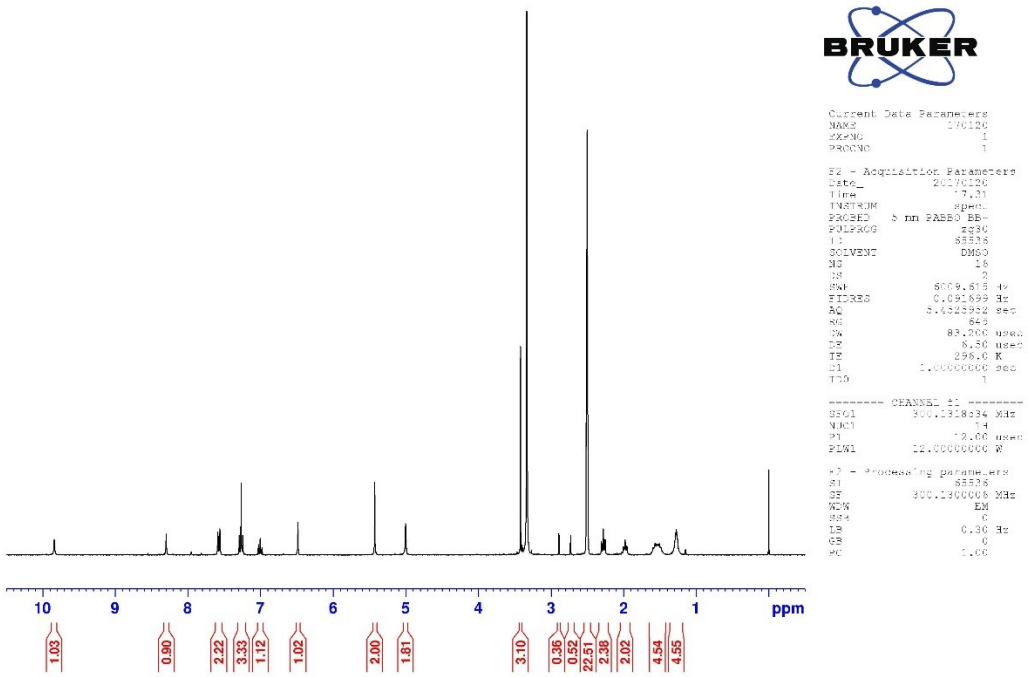
[5] N. Ieda, S. Yamada, M. Kawaguchi, N. Miyata and H. Nakagawa, *Bioorg. Med. Chem.*, **2016**, *24*, 2789-2793.



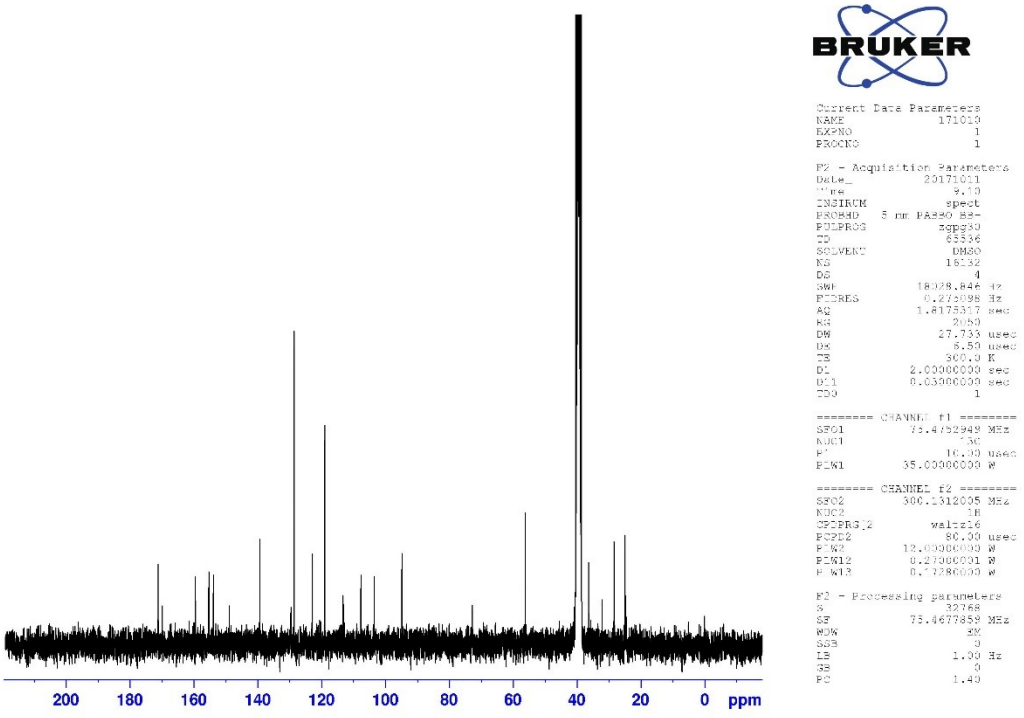
^{1}H NMR spectrum of **1**



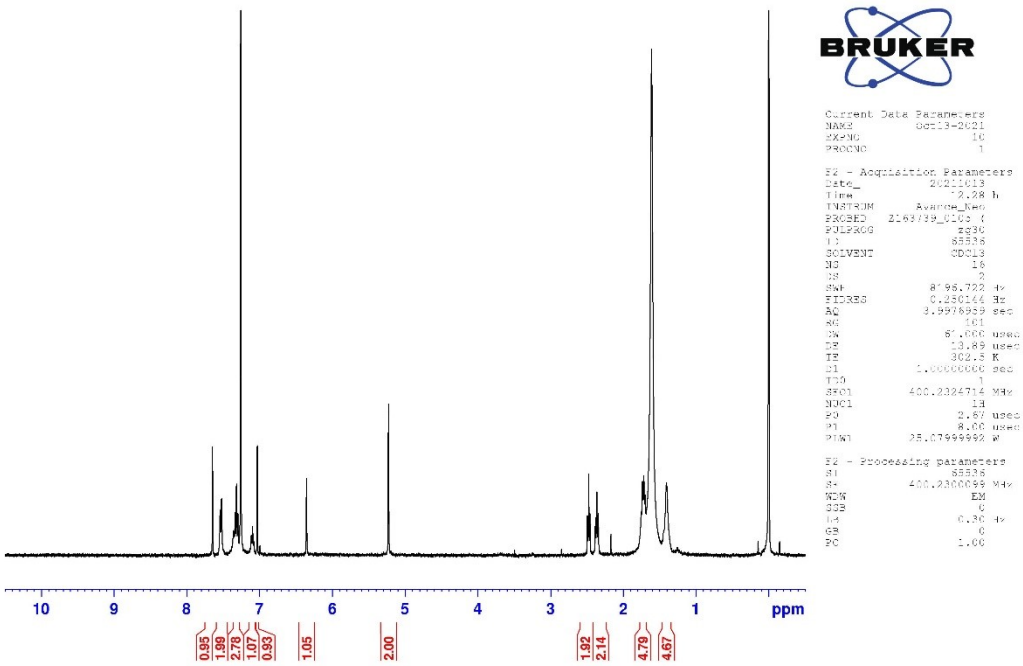
^{13}C NMR spectrum of **1**



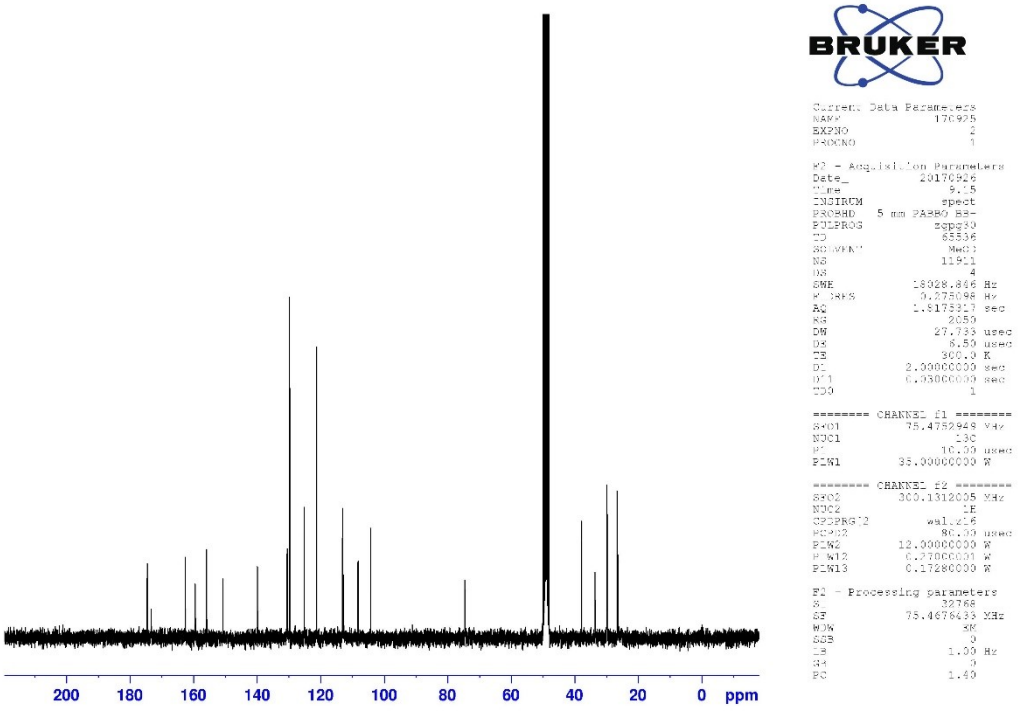
^{1}H NMR spectrum of **MOM-Bhc-SAHA**



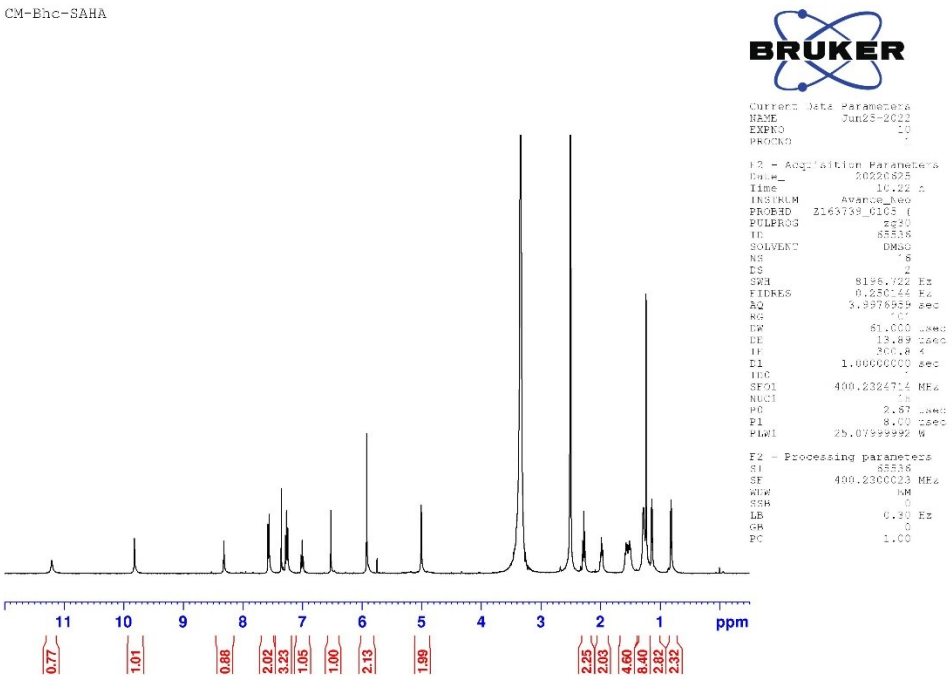
^{13}C NMR spectrum of **MOM-Bhc-SAHA**



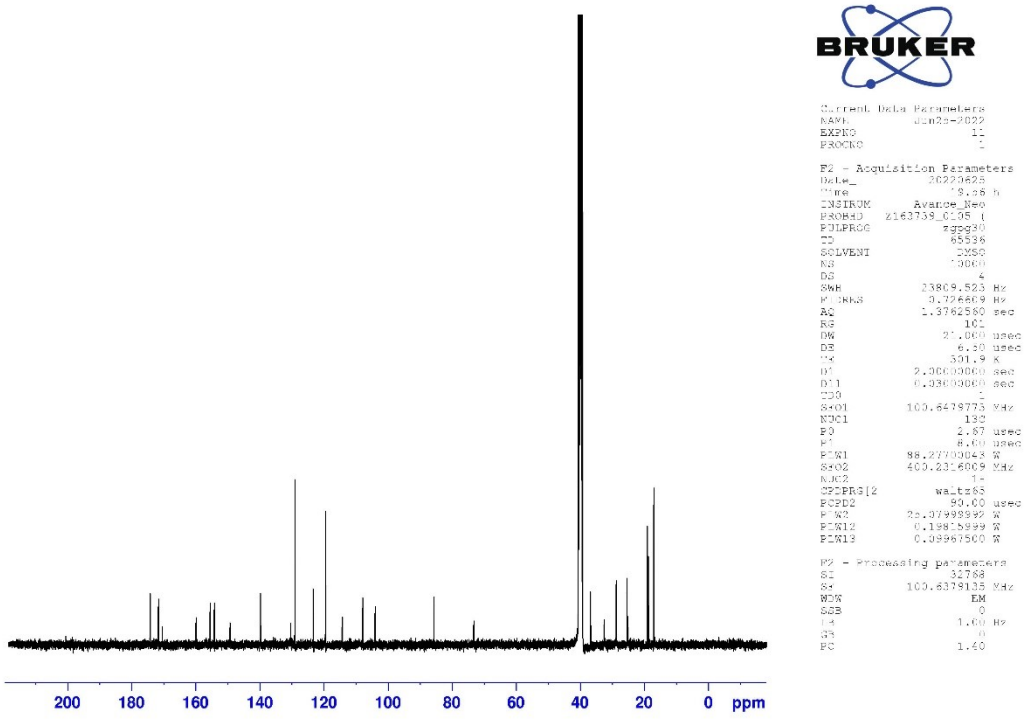
^{1}H NMR spectrum of **2**



^{13}C NMR spectrum of **2**



^{1}H NMR spectrum of **3**



^{13}C NMR spectrum of **3**