Supporting Information

Development of A New Benzophenone-Diketopiperazine-Type Potent Anti-Microtubule Agent Possessing a 2-Pyridine Structure

Yoshiki Hayashi†, Haruka Takeno†, Takumi Chinen‡, Kyohei Muguruma†, Kohei Okuyama[§,‖], Akihiro Taguchi†, Kentaro Takayama†, Fumika Yakushiji†, Masahiko Miura[§], Takeo Usui‡ and Yoshio Hayashi†*

†Department of Medicinal Chemistry, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

‡Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

[§]Section of Oral Radiation Oncology, Department of Oral Health Science, and [‖]Section of Maxillofacial Surgery, Department of Maxillofacial and Neck Reconstruction, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8549, Japan

Contents

Experimental Methods

1. Chemistry

1-1. General

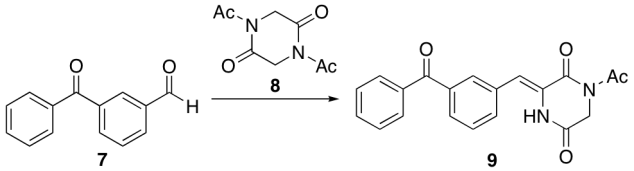
Reagents and solvents were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), and Aldrich Chemical Co., Inc. (Milwaukee, WI), and were used without further purification. Column chromatography was performed using silica gel 60N (Kanto Chemical Co., Inc., spherical natural, 40–50 μm). TLC was performed using Merck Silica gel 60F254 precoated plates. Melting points were measured on a Yanaco MP-500D micro melting point apparatus without correction. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; SunFire^{TM} C18 5 μM 4.6 × 150 nm) and a binary solvent system that formed a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL/min. The UV absorption was detected at 230 nm. Solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. ^1H and ^{13}C NMR spectra were obtained using a BRUKER DPX-400 spectrometer (400 MHz) with tetramethylsilane as an internal standard. Chemical shifts are reported in parts-per-million (ppm) and are referenced relative to tetramethylsilane (0.00 ppm), the corresponding deuterated solvent (DMSO-d_6, 2.50 ppm, for the ^1H spectra; or DMSO-d_6, 39.52 ppm or $CDCl_3$, 77.16 ppm, for the ^{13}C spectra). High-resolution mass spectra (ESI-TOF MS) were recorded using a micromass Q-Tof Ultima API mass spectrometer.

1-2. Synthesis

1-2-1. (*Z*)-*N*-acetyl-3-(3-benzoylbenzylidene)piperazine-2,5-dione (9)

To a solution of 3-benzoylbenzaldehyde **7** (10.0 g, 47.6 mmol) in DMF (420 mL) was added *N*,*N*'-diacetylpiperazine-2,5-dione **8** (11.3 g, 57.1 mmol), and the solution was repeatedly evacuated over a short period of time to remove oxygen and flush the solution with Ar. A solution of potassium *tert*-butoxide (5.60 g, 50.0 mmol) in DMF (50.0 mL) and *tert*-butanol (50.0 mL) was then added dropwise under an Ar atmosphere at –15 °C. The resultant mixture was stirred at –15 °C. After 2.5 h stirring, the mixture was added to 10% citric acid (120 mL) and stirred for an additional 30 min. The solvent was removed by evaporation, and the residue was dissolved in $CHCl_3$, washed with 10% citric acid and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was purified by column chromatography on silica-gel using $CHCl_3$-MeOH (200:1 to 30:1) as an eluent. The product was recrystallized from EtOAc-Et_2O to give a white solid of the desired compound **9** (10.7 g, 64%); mp 182–184 °C; IR (KBr, cm^{-1}) 3206, 3060, 2937, 1696, 1678, 1648,

1637, 1597; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (s, 1H), 7.84 (s, 1H), 7.81–7.72 (m, 3H), 7.65–7.53 (m, 3H), 7.49 (t, *J*= 7.7 Hz, 2H), 7.20 (s, 1H), 4.44 (s, 2H), 2.65 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.9, 172.5, 163.3, 160.0, 138.8, 137.0, 133.08, 133.06, 132.4, 130.7, 130.2, 130.1, 129.5, 128.6, 126.8, 118.9, 46.2, 27.4; HRMS (ESI): *m/z* 349.1180 $[M+H]^+$ (Calcd for $C_{20}H_{17}N_2O_4$: 349.1188).



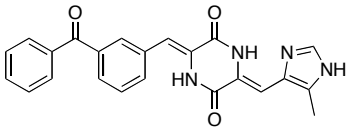
Scheme S1. Reagents and conditions: *t*-BuOK, *t*-BuOH, DMF, –15 °C, 2.5 h.

1-2-2. (*Z*)-3-(3-benzoylbenzylidene)piperazine-2,5-dione (10)

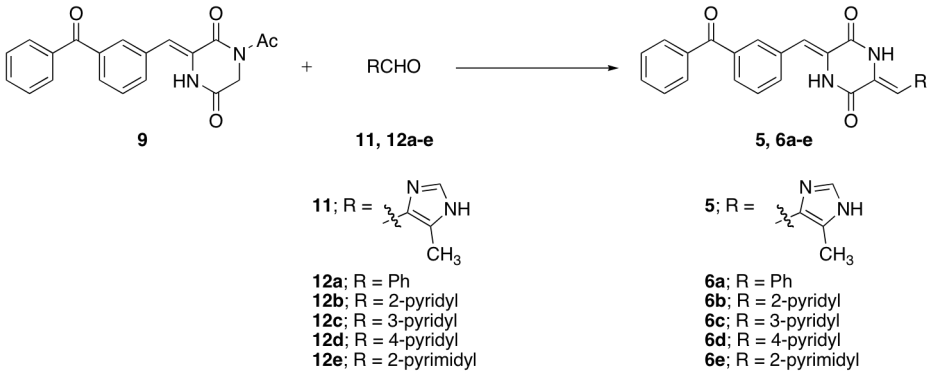
To a solution of (*Z*)-*N*-acetyl-3-(3-benzoylbenzylidene)piperazine-2,5-dione **9** (13 mg, 0.0373 mmol) in DMF (2 mL) was added 28% NH_4OH (0.26 mL, 4.29 mmol). The mixture was then stirred for 2 h at room temperature. After the solvent had been removed by evaporation, the residue was dissolved in AcOEt, washed with 10% $NaHCO_3$ and brine, and dried over Na_2SO_4. Concentration *in vacuo* gave a white solid of the desired compound **10** (6.0 mg, 53%); mp 205–207 °C; IR (KBr, cm^{-1}) 3198, 3058, 1682, 1661, 1632, 1594, 1448, 1277; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (br s, 1H), 7.83–7.74 (m, 4H), 7.65–7.47 (m, 5H), 7.01 (s, 1H), 6.64 (s, 1H), 4.24 (d, *J* = 1.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.0, 162.9, 159.5, 138.9, 137.2, 133.2, 133.0, 132.1, 130.3, 130.2, 130.0, 129.6, 128.7, 126.2, 115.4, 45.6; HRMS (ESI): *m/z* 329.0896 $[M+Na]^+$ (Calcd for $C_{18}H_{14}N_2O_3Na$: 329.0902).

Scheme S2. Reagents and conditions: aqueous 28% NH_4OH, DMF, rt, 1 h.

1-2-3. (3*Z*,6*Z*)-3-(3-benzoylbenzylidene)-6-((5-methyl-1*H*-imidazol-4-yl)methylene)piperazine-2,5-dione (5)



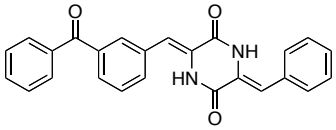
To a solution of (*Z*)-*N*-acetyl-3-(3-benzoylbenzylidene)piperazine-2,5-dione **9** (50 mg, 0.144 mmol) in DMF (2.0 mL) was added 4-methyl-5-imidazolecarboxaldehyde **11** (19.0 mg, 0.173 mmol). The solution was repeatedly evacuated over a short period of time to remove oxygen. The solution was then flushed with Ar. Cs_2CO_3 was then added (70.4 mg, 0.216 mmol) and the evacuation–flushing process was repeated again. The resultant mixture was stirred at 110 °C for 2 h, and the progression of the reaction was monitored by TLC. After the reaction had finished, the solvent was removed by evaporation and the residue was dissolved in $CHCl_3$, washed with 10% $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The product was purified by column chromatography using $CHCl_3$-MeOH as an eluent to give a yellow solid **5** (24.7 mg, 43%); mp 191–194 °C; IR (KBr, cm^{-1}) 3196, 3062, 1684, 1661, 1635, 1596, 1419; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.52 (br s, 1H), 11.99 (s, 1H), 10.31 (br s, 1H), 7.87 (s, 1H), 7.85–7.79 (m, 3H), 7.78–7.74 (m, 1H), 7.74–7.67 (m, 1H), 7.66–7.62 (m, 1H), 7.61–7.56 (m, 3H), 6.80 (s, 1H), 6.59 (s, 1H), 2.32 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.6, 157.6, 156.1, 137.4, 136.9, 135.1, 133.6, 133.3, 132.8, 132.5, 130.2, 129.82, 129.79, 129.2, 128.8, 128.6, 127.8, 123.5, 112.6, 104.2, 9.1; HRMS (ESI): *m/z* 399.1453 $[M+H]^+$ (Calcd for $C_{23}H_{19}N_4O_3$: 399.1457).



Scheme S3. Reagents and conditions: Cs_2CO_3, degassed DMF, 110 °C.

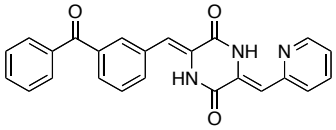
Compounds **6a-d** were prepared from compound **9** and corresponding aldehydes **12a-d** according to the procedure described for the synthesis of **5**.

1-2-4. (3*Z*,6*Z*)-3-(3-benzoylbenzylidene)-6-benzylidenepiperazine-2,5-dione (6a)



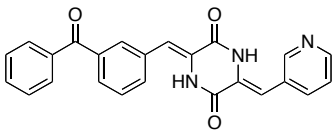
Yield of 58% from **9** and benzaldehyde **12a**; mp 247–249 °C; IR (KBr, cm^{-1}) 3212, 3060, 1686, 1655, 1630, 1597, 1412; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.54 (br s, 1H), 10.30 (br s, 1H), 7.88–7.80 (m, 3H), 7.80–7.75 (m, 1H), 7.74–7.64 (m, 2H), 7.63–7.51 (m, 5H), 7.48–7.37 (m, 2H), 7.37–7.29 (m, 1H), 6.84 (s, 1H), 6.78 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.5, 158.1, 157.7, 137.4, 136.8, 133.42, 133.37, 133.1, 132.8, 130.2, 129.8, 129.3, 129.0, 128.8, 128.7, 128.6, 128.2, 127.5, 126.3, 115.1, 113.8; HRMS (ESI): *m/z* 417.1209 $[M+Na]^+$ (Calcd for $C_{25}H_{18}N_2O_3Na$: 417.1215).

1-2-5. (3*Z*,6*Z*)-3-(3-benzoylbenzylidene)-6-(2-pyridylmethylene)piperazine-2,5-dione (6b)



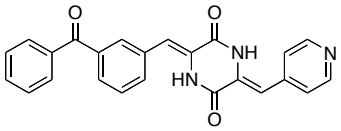
Yield of 31% from **9** and 2-pyridinecarboxaldehyde **12b**; mp 263–266 °C; IR (KBr, cm^{-1}) 3073, 3053, 1692, 1653, 1643, 1587, 1355; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.60 (s, 1H), 10.64 (s, 1H), 8.73 (d, *J* =4.8 Hz, 1H), 7.95–7.76 (m, 5H), 7.74–7.64 (m, 3H), 7.64–7.56 (m, 3H), 7.41–7.35 (m, 1H), 6.91 (s, 1H), 6.73 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.5, 156.9, 156.6, 154.6, 148.5, 137.8, 137.4, 136.8, 133.5, 133.2, 132.8, 131.0, 130.3, 129.8, 129.1, 128.8, 128.6, 127.1, 126.5, 122.5, 114.4, 107.7; HRMS (ESI): *m/z* 418.1171 $[M+Na]^+$ (Calcd for $C_{24}H_{17}N_3O_3Na$: 418.1168).

1-2-6. (3*Z*,6*Z*)-3-(3-benzoylbenzylidene)-6-(3-pyridylmethylene)piperazine-2,5-dione (6c)



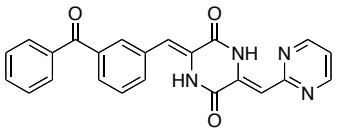
Yield of 86% from **9** and 3-pyridinecarboxaldehyde **12c**; mp 258–260 °C; IR (KBr, cm^{-1}) 3204, 3055, 3036, 1686, 1654, 1630, 1595, 1413; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 2H), 8.69 (d, *J* = 2.1 Hz, 1H), 8.48 (dd, *J* = 4.8, 1.5 Hz, 1H), 7.93 (dt, *J* = 8.1, 1.8 Hz, 1H), 7.88–7.74 (m, 4H), 7.73–7.64 (m, 2H), 7.63–7.55 (m, 3H), 7.42 (dd, *J* = 7.9, 4.9 Hz, 1H), 6.85 (s, 1H), 6.76 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.5, 157.9, 157.8, 150.2, 148.4, 137.4, 136.8, 136.2, 133.4, 132.8, 131.9, 130.2, 129.8, 129.4, 129.0, 128.9, 128.6, 128.2, 127.5, 123.5, 114.0, 111.4; HRMS (ESI): *m/z* 396.1351 $[M+H]^+$ (Calcd for $C_{24}H_{18}N_3O_3$: 396.1348)

1-2-7. (3*Z*,6*Z*)-3-(3-benzoylbenzylidene)-6-(4-pyridylmethylene)piperazine-2,5-dione (6d)



Yield of 14% from **9** and 4-pyridinecarboxaldehyde **12d**; mp 258–260 °C; IR (KBr, cm^{-1}) 3215, 3056, 1692, 1657, 1633, 1593, 1415; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.63 (br s, 2H), 8.57 (d, *J* = 6.0 Hz, 2H), 7.88–7.74 (m, 4H), 7.73–7.64 (m, 2H), 7.63–7.55 (m, 3H), 7.50–7.44 (m, 2H), 6.87 (s, 1H), 6.69 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.5, 157.9, 157.6, 149.8, 140.6, 137.4, 136.8, 133.4, 133.3, 132.9, 130.3, 129.8, 129.6, 129.1, 128.9, 128.6, 127.4, 123.6, 114.4, 111.5; HRMS (ESI): *m/z* 396.1341 $[M+H]^+$ (Calcd for $C_{24}H_{18}N_3O_3$: 396.1348).

1-2-8. (3*Z*,6*Z*)-3-(3-benzoylbenzylidene)-6-(2-pyrimidylmethylene)piperazine-2,5- dione (6e)



To a solution of 2-cyanopyrimidine **13** (208 mg, 2.0 mmol) in anhydrous THF (20 mL) was added dropwise DIBAL-H (a 1.0 M solution in toluene 2.4 mL, 2.4 mmol) at –78 °C under an Ar atmosphere. The cooling bath was then removed and the reaction mixture was stirred for 2 h at 0 °C. The reaction mixture was quenched by the addition of MeOH-AcOH (2:1), and a saturated solution of Rochelle salt was added. The mixture was then stirred for an additional 30 min at room temperature, extracted with $CHCl_3$, and washed with 10% $NaHCO_3$ and brine. The organic layer was then dried over Na_2SO_4 and concentrated *in vacuo*. The resultant aldehyde was difficult to purify; therefore, the residue was used directly without further purification. The residue was dissolved in DMF (0.5 mL), and (*Z*)-*N*-acetyl-3-(3-benzoylbenzylidene)piperazine-2,5-dione **9** (30 mg, 0.086 mmol) was added to the solution. The mixture was repeatedly evacuated over a short period of time to remove the oxygen, and the flask was flushed with Ar. Cs_2CO_3 was then added (53.3 mg, 0.16 mmol) and the evacuation–flushing process was repeated again. The resultant mixture was stirred at 110 °C for 2 h, and the progression of the reaction was monitored using TLC. After the reaction had finished, the solvent was removed by evaporation, and the residue was dissolved in $CHCl_3$, washed with 10% $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was purified by column chromatography using $CHCl_3$-MeOH as an eluent to give a yellow solid **6e** (7.6 mg, 2%); mp 256–259 °C; IR (KBr, cm^{-1}) 3204, 3064, 1697,

1678, 1645, 1568, 1556, 1403; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.02 (s, 1H), 10.81 (br s, 1H), 8.95 (d, J = 4.9 Hz, 2H), 7.89 (s, 1H), 7.87–7.79 (m, 3H), 7.73–7.65 (m, 2H), 7.65–7.56 (m, 3H), 7.42 (t, J = 5.0 Hz, 1H), 6.94 (s, 1H), 6.59 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.5, 163.5, 157.4, 156.9, 156.5, 137.4, 136.8, 134.9, 133.6, 132.9, 130.4, 129.8, 129.2, 128.8, 128.6, 118.7, 115.2, 107.1; HRMS (ESI): *m/z* 419.1117 $[M+Na]^+$ (Calcd for $C_{23}H_{16}N_4O_3Na$: 419.1120).

1-3. Crystal data

The crystal data of **6b** was shown in below. CCDC 998560 contains these supplementary crystallographic data and these data can be obtain free of charge from the Cambridge Crystallographic Data Sentre via www.ccdc.cam.ac.uk/data_request/cif.

Identification code	KPU-300
Chemical formula	$C_{24}H_{17}N_3O_3$
Formula weight	395.41
Temperature	90 K
Wavelength	0.71073 Å
Crystal system	monoclinic
Space group	C 1 2/c 1
Unit cell dimensions	a = 35.615(11) Å α = 90°
	b = 7.070(2) Å β = 112.863(4)°
	c = 15.551(5) Å γ = 90°
Volume	3608.1(19) Å^3
Z	8
Density (calculated)	1.456 g/cm^3
Absorption coefficient	0.098 mm^{-1}
F(000)	1648
Crystal size	0.020 x 0.100 x 0.170 mm
Theta range for data collection	2.48 to 25.03°
Index ranges	-42<=h<=42, -5<=k<=8, -18<=l<=18
Reflections collected	8160
Independent reflections	3189 [R(int) = 0.0329]
Coverage of independent reflections	99.7%
Absorption correction	multi-scan
Max. and min. transmission	0.9980 and 0.9835

Refinement method		Full-matrix least-squares on F2
Data / restraints / parameters		3189 / 0 / 277
Goodness-of-fit on F2		1.040
Final R indices	2430 data; $I>2\sigma(I)$	R1 = 0.0396, wR2 = 0.0927
	all data	R1 = 0.0580, wR2 = 0.1002
Largest diff. peak and hole		0.213 and -0.237 $eÅ^{-3}$

2. Biological evaluation

2-1. HT-29 cell culture conditions

HT-29 cells were purchased from ATCC and maintained in McCoy's 5A medium containing 10% fetal bovine serum supplemented with 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2-2. XTT/PMS-based cytotoxicity assay

The XTT/PMS assay was performed as described previously.[1] Briefly, HT-29 cells were plated in 96-well plates at 2500 cells/well the day before the test compound addition step. Stock solutions of the compounds were prepared in DMSO. Serially diluted compounds were added to the cells, resulting in a final concentration that ranged from 0.1 to 100 nM for **6b**, 1 to 1000 nM for **5** and **6a**, **6c–e**, or 0.002 to 20000 nM for **9** and **10**. Forty-eight hours later, a 0.1 mg/mL XTT solution in PBS buffer containing 25 μM phenazine methosulfate was added to each well, and the cells were incubated for an additional 2 h. The absorbance of the formazan product was measured at 492 nm using a plate reader (TECAN SAFIRE). To compensate for the non-specific absorption, the absorbance at 690 nm was also measured.

2-3. Tubulin binding assay

Fluorescence spectra were measured at 37 °C as described previously.[2,3] Porcine tubulin (0.5 μM) in MES buffer (0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, pH 6.8) was incubated with different concentrations of the test compounds (0–20 μM, 1% DMSO) at 37 °C for 1 h. After incubation, the fluorescence of each solution was measured (excitation at 295 nm, emission at 300–450 nm) using an FP-6200 Spectrofluorometer (JASCO, JAPAN).

2-4. *In vitro* tubulin polymerization assay

Tubulin was purified from porcine brain using high-molarity buffer.[4] Turbidity assays were performed by incubating 1 mg/mL tubulin in RB buffer (100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.8) with 1 mM GTP and 1 M glutamate. The absorbance at 350 nm was monitored at 37 °C using a thermostatic spectrophotometer (Beckman Coulter Inc., Brea, CA)

2-5. HeLa and A549 cell culture conditions and WST-8-based cytotoxicity assay

The human cervix epidermoid carcinoma cell line, HeLa, and the human lung carcinoma cell line, A549, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (Nichirei Bioscience Inc., Tokyo, Japan). Cells were grown at 37 °C in a 5% CO_2 atmosphere. The IC_{50} values were calculated based on the measurements obtained before and after treating the cells (3×10^4 cells/mL) with various concentrations of each compound for 48 h. Cell growth was measured using the WST-8 cell counting kit.

2-6. NHSF46 cell culture conditions and XTT/PMS-based cytotoxicity assay

The human diploid fibroblasts cells, NHSF46, were purchased from Riken BioResource Center (Ibaraki, JAPAN) and cultured in MEM Alpha medium containing 10% fetal bovine serum supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C under a humidified 5% CO_2 atmosphere. The XTT/TMS assay was performed by the same procedure as that used for HT-29 cells with a final concentration ranging from 1 to 100 nM of derivative **6b**.[1] The absorbance of the formazan product was measured at 490 nm on a Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, USA). The IC_{50} value was obtained as an average from two independent dose response curves.

2-7. HMNC cell culture conditions and WST-1-based cytotoxicity assay

The human peripheral blood mononuclear cells (HMNC) were purchased from Cell Applications, Inc. (San Diego, USA) and cultured in blood cell culture medium at 37 °C under a humidified 5% CO_2 atmosphere. For calculation of IC_{50} values, cells were plated in 96-well plates at 2.5×10^4 cells/well. Derivative **6b** was added to the cells, resulting in a final concentration ranging from 2 to 2000 nM. 48 h later, 10 μL of WST-1 reagent (Roche Diagnostics GmbH, Germany) was added to each well and the cells were incubated for an additional 2 h. The absorbance of the formazan product was measured at 450 nm on Multiskan FC (Thermo Fisher Scientific, K.K., USA). To compensate for the non-specific absorption, the absorbance at 620 nm was also measured. The IC_{50} value was calculated as the mean ± SD from three independent dose response curves.

2-8. Immunofluorescence

HeLa cells (3×10^4 cells /mL) were placed on sterile coverslips and were treated with a given concentration of each compound for 6 h. Coverslips were fixed using –20 °C MeOH for 5 min and were washed in PBS-B (PBS containing 0.5% w/v BSA). Next, the coverslips were coated with an anti-α-tubulin antibody (sc-32293, Santa Cruz Biotechnology Inc., Santa Cruz, CA) in PBS-B, and placed in a humidified container at 37 °C for 1 h. The coverslips were then washed twice with PBS-B, coated with a solution of Alexa Fluor 488−conjugated anti-mouse IgG antibody (Invitrogen) in PBS-B, and incubated for 30 min. Finally, coverslips were washed with PBS and mounted using 0.1 μg/mL DAPI solution (Dojindo, Kumamoto, Japan). The morphologies of the chromosomes and microtubules were observed under a Leica LAS AF 6000 fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany).

References

(1) Yamazaki, Y.; Kido, Y.; Hidaka, K.; Yasui, H.;Kiso, Y.; Yakushiji, F.; Hayashi, Y. Tubulin photoaffinity labeling study with a plinabulin chemical probe possessing a biotin tag at the oxazole. *Bioorg. Med. Chem.* **2011**, 19, 595-602

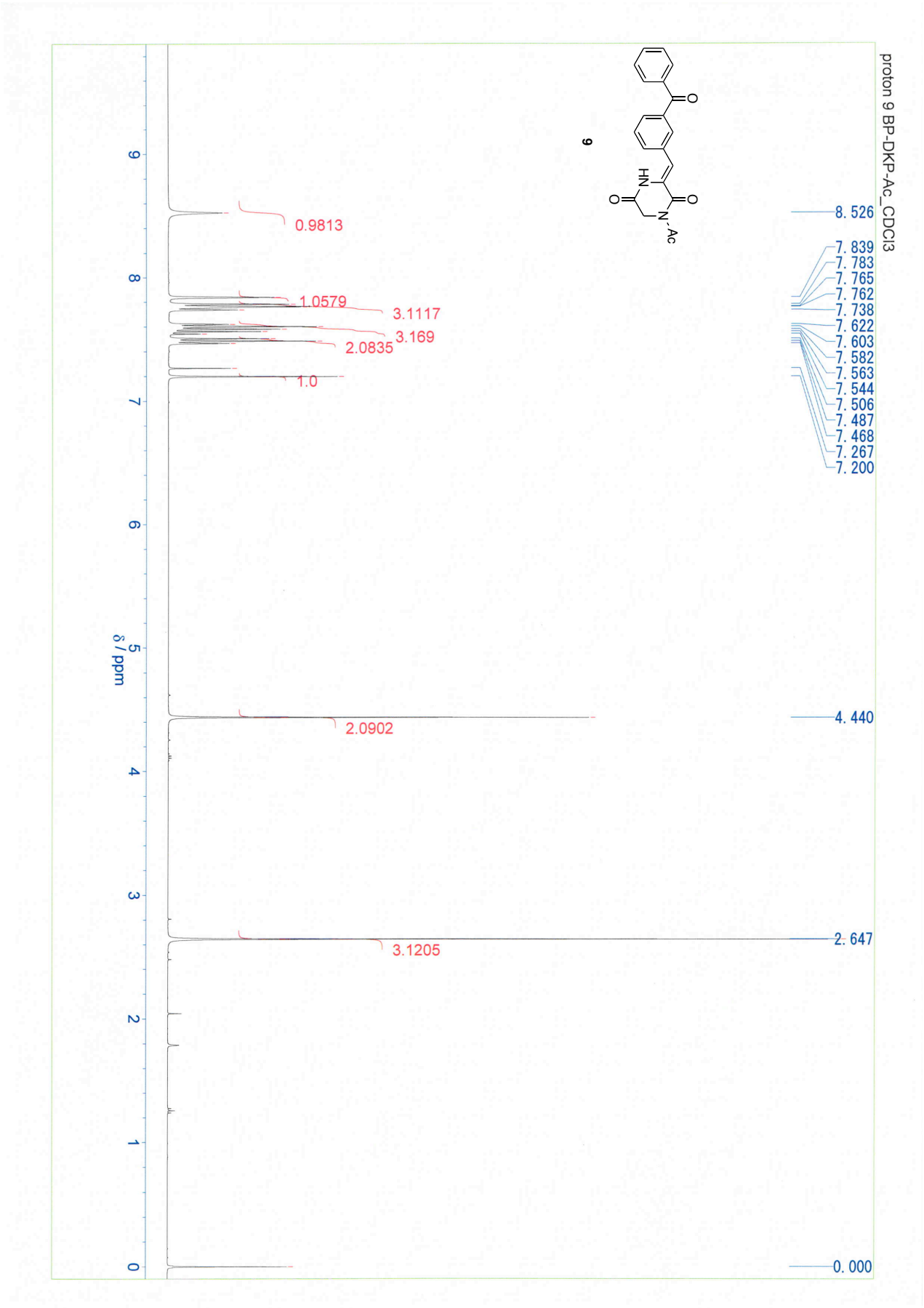
(2) Yamazaki, Y.; Kohno, K.; Yasui, H.; Kiso, Y.; Akamatsu, M.; Nicolson, B.; Deyanat-Yazdi, G.; Neuteboom, S.; Potts, B.; Lloyd, G. K.; Hayashi, Y. Tubulin photoaffinity labeling with biotin-tagged derivatives of potent diketopiperazine antimicrotubule agents. *ChemBioChem* **2008**, 9, 3074-3081

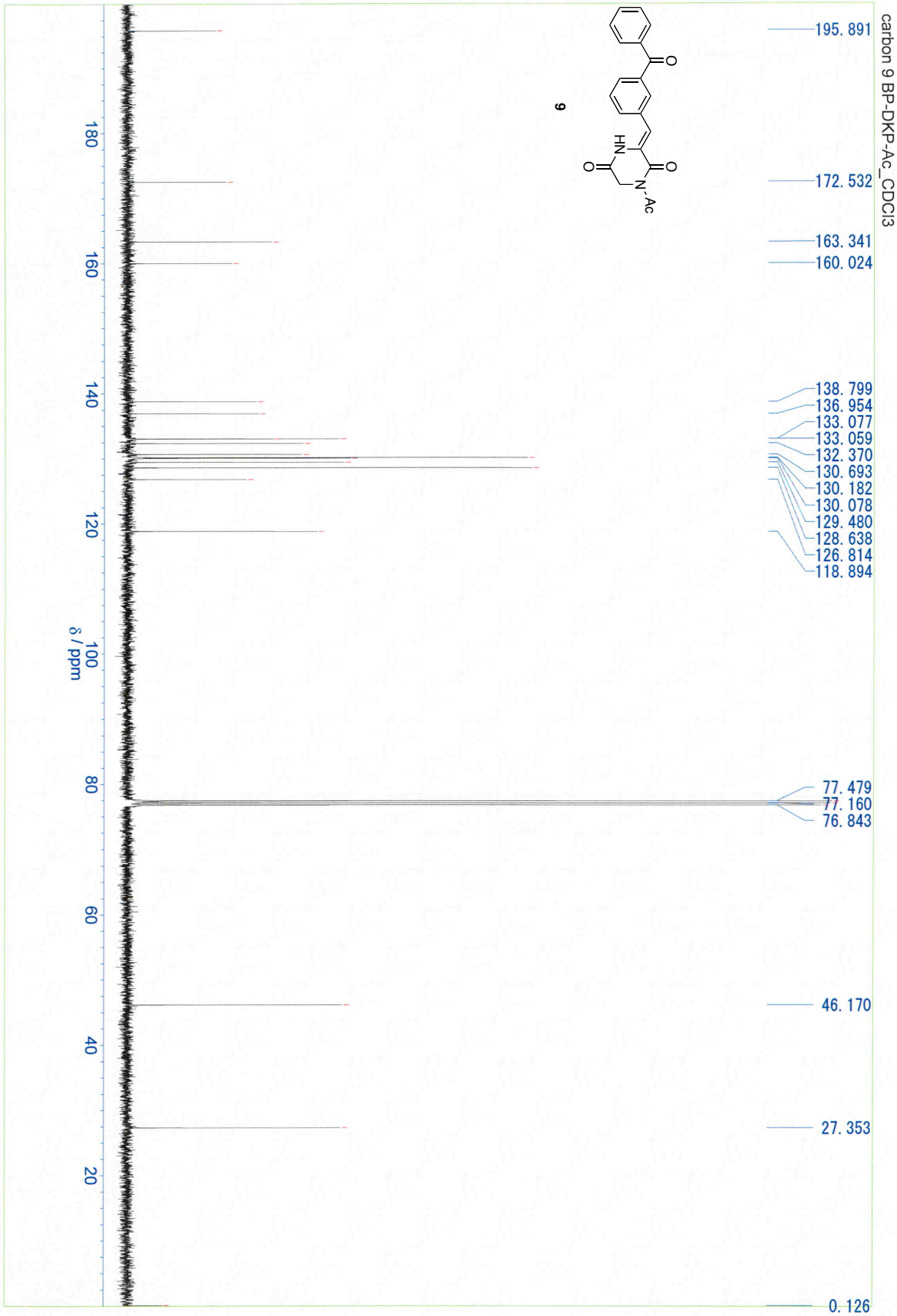
(3) Yamazaki, Y.; Tanaka, K.; Nicholson, B.; Deyanat-Yazdi, G.; Potts, B.; Yoshida, T.; Oda, A.; Kitagawa, T.; Orikasa, S.; Kiso, Y.; Yasui, H.; Akamatsu, M.; Chinen, T.; Usui, T.; Shinozaki, Y.; Yakushiji, F.; Miller, B. R.; Neuteboom, S.; Palladino, M.; Kanoh, K.; Lloyd, G. K.; Hayashi, Y. Synthesis and Structure–Activity Relationship Study of Antimicrotubule Agents Phenylahistin Derivatives with a Didehydropiperazine-2,5-dione Structure. *J. Med. Chem.* **2012**, 55, 1056-1071.

(4) Castoldi, M. and Popov. A. V. Purification of brain tubulin throughtwo cycles of polymerization-depolymerization in a high-molarity buffer. *Protein Expr. Purif.,* **2003**. 32, 83-88.

3. ^{1}H and ^{13}C NMR spectra

compd 9

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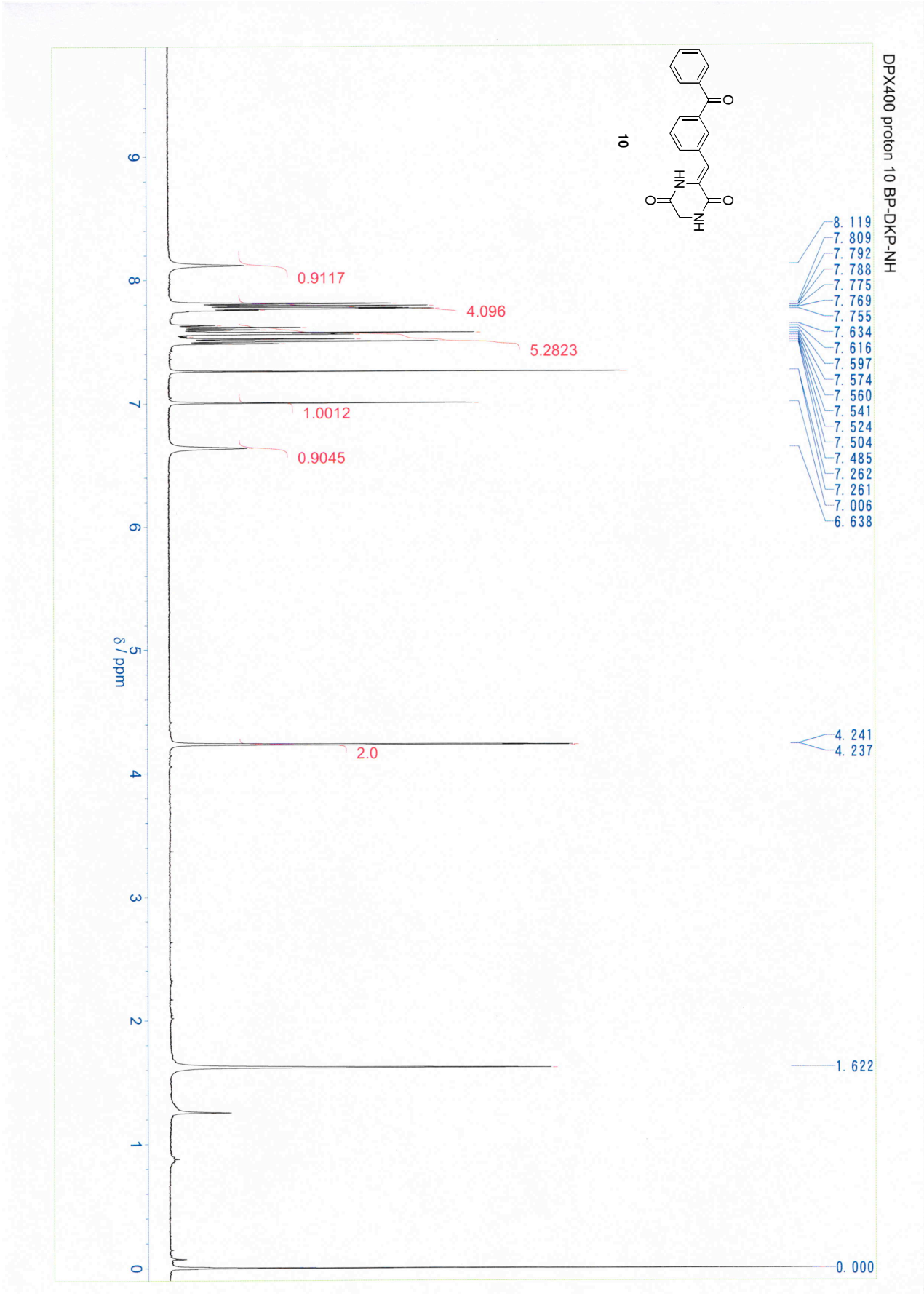
172.532
163.341
160.024
138.799
136.954
133.077
133.059
132.370
130.693
130.182
130.078
129.480
128.638
126.814
118.894
77.479
77.160
76.843
46.170
27.353
0.126
δ / ppm
9

compd 10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10
δ / ppm
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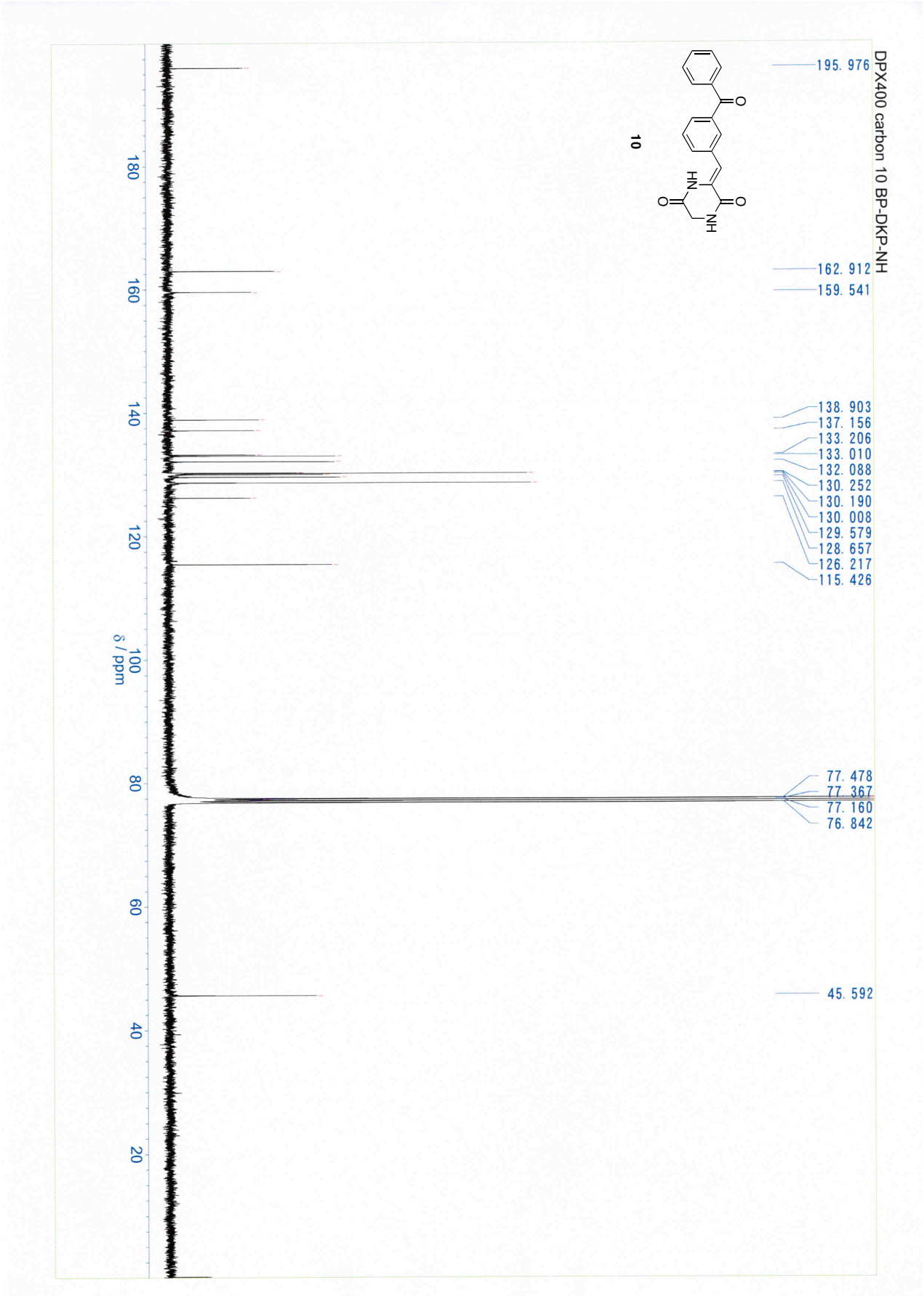
5.2823
1.0012
0.9045
2.0
8. 119
7. 809
7. 792
7. 788
7. 775
7. 769
7. 755
7. 634
7. 616
7. 597
7. 574
7. 560
7. 541
7. 524
7. 504
7. 485
7. 262
7. 261
7. 006
6. 638
4. 241
4. 237
1. 622
0. 000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10
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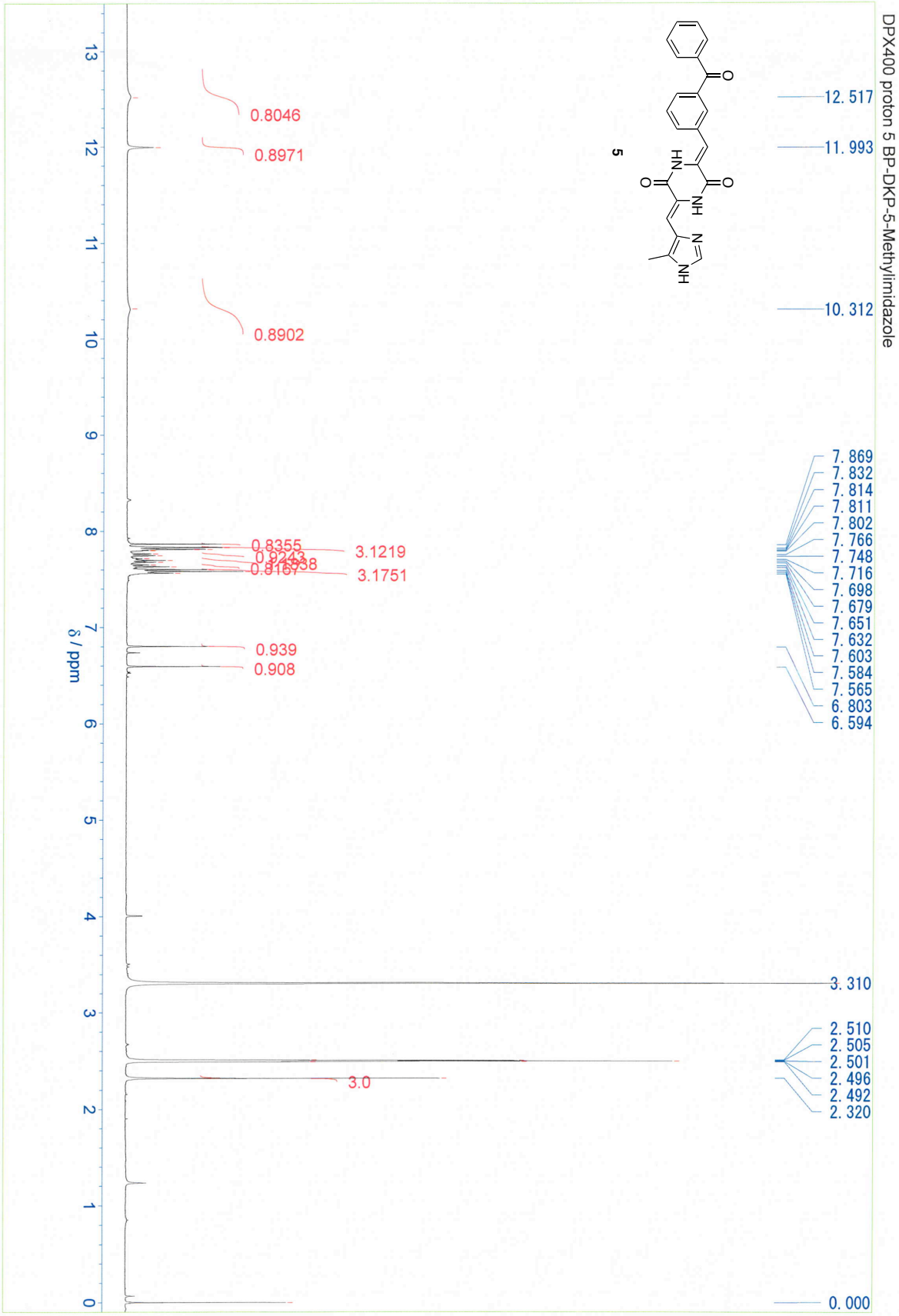
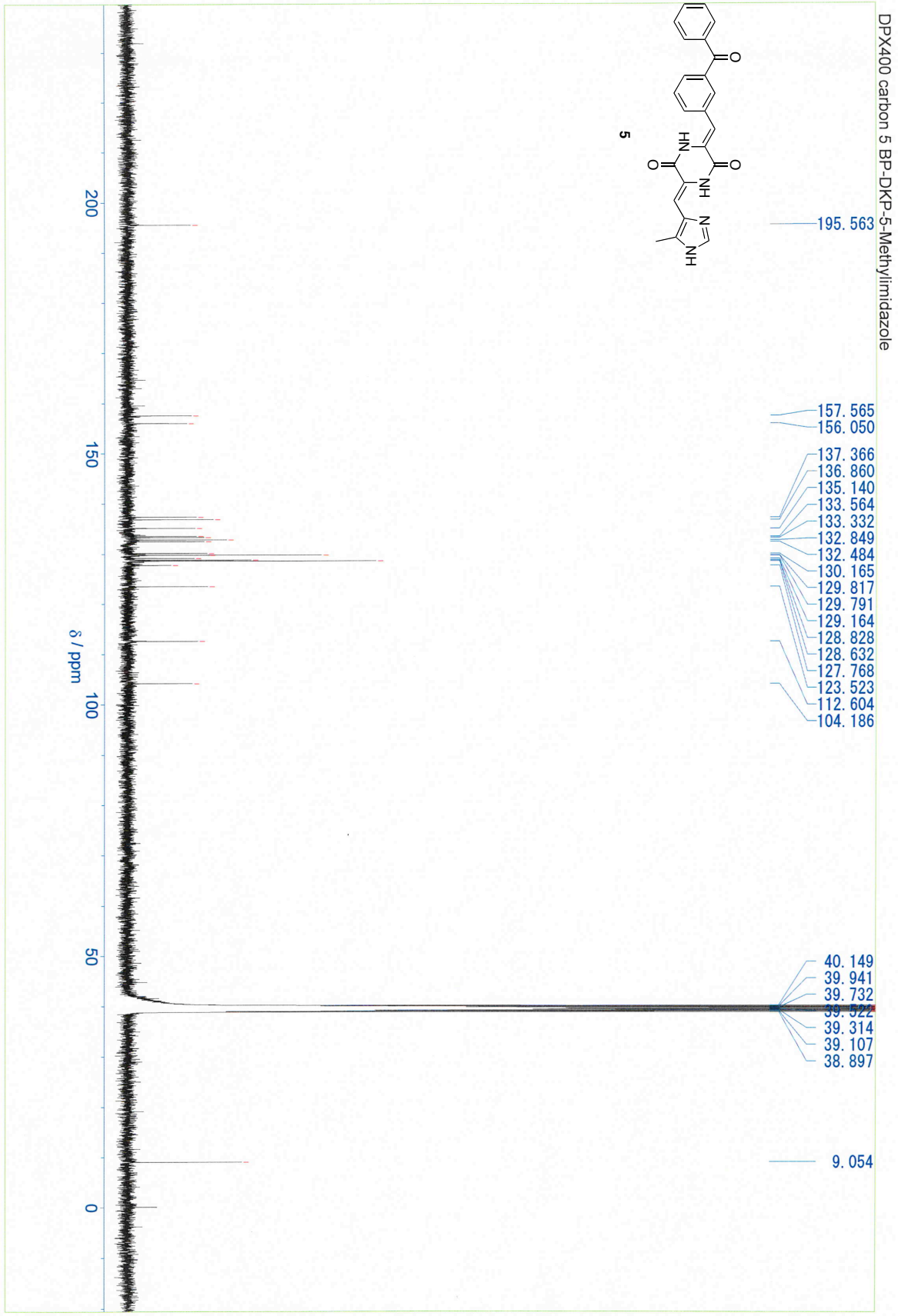
159.541
138.903
137.156
133.206
133.010
132.088
130.252
130.190
130.008
129.579
128.657
126.217
115.426
77.478
77.367
77.160
76.842
45.592
δ / ppm
180
160
140
120
100
80
60
40
20

compd 5


DPX400 proton 5 BP-DKP-5-Methylimidazole
δ / ppm
0.8046
0.8971
0.8902
0.8355
0.9243
0.8167
3.1219
3.1751
0.939
0.908
3.0
12.517
11.993
10.312
7.869
7.832
7.814
7.811
7.802
7.766
7.748
7.716
7.698
7.679
7.651
7.632
7.603
7.584
7.565
6.803
6.594
3.310
2.510
2.505
2.501
2.496
2.492
2.320
0.000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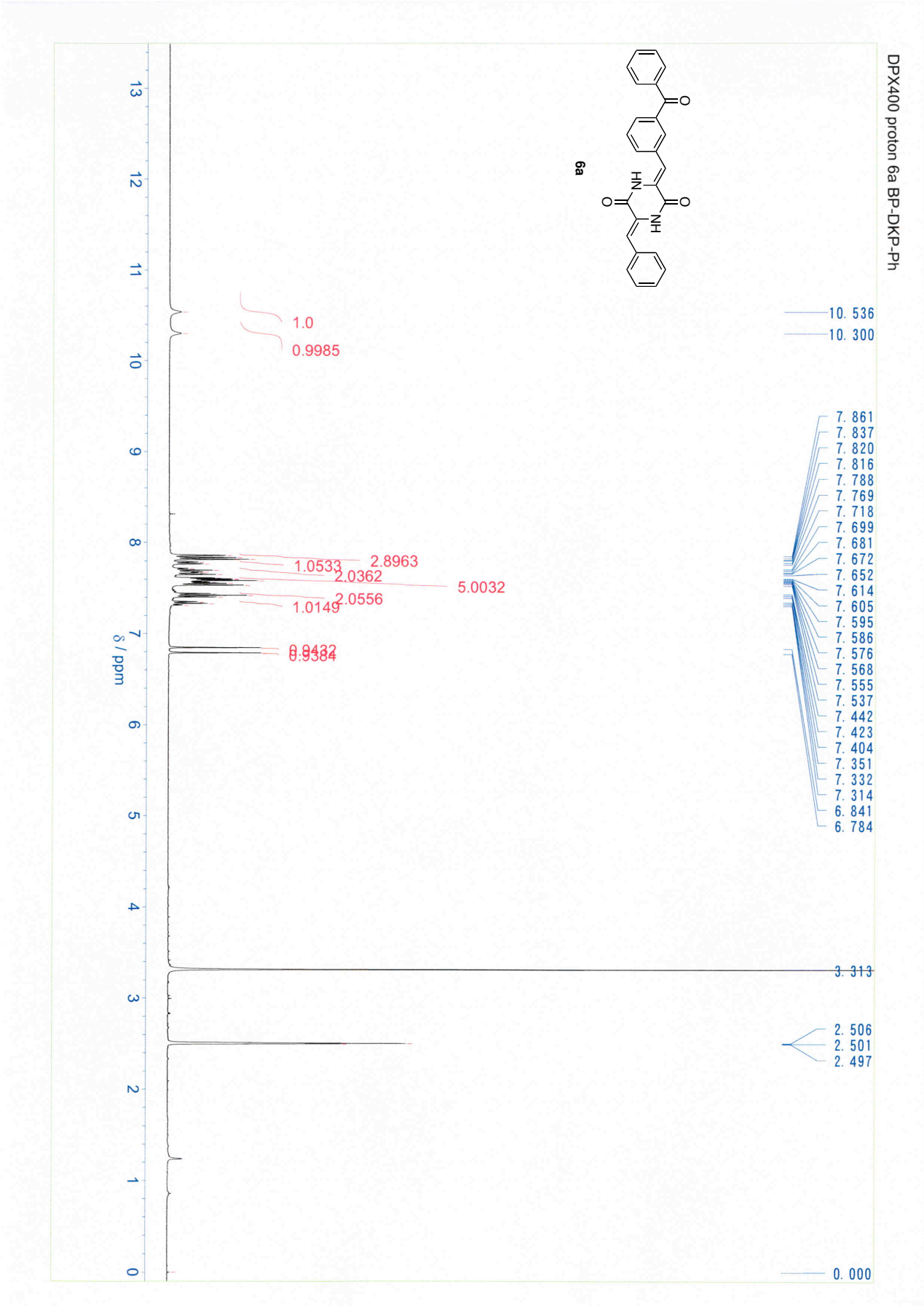
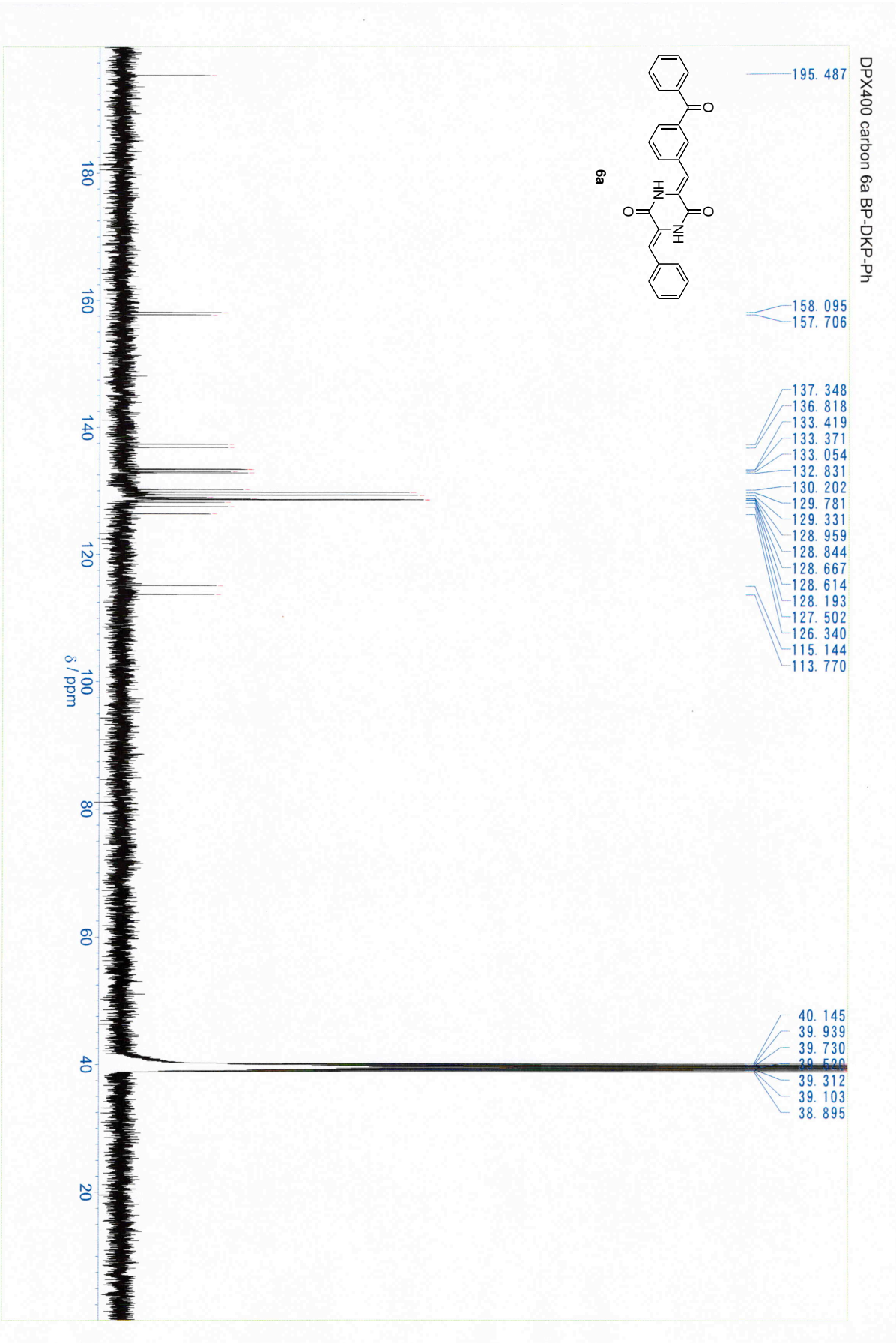
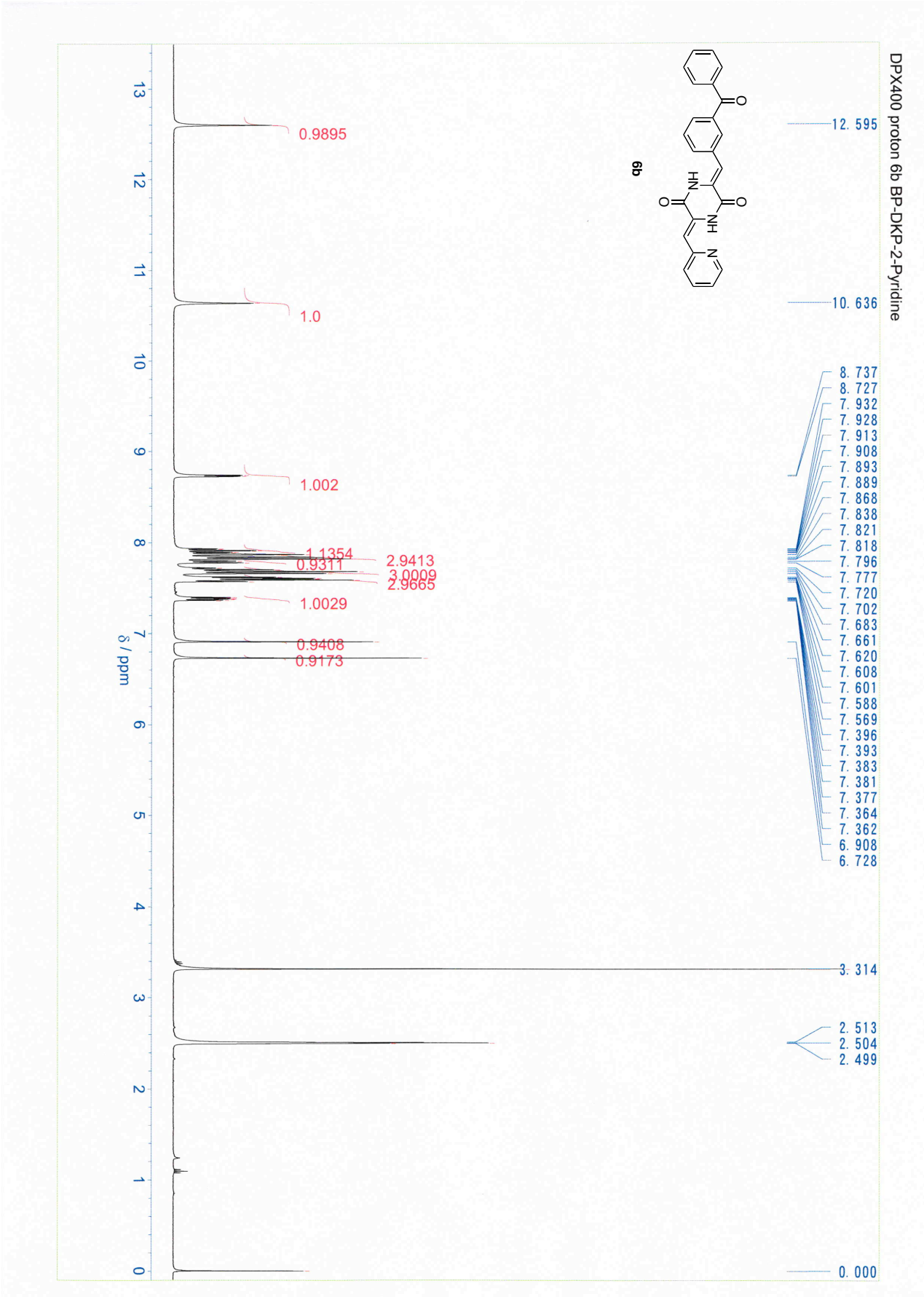
5
195.563
157.565
156.050
137.366
136.860
135.140
133.564
133.332
132.849
132.484
130.165
129.817
129.791
129.164
128.828
128.632
127.768
123.523
112.604
104.186
40.149
39.941
39.732
39.314
39.107
38.897
9.054
δ / ppm
200
150
100
50
0

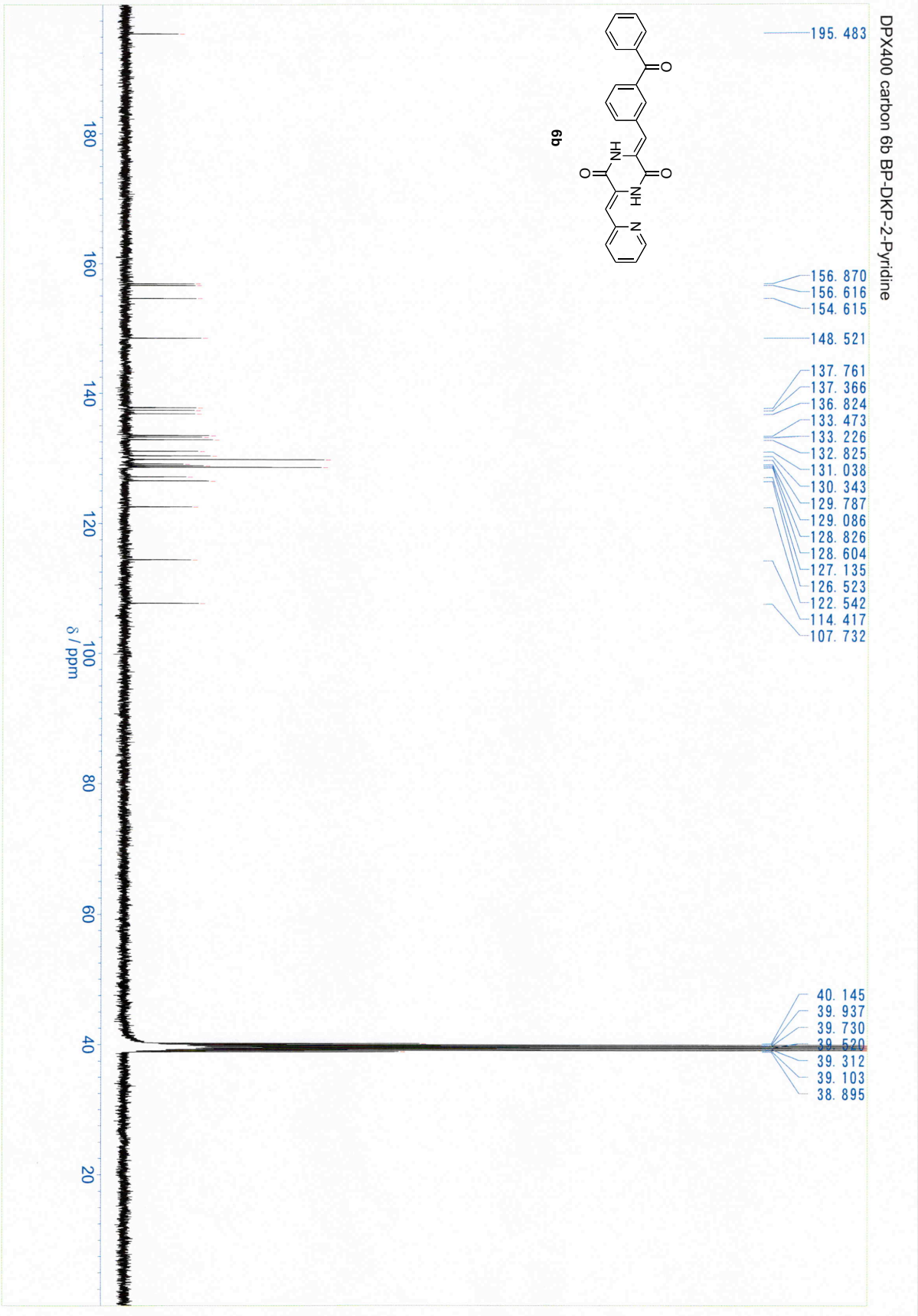
compd 6a


DPX400 proton 6a BP-DKP-Ph
6a
δ / ppm
10. 536
10. 300
7. 861
7. 837
7. 820
7. 816
7. 788
7. 769
7. 718
7. 699
7. 681
7. 672
7. 652
7. 614
7. 605
7. 595
7. 586
7. 576
7. 568
7. 555
7. 537
7. 442
7. 423
7. 404
7. 351
7. 332
7. 314
6. 841
6. 784
3. 313
2. 506
2. 501
2. 497
0. 000
1.0
0.9985
1.0533
2.8963
2.0362
5.0032
2.0556
1.0149
0.9432
0.9384

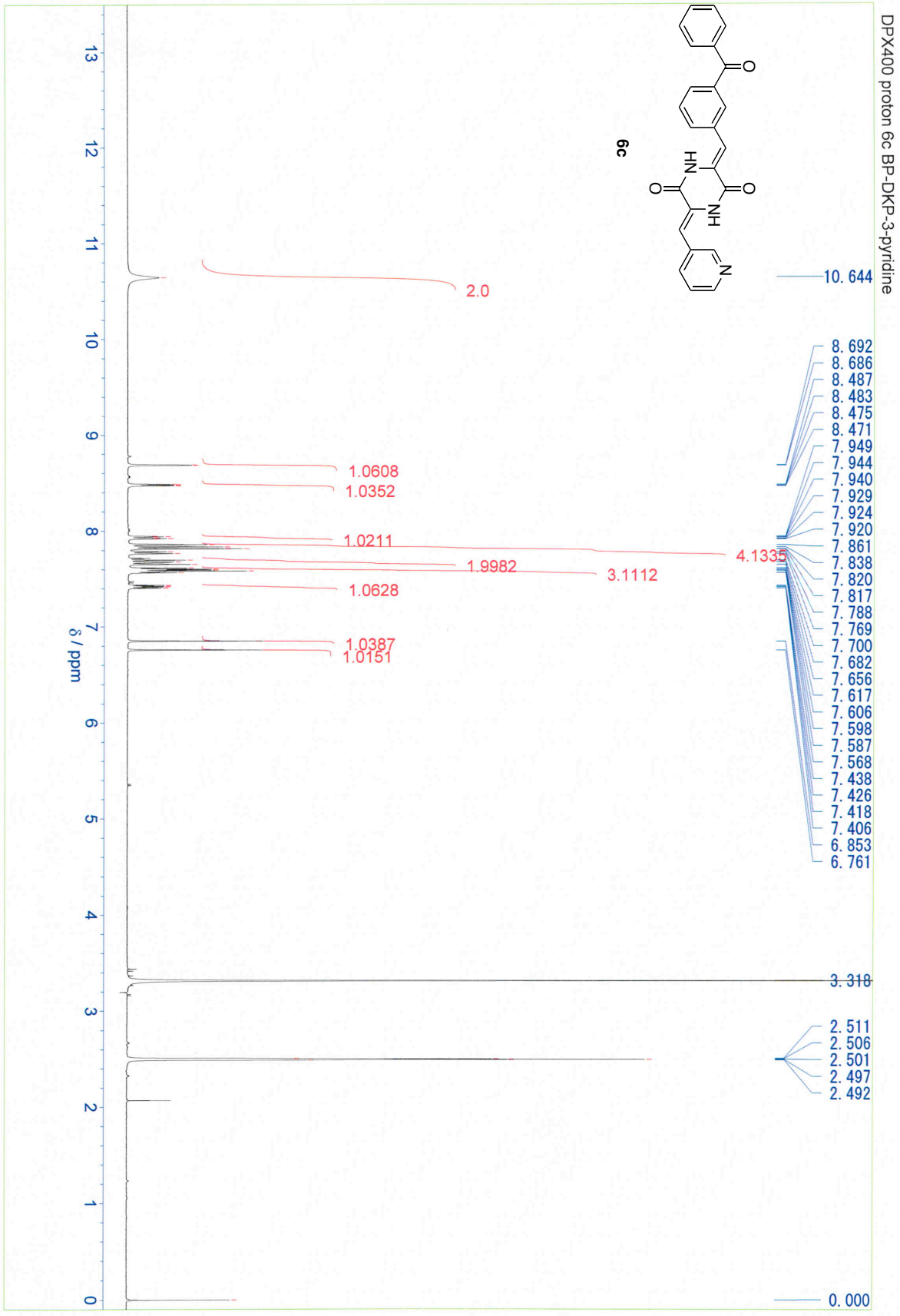

DPX400 carbon 6a BP-DKP-Ph
6a
195.487
158.095
157.706
137.348
136.818
133.419
133.371
133.054
132.831
130.202
129.781
129.331
128.959
128.844
128.667
128.614
128.193
127.502
126.340
115.144
113.770
40.145
39.939
39.730
39.312
39.103
38.895
δ / ppm

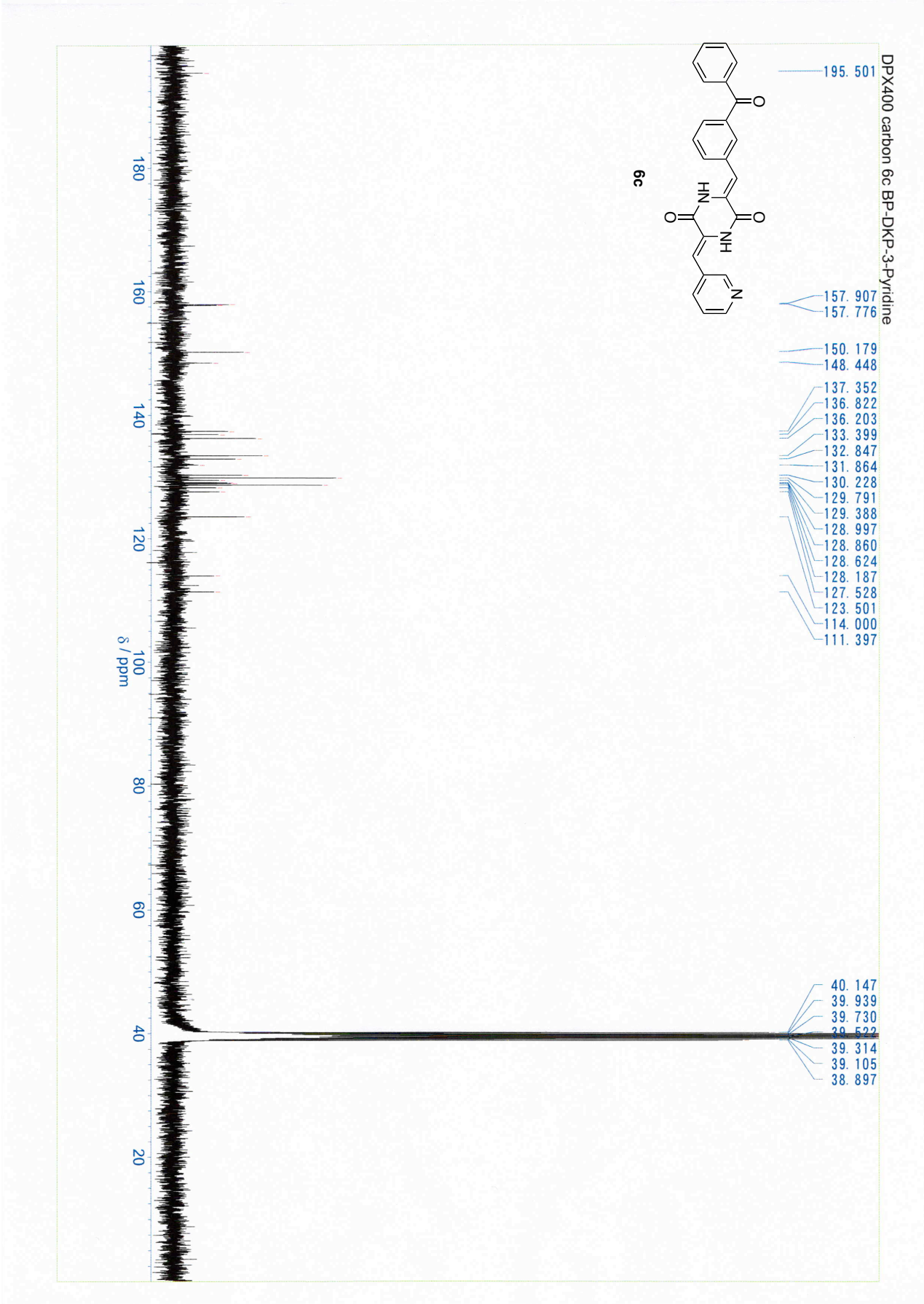
compd 6b


DPX400 proton 6b BP-DKP-2-Pyridine
6b
δ / ppm
0.9895
1.0
1.002
1.1354
0.9311
2.9413
3.0009
2.9665
1.0029
0.9408
0.9173
12.595
10.636
8.737
8.727
7.932
7.928
7.913
7.908
7.893
7.889
7.868
7.838
7.821
7.818
7.796
7.777
7.720
7.702
7.683
7.661
7.620
7.608
7.601
7.588
7.569
7.396
7.393
7.383
7.381
7.377
7.364
7.362
6.908
6.728
3.314
2.513
2.504
2.499
0.000

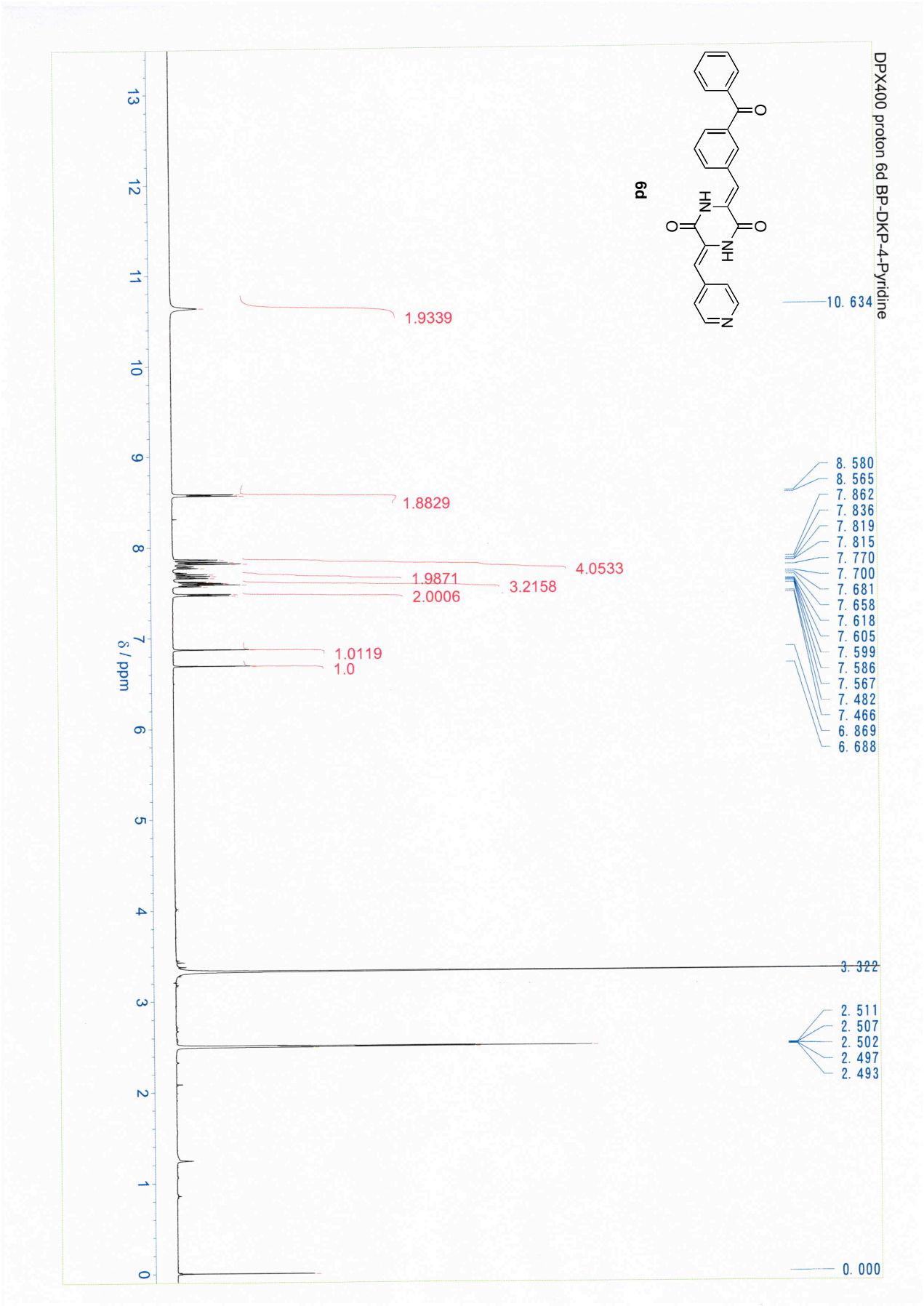

DPX400 carbon 6b BP-DKP-2-Pyridine
6b
195.483
156.870
156.616
154.615
148.521
137.761
137.366
136.824
133.473
133.226
132.825
131.038
130.343
129.787
129.086
128.826
128.604
127.135
126.523
122.542
114.417
107.732
40.145
39.937
39.730
39.520
39.312
39.103
38.895
δ / ppm

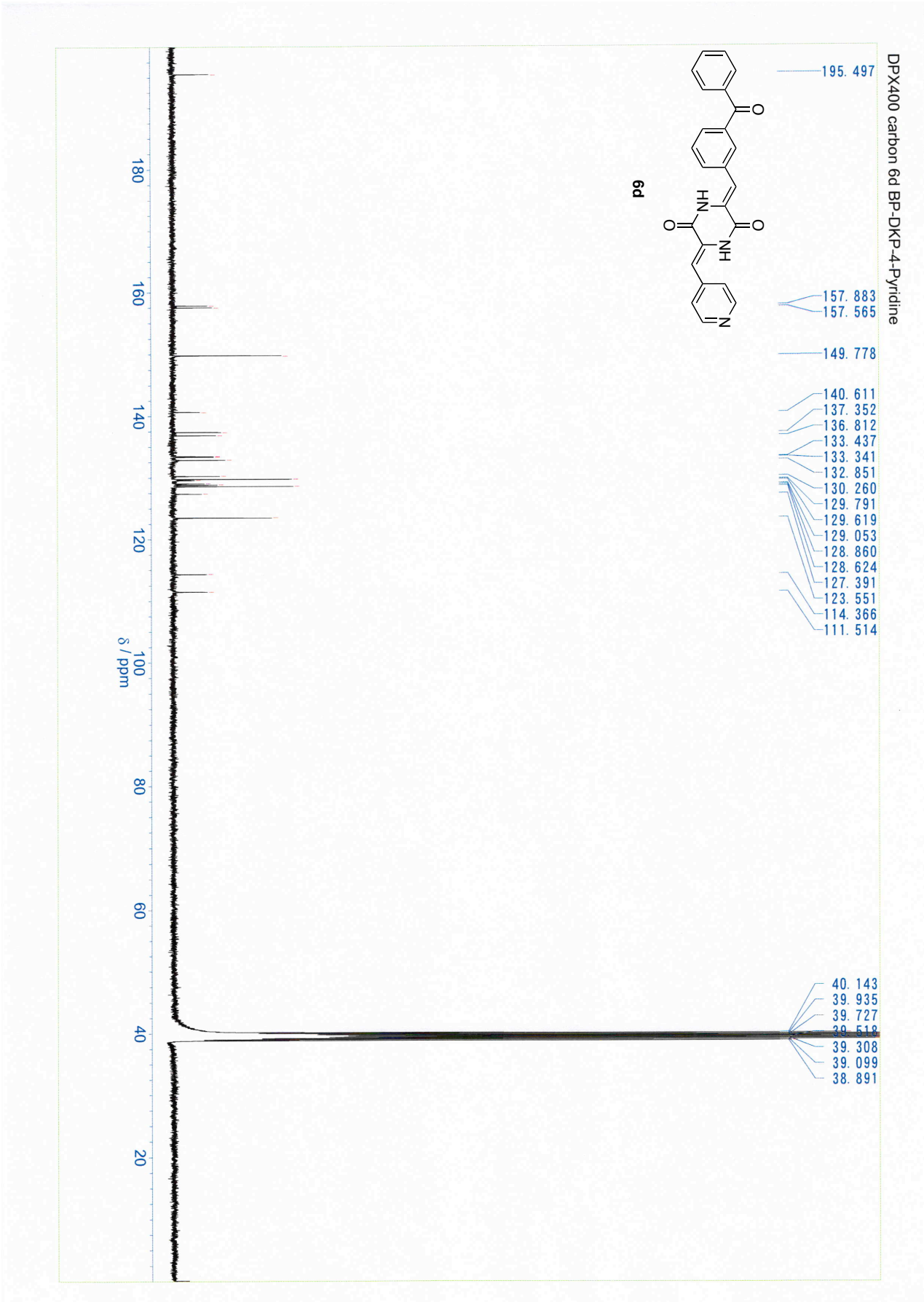
compd 6c


DPX400 proton 6c BP-DKP-3-pyridine
6c
δ / ppm
2.0
1.0608
1.0352
1.0211
1.9982
3.1112
4.1335
1.0628
1.0387
1.0151
10.644
8.692
8.686
8.487
8.483
8.475
8.471
7.949
7.944
7.940
7.929
7.924
7.920
7.861
7.838
7.820
7.817
7.788
7.769
7.700
7.682
7.656
7.617
7.606
7.598
7.587
7.568
7.438
7.426
7.418
7.406
6.853
6.761
3.318
2.511
2.506
2.501
2.497
2.492
0.000

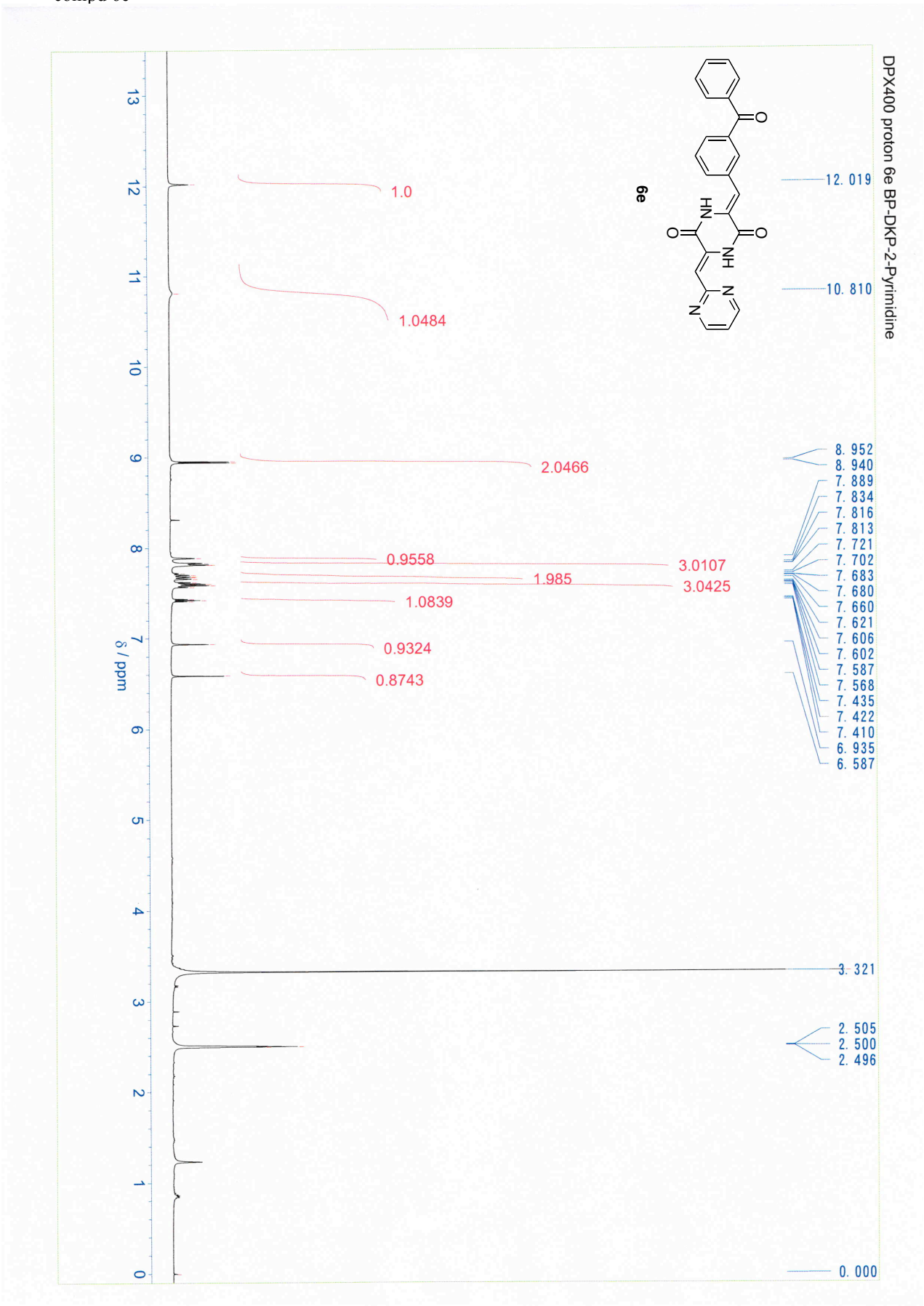

DPX400 carbon 6c BP-DKP-3-Pyridine
6c
195.501
157.907
157.776
150.179
148.448
137.352
136.822
136.203
133.399
132.847
131.864
130.228
129.791
129.388
128.997
128.860
128.624
128.187
127.528
123.501
114.000
111.397
40.147
39.939
39.730
39.522
39.314
39.105
38.897
δ / ppm

compd 6d


DPX400 proton 6d BP-DKP-4-Pyridine
6d
HN
NH
O
O
O
N
10. 634
1.9339
1.8829
4.0533
1.9871
3.2158
2.0006
1.0119
1.0
8. 580
8. 565
7. 862
7. 836
7. 819
7. 815
7. 770
7. 700
7. 681
7. 658
7. 618
7. 605
7. 599
7. 586
7. 567
7. 482
7. 466
6. 869
6. 688
3. 322
2. 511
2. 507
2. 502
2. 497
2. 493
0. 000
δ / ppm
13
12
11
10
9
8
7
6
5
4
3
2
1
0


DPX400 carbon 6d BP-DKP-4-Pyridine
6d
195.497
157.883
157.565
149.778
140.611
137.352
136.812
133.437
133.341
132.851
130.260
129.791
129.619
129.053
128.860
128.624
127.391
123.551
114.366
111.514
40.143
39.935
39.727
39.518
39.308
39.099
38.891
δ / ppm

compd 6e


DPX400 proton 6e BP-DKP-2-Pyrimidine
6e
δ / ppm
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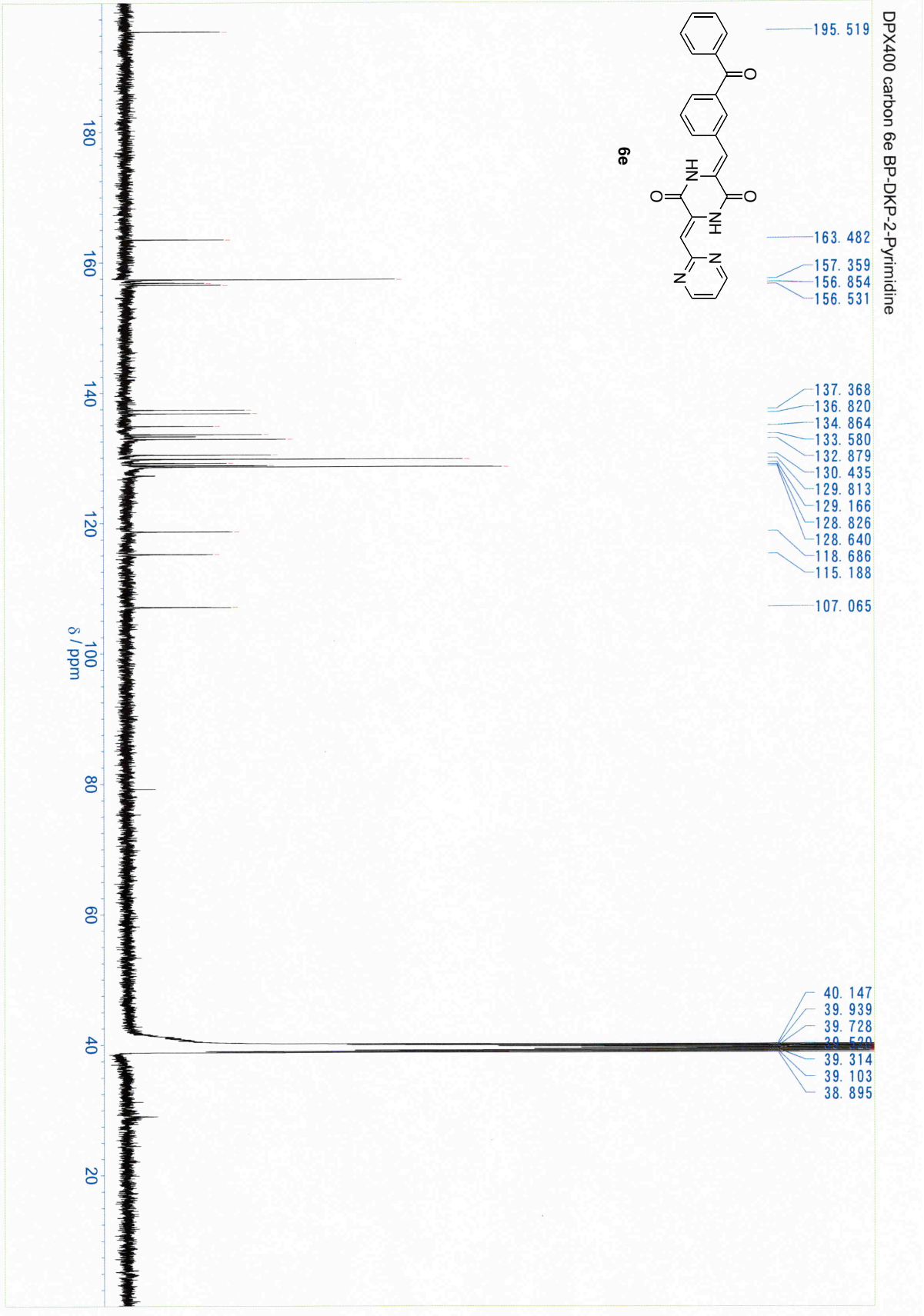
1.0484
2.0466
0.9558
1.985
1.0839
0.9324
0.8743
3.0107
3.0425
12. 019
10. 810
8. 952
8. 940
7. 889
7. 834
7. 816
7. 813
7. 721
7. 702
7. 683
7. 680
7. 660
7. 621
7. 606
7. 602
7. 587
7. 568
7. 435
7. 422
7. 410
6. 935
6. 587
3. 321
2. 505
2. 500
2. 496
0. 000


DPX400 carbon 6e BP-DKP-2-Pyrimidine
6e
195.519
163.482
157.359
156.854
156.531
137.368
136.820
134.864
133.580
132.879
130.435
129.813
129.166
128.826
128.640
118.686
115.188
107.065
40.147
39.939
39.728
39.314
39.103
38.895
δ / ppm
180
160
140
120
100
80
60
40
20