

CHEMBIOCHEM

Supporting Information

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Supporting Information

for

Tubulin Photoaffinity Labeling by Biotin-Tagged Derivatives of Potent Diketopiperazine Anti-Microtubule Agents

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1. Superimposition study

The superimposition study was performed using the three-dimensional (3D) structures of colchicine and NPI-2358, which were obtained from the crystallographic data of tubulin-colchicine complex (PDB ID, 1SA0) and the in-house X-ray crystallographic data of NPI-2358, respectively, using the Molecular Operating Environment modeling package (MOE 2006.08, Chemical Computing Group, Inc., Montreal, Canada) with MMFF94x force field. Both structures were superimposed at the phenyl ring of colchicine and the diketopiperazine ring of NPI-2358, and were energy minimized each other for several times. As shown in Figure S1, no favorable superimposition between NPI-2358 and colchicine was observed.

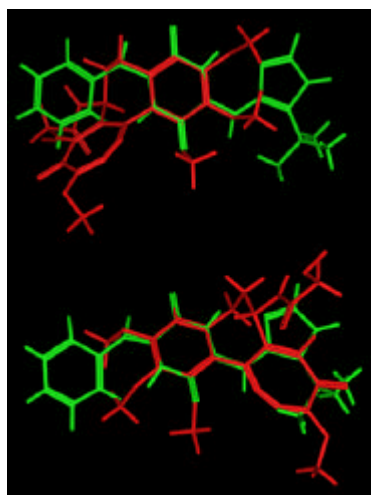


Figure S1. Superimposition study of colchicine and NPI-2358.

2. Tubulin polymerization assay.

Various concentrations of drugs were mixed with MAP-rich tubulin (2 mg/mL) at 0 °C and incubated at 37 °C. Changes in turbidity were monitored at 340 nm.

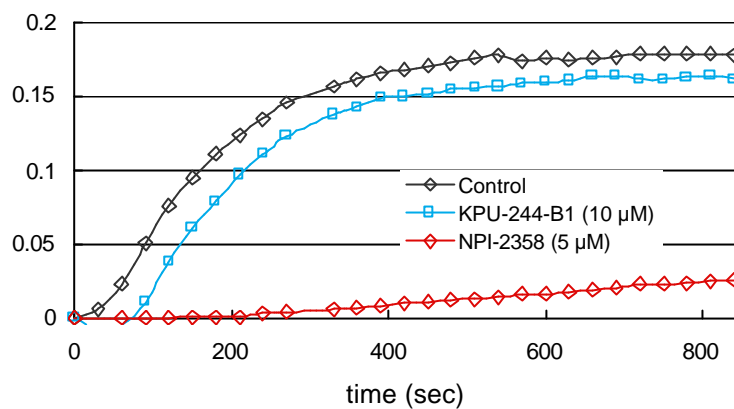


Figure S2. Tubulin polymerization assay of NPI-2358 and compound 3 (KPU-244-B1).

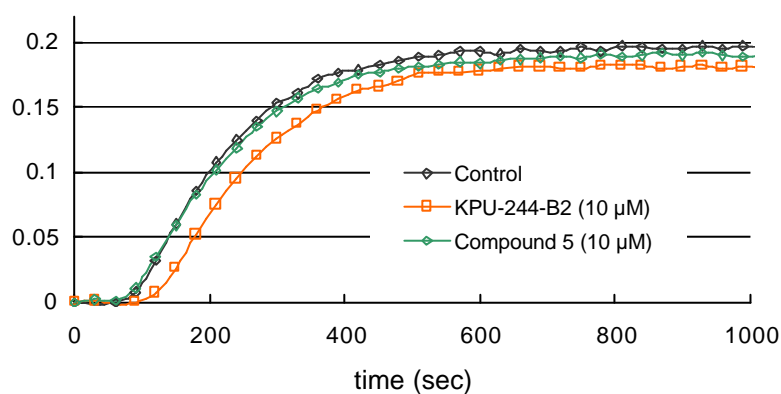


Figure S3. Tubulin polymerization assay of compound 4 (KPU-244-B2) and compound 5.

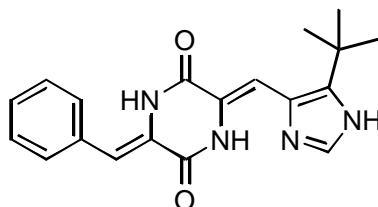
3. Tubulin binding assay based on the fluorescence quenching

For tubulin binding assay, tubulin (800 μL , 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 concentration of the test compounds (0–20 μM , 1 % DMSO) at 37 $^\circ\text{C}$ for 1 h. After incubation, the fluorescence of each solution was measured (excitation 295 nm, emission 300–450 nm) by FP-750 Spectrofluorometer (JASCO). The fluorescence of all samples was corrected for inner filter effects. The concentration of tubulin–compound complex were estimated from the decreased fluorescence intensity at 355 nm, and the dissociation constant between tubulin and compounds were calculated using the following binding equation of Langmuir model.

$$\Delta\text{FL} = \theta \cdot C_b$$

$$C_b = 0.5 \cdot [(C_t + n \cdot P_t + K_d) - \{(C_t - n \cdot P_t - K_d)^2 + 4 \cdot K_d \cdot C_t\}^{1/2}]$$

ΔFL ; The decreased fluorescence intensity of tubulin, C_b ; The concentration of tubulin–compound complex (μM), θ ; The molar fluorescence intensity of tubulin (arbitrary unit/ $\mu\text{M}/\text{cm}$), C_t ; The total concentration of compounds (μM), P_t ; The total concentration of tubulin (μM), n ; The number of binding sites, K_d ; The dissociation constant (μM).



NPI-2358 (KPU-2), 1

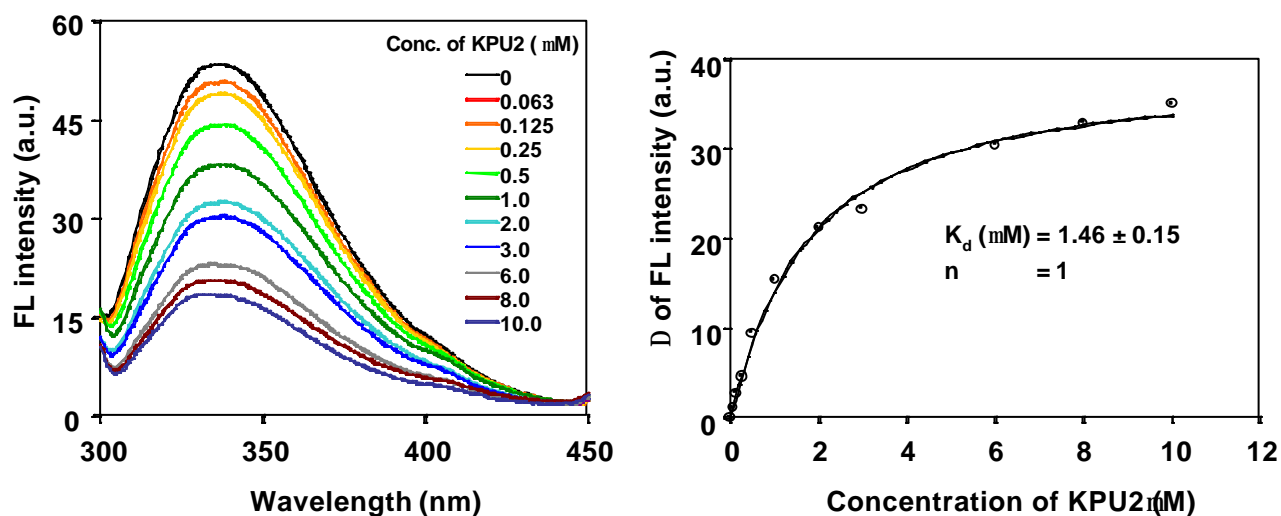


Figure S4. Binding assay of compound 1 (NPI-2358/KPU-2) with tubulin at 37 $^\circ\text{C}$.

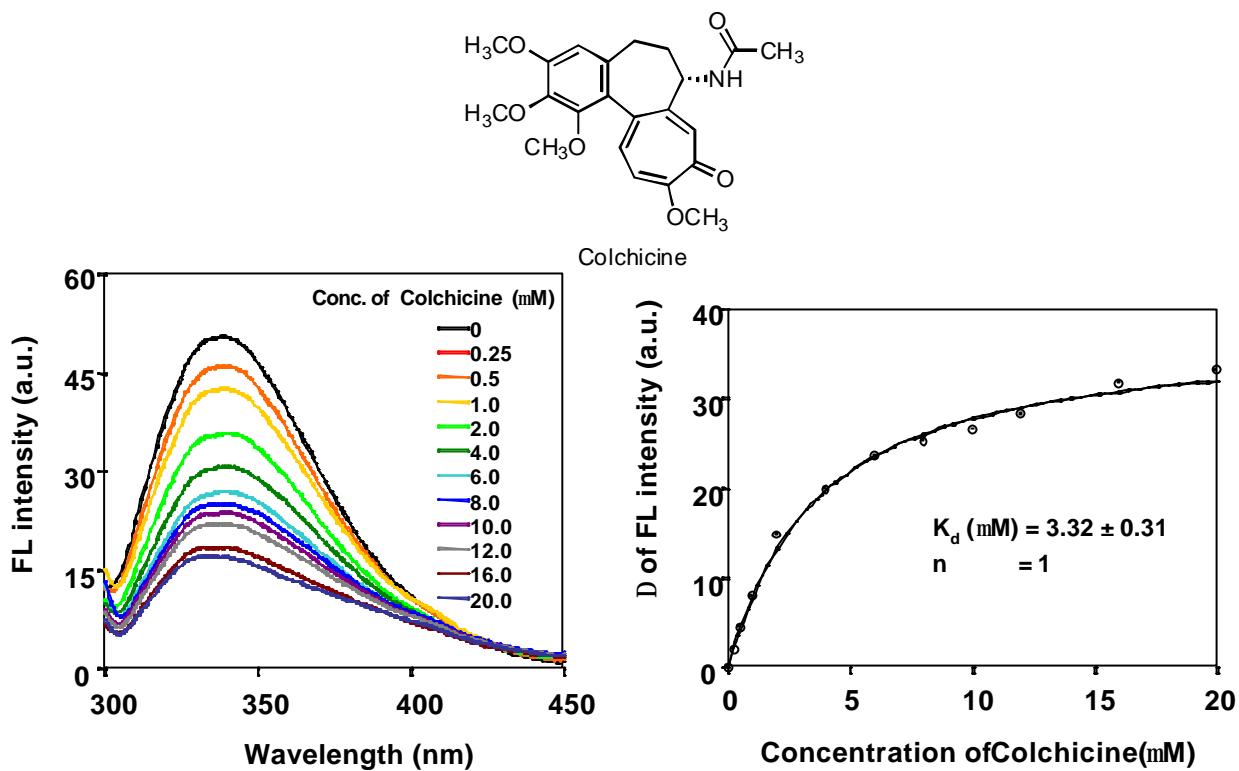


Figure S7. Binding assay of colchicine with tubulin at 37°C.

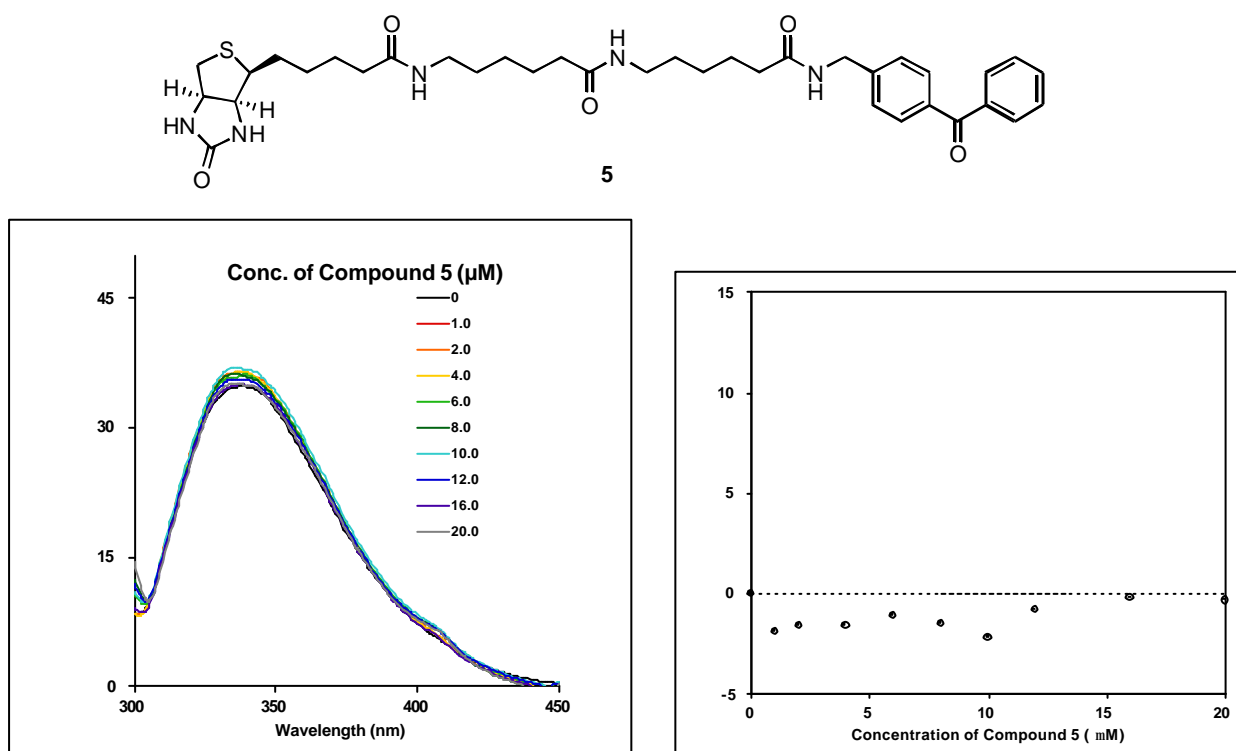
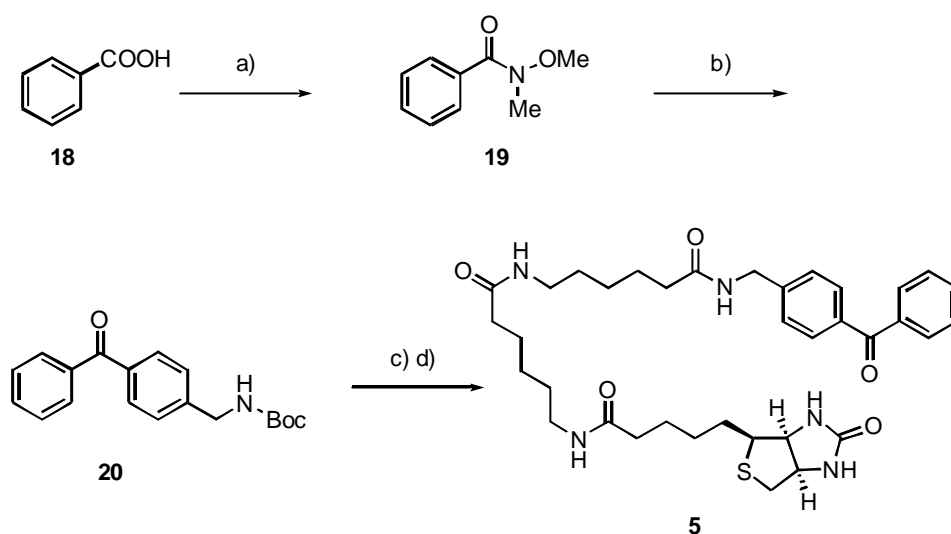


Figure S8. Binding assay of compound 5 with tubulin at 37°C.

4. Synthesis of photoaffinity negative control compound 5



Scheme S1. Synthesis of compound **5** a) MeONMe•HCl, EDC•HCl, Et₃N, DMF, RT, 73%; b) *n*-BuLi, 4-*N*-Boc-aminomethylbromobenzene **8**, Et₂O-THF, -78 °C, 34%; c) 4 M HCl-dioxane, RT; d) EZ-Link™ NHS-LC-LC-Biotin, Et₃N, DMF, rt, then HPLC purification 25% in 2 steps.

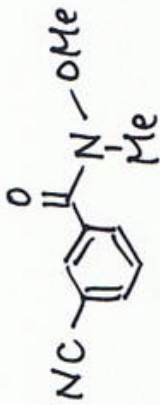
***N*-methoxy-*N*-methylbenzamide 19:** To a solution of benzoic acid **18** (1.0 g, 8.19 mmol) in DMF (80 mL) was added *N,O*-dimethylhydroxylamine hydrochloride (838.6 mg, 8.60 mmol), Et₃N (1.6 mL, 11.5 mmol) and EDC•HCl (1.65 g, 8.60 mmol). After the mixture was stirred for 3 h at room temperature, the solvent was removed in vacuo and the residue was dissolved in EtOAc, washed with 10% citric acid, 5% NaHCO₃ and saturated NaCl, dried over Na₂SO₄, Then the solvent was removed to give a colorless oil of compound **19**; (1.0 g, 73%); ¹H NMR (300 MHz, CDCl₃) δ 3.36 (s, 3H), 3.58 (s, 3H), 7.36-7.45 (m, 3H), 7.67 (m, 2H); HRMS (EI) : *m/z* 165.0791 (M⁺) (calcd for C₉H₁₁NO₂: 165.0790).

***tert*-Buthyl 4(3-cyanobenzoyl)benzylcarbamate 20:** To a solution of 4-*N*-Boc-aminomethylbromobenzene **8** (611 mg, 2.13 mmol) in anhydrous ether (6 mL) was added dropwise *n*-BuLi (1.58 M solution in *n*-hexane, 3.4 mL, 4.27 mmol) at 0 °C under Ar atmosphere. After the mixture was stirred for 1 h at the same temperature, the mixture was slowly added to a solution of compound **19** (288.5 mg, 2.13 mmol) in THF (6 mL) at -78 °C under Ar atmosphere, then cooling bath was removed and the reaction mixture was stirred for overnight at room temperature. The solution was poured into ice-cold 1 M HCl (30 mL), neutralized with a powder of NaHCO₃ and the organic phased was extracted with AcOEt, washed with brine, and dried over Na₂SO₄.

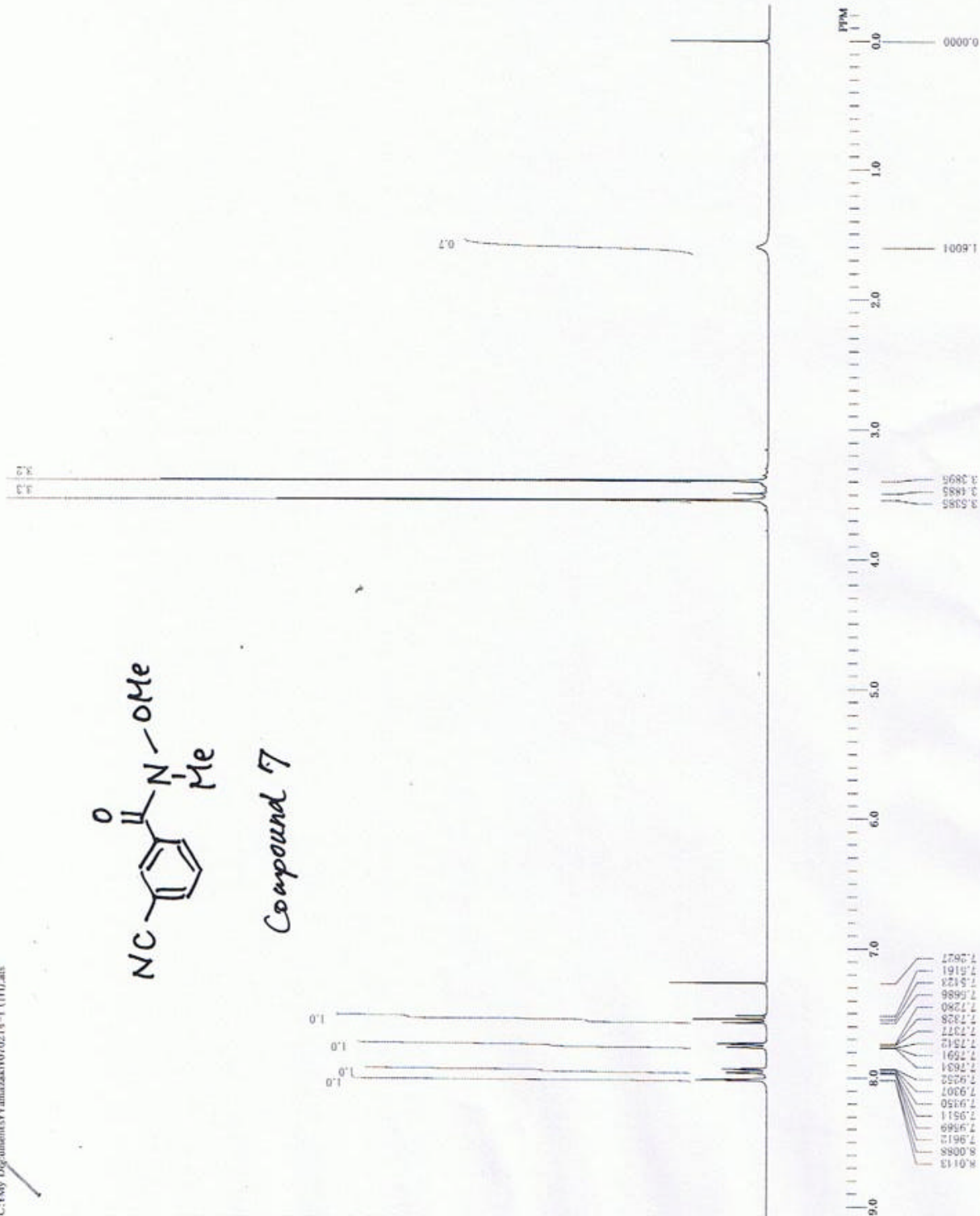
Then the solvent was removed under reduced pressure and the resulting brown oil was purified by silica-gel column chromatography (*n*-hexane / EtOAc = 4:1) to yield benzophenone derivative **20** as a white solid (224 mg, 34%); mp 127-130 °C; ¹H NMR (300 MHz, CDCl₃) δ 1.47 (s, 9H), 4.42 (d, 2H, *J* = 6.0 Hz), 4.94 (br s, 1H), 7.39 (d, 2H, *J* = 8.3 Hz), 7.48 (t, 2H, *J* = 7.3 Hz), 7.59 (m, 1H), 7.78 (m, 4H); HRMS (EI): *m/z* 311.1518 (M⁺) (calcd for C₁₉H₂₁NO₃: 311.1521).

4-N-(D-biotinylaminohexanoylamino)aminomethylbenzophenone 5: For Boc-deprotecton, compound **20** (5.6 mg, 0.018 mmol) was dissolved in 4 M HCl / dioxane (0.7 mL), stirred at room temperature for 1 h. After the solvent was removed by evaporation, the residue was washed with ether trice. To a solution of this residue in DMF (1 mL) were added EZ-Link™NHS-LC-LC-Biotin (PIERCE) (3.4 mg, 0.006 mmol) and Et₃N (2.5 μL, 0.018 mmol) in turn, and the mixture was stirred at room temperature for 11 h. After the solvent was removed by evaporation in vacuo, the residue was extracted with AcOEt, washed with 10% citric acid, 5% NaHCO₃ and saturated NaCl, and dried over anhydrous Na₂SO₄. Then the solvent was concentrated in vacuo and the resultant residue was purified by preparative HPLC (with a linear gradient of 20-100% CH₃CN in 0.1% aqueous TFA over 40 min) to give a white powder (1.0 mg, 25%); ¹H NMR (400 MHz DMSO-*d*₆) δ 1.16-1.64 (m, 18H), 2.02 (q, 4H, *J* = 7.1 Hz), 2.16 (t, 2H, *J* = 7.5 Hz), 2.57 (d, 1H, *J* = 12.4 Hz), 2.81 (dd, 1H, *J* = 5.2, 12.4 Hz), 3.00 (quintet, 5H, *J* = 6.4 Hz), 3.06-3.11 (m, 1H), 4.12 (dd, 1H, *J* = 4.4, 7.6 Hz), 4.30 (dd, 1H, *J* = 4.4, 7.6 Hz), 4.36 (d, 2H, *J* = 6.0 Hz), 6.35 (br, 1H), 6.40 (br s, 1H), 7.41 (d, 2H, *J* = 8.6 Hz), 7.56 (t, 2H, *J* = 7.5 Hz), 7.68-7.74 (m, 7H), 8.42 (t, 1H, *J* = 6.0 Hz); HRMS (FAB) : *m/z* 664.3530 (M+H⁺) (calcd for C₃₆H₄₉N₅O₅S: 663.3454).

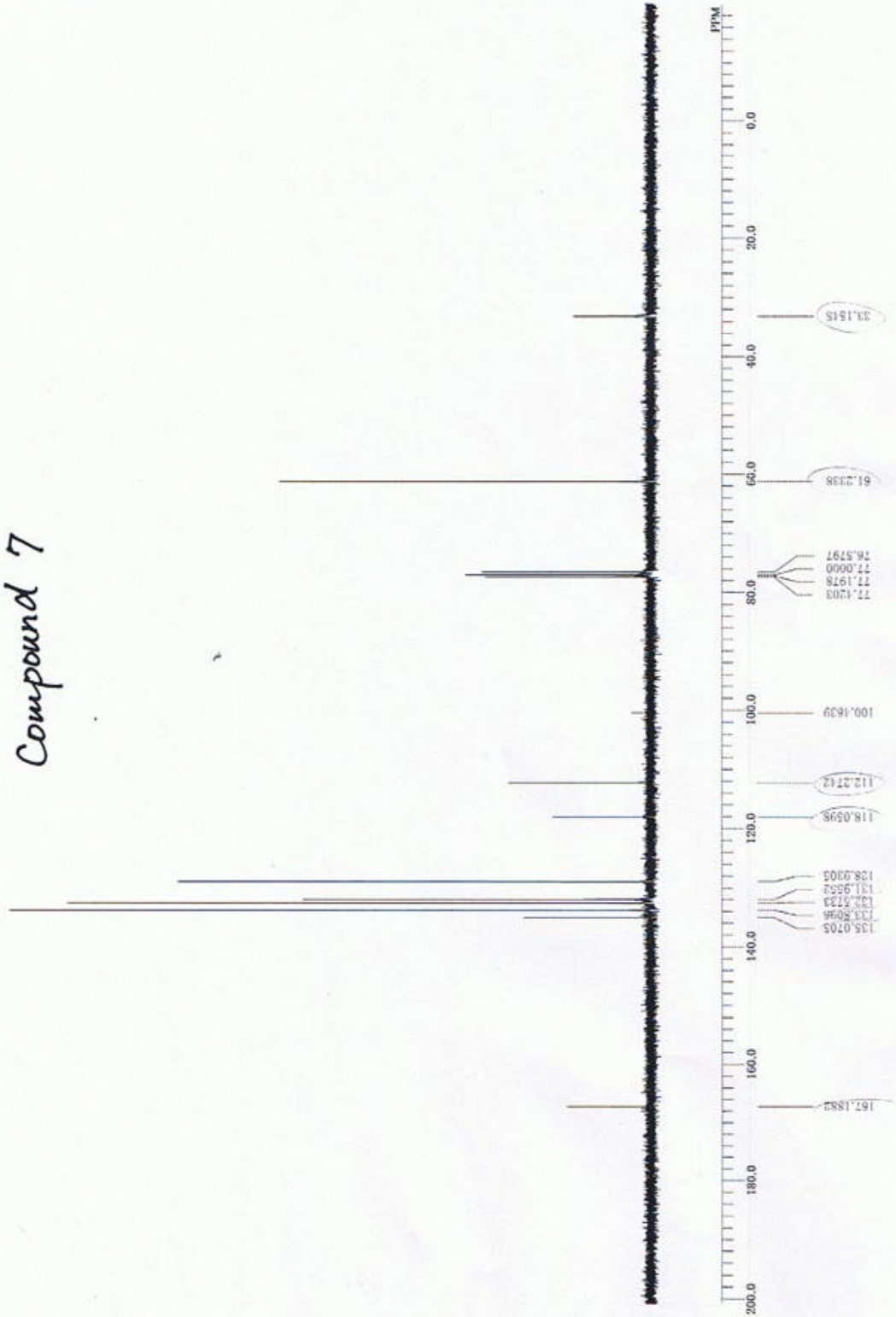
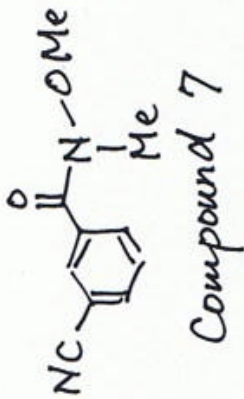
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Compound 7



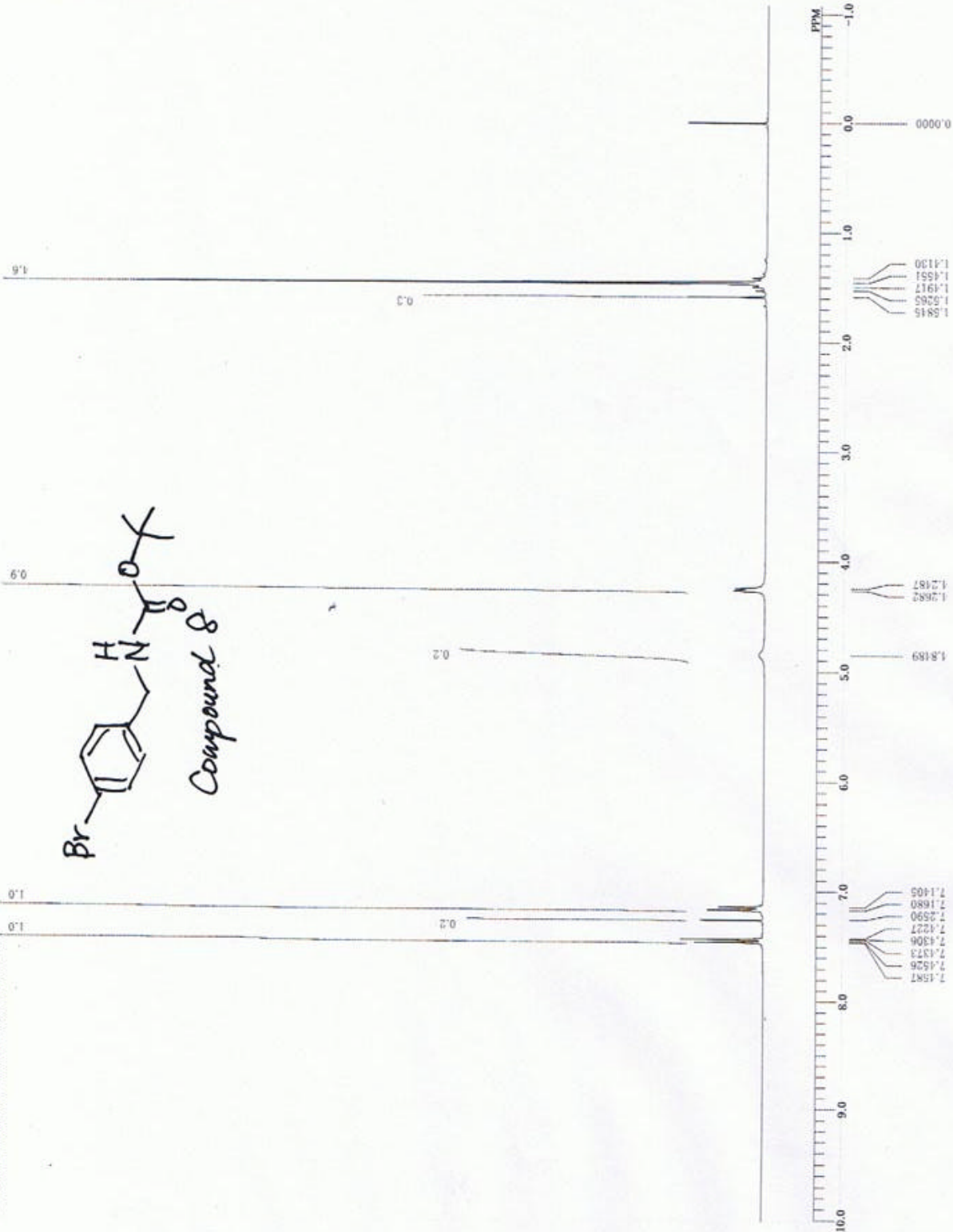
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RGAIN



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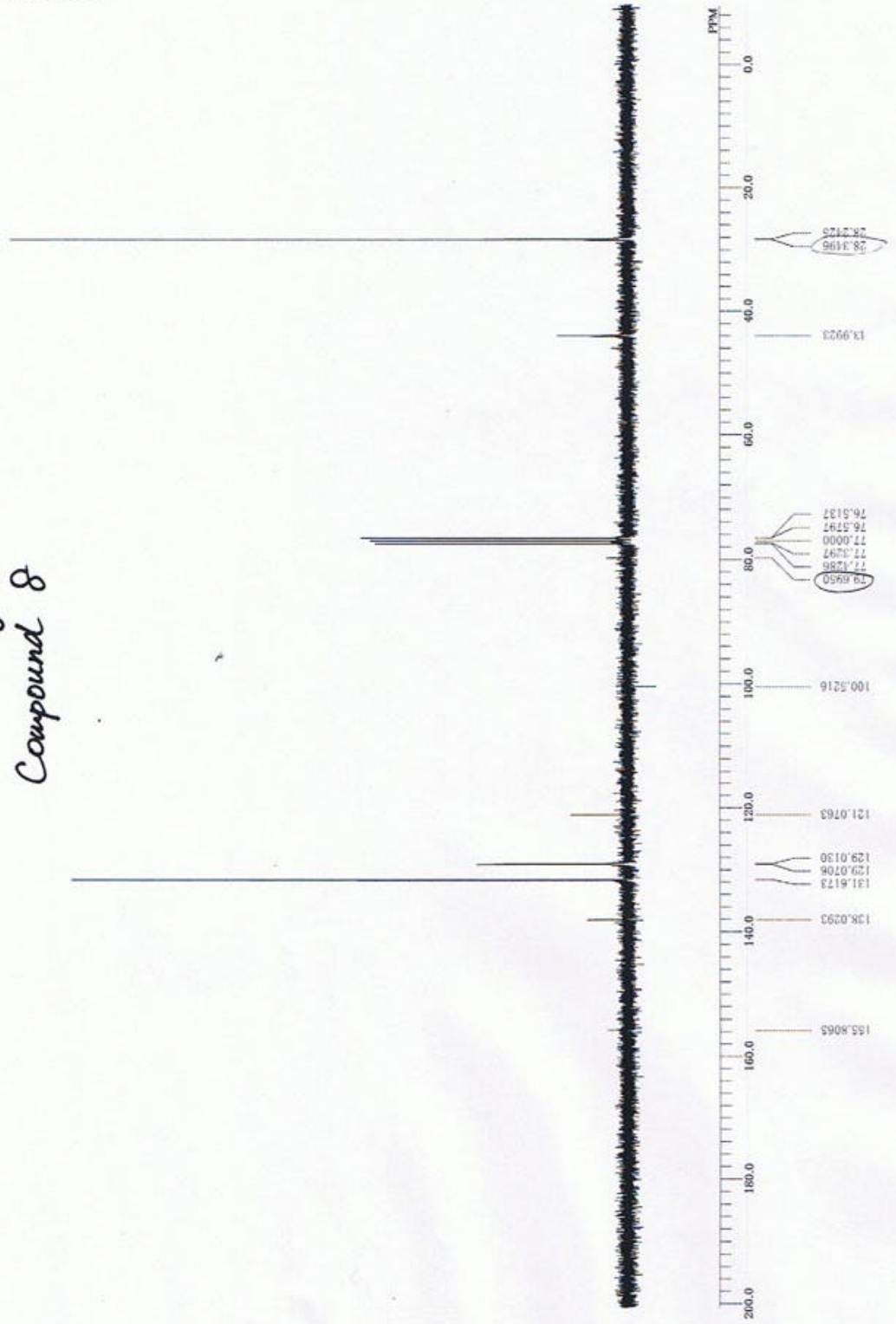
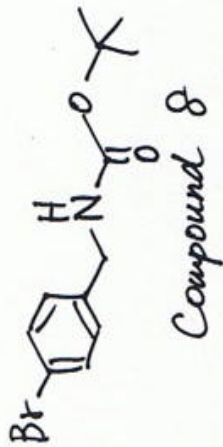
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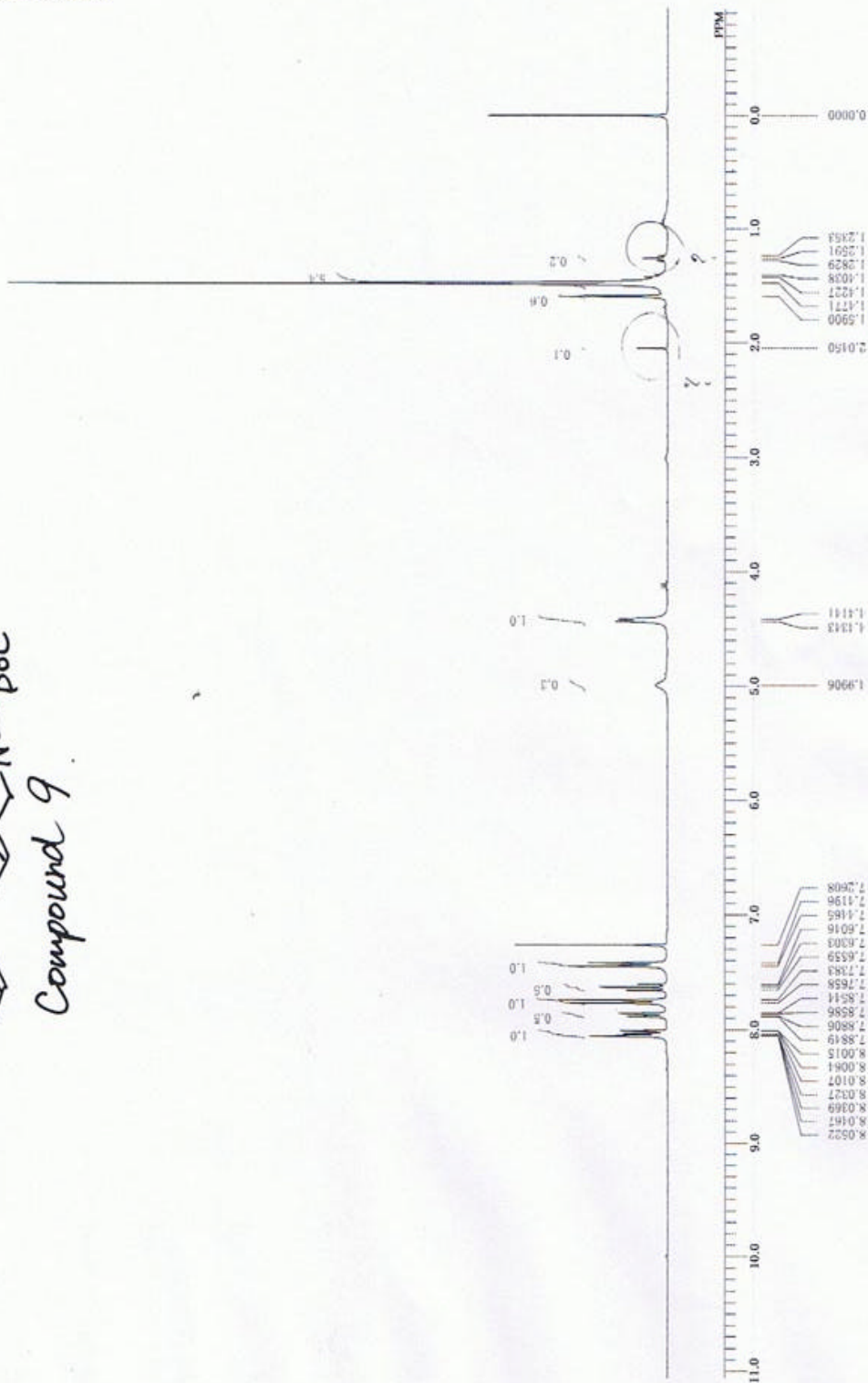
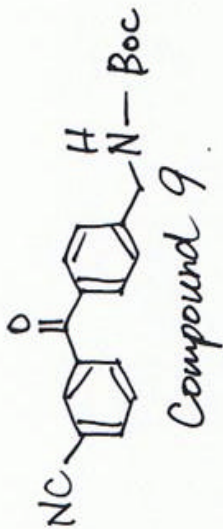
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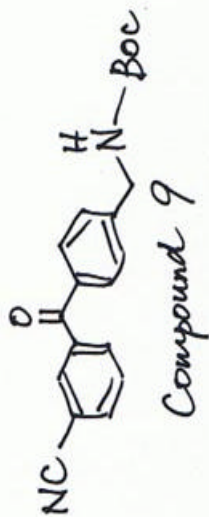
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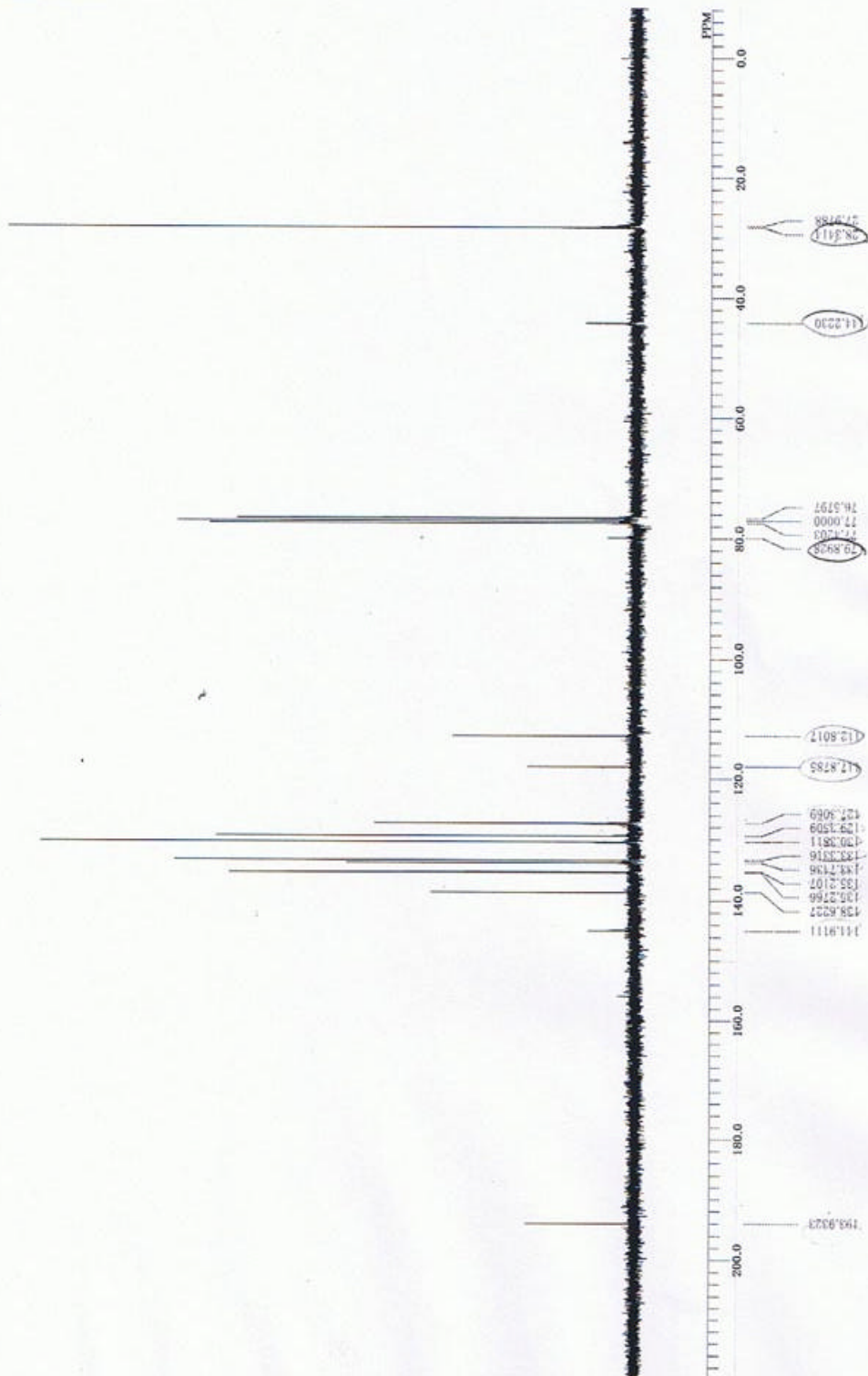
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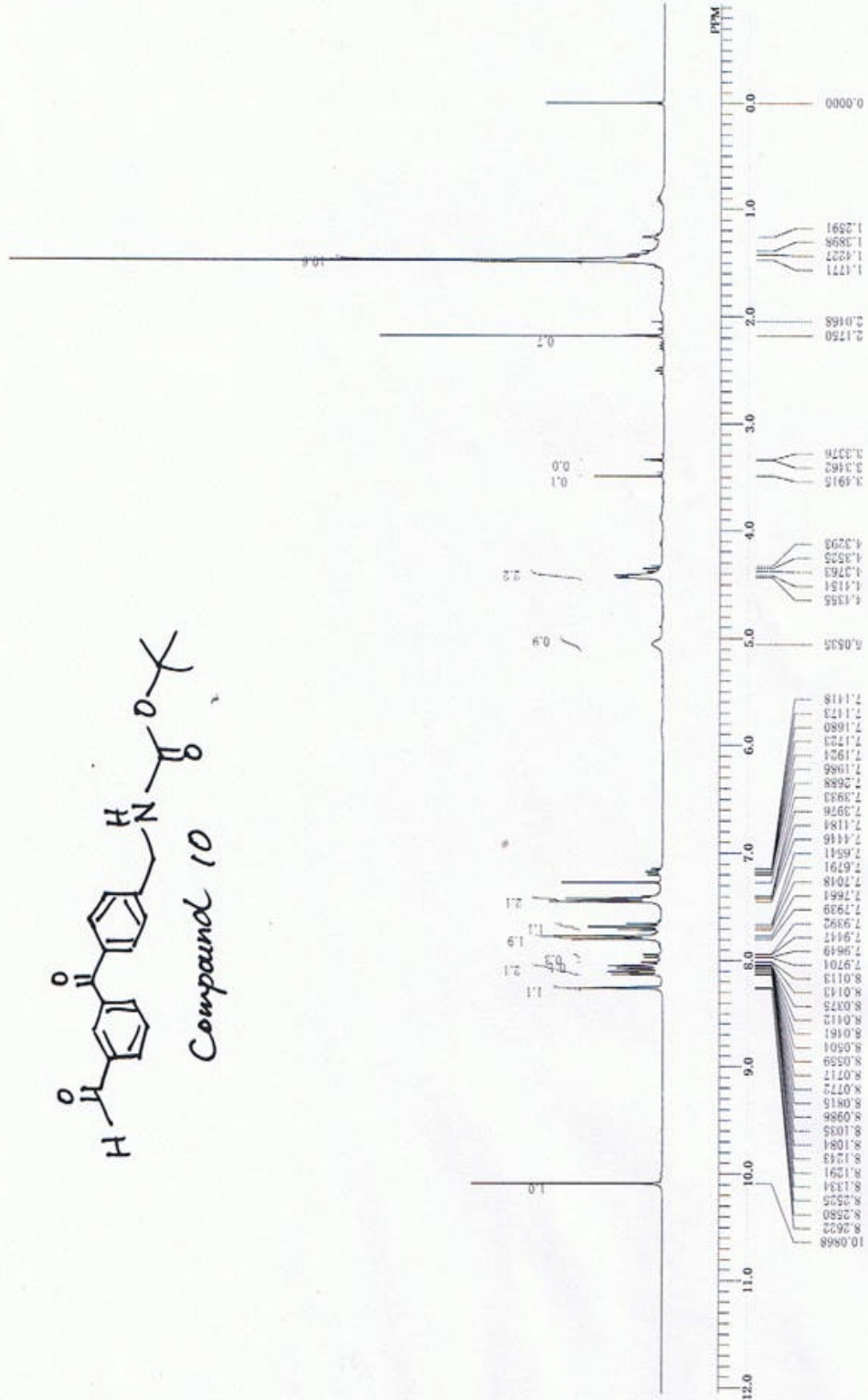
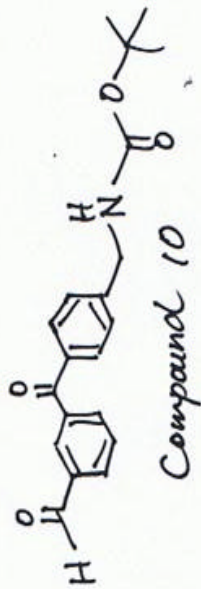


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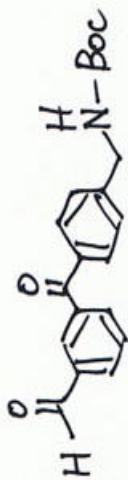


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 BF
 RGAIN

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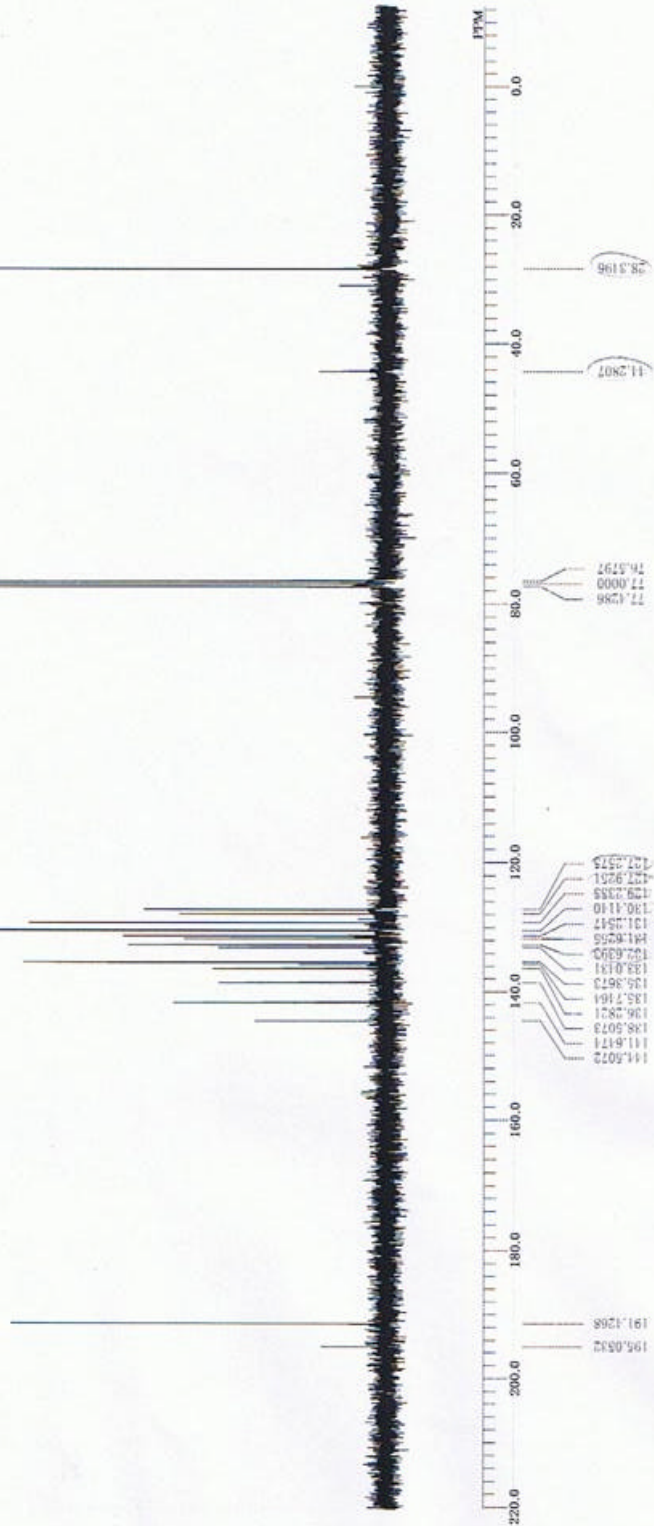
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Compound 10

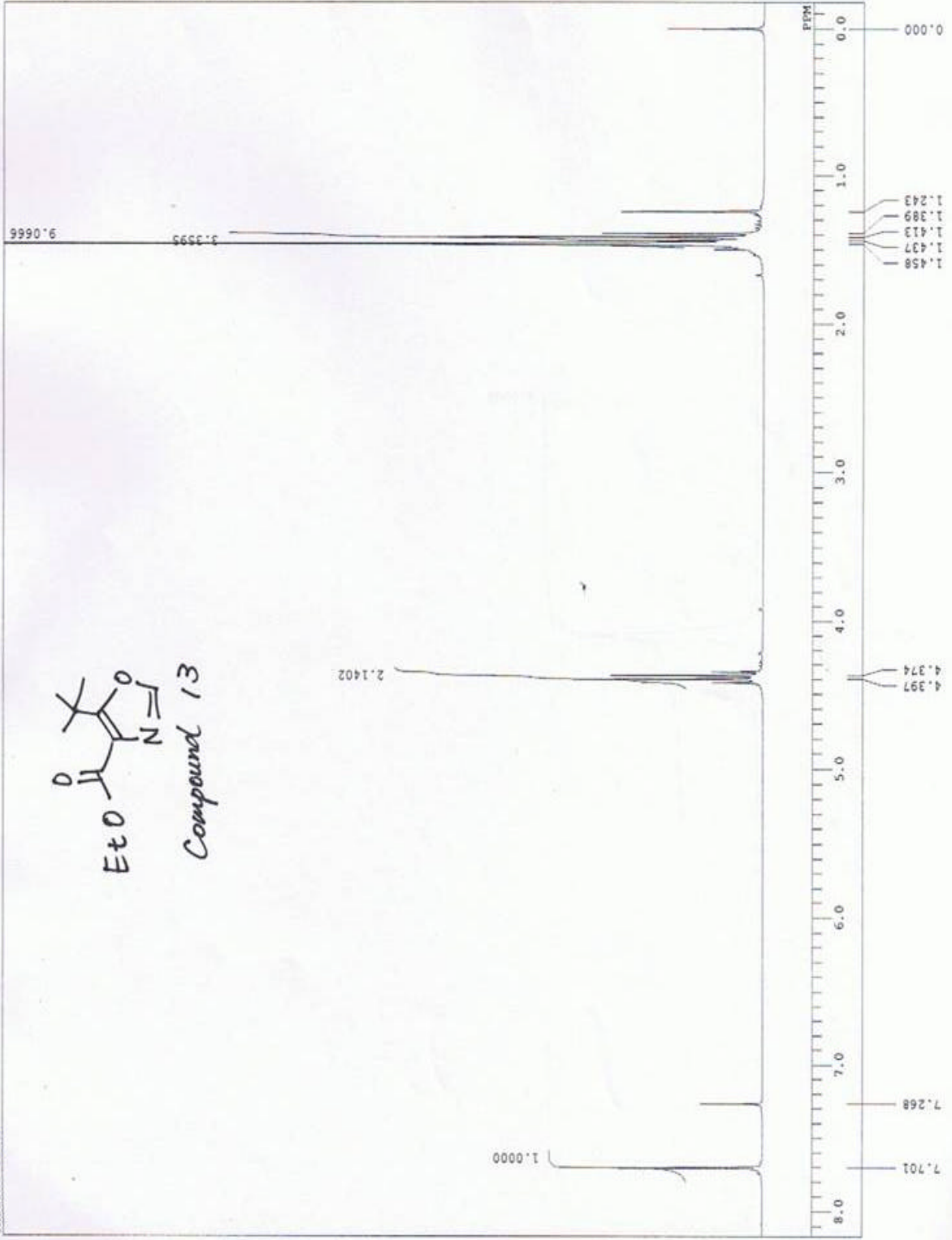
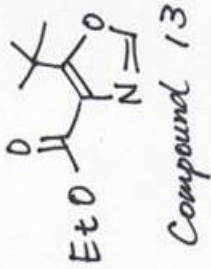
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BF 24
RGAIN

DFILE
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DATIM
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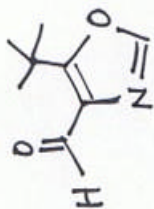
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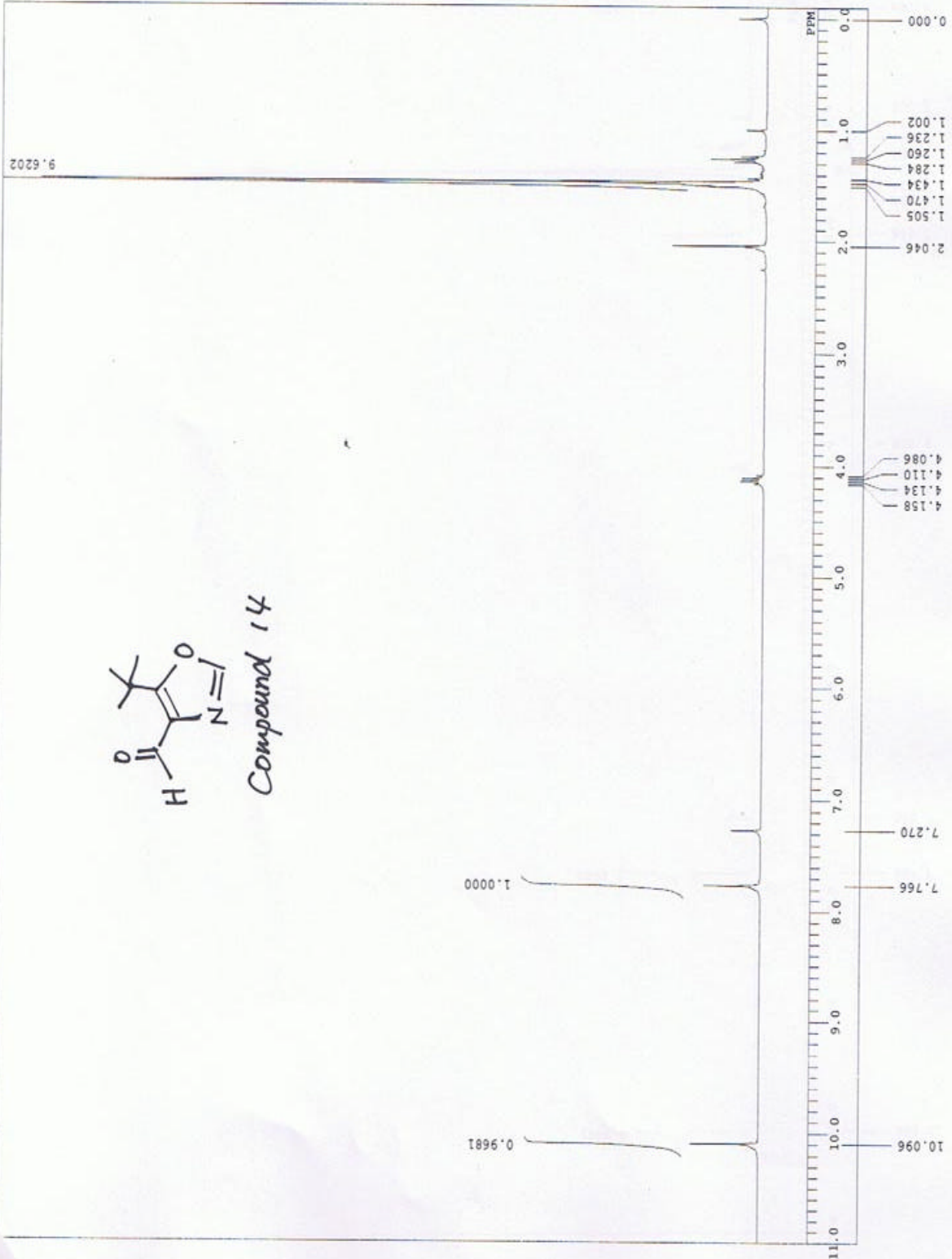


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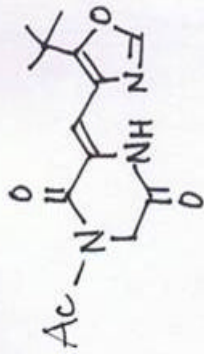
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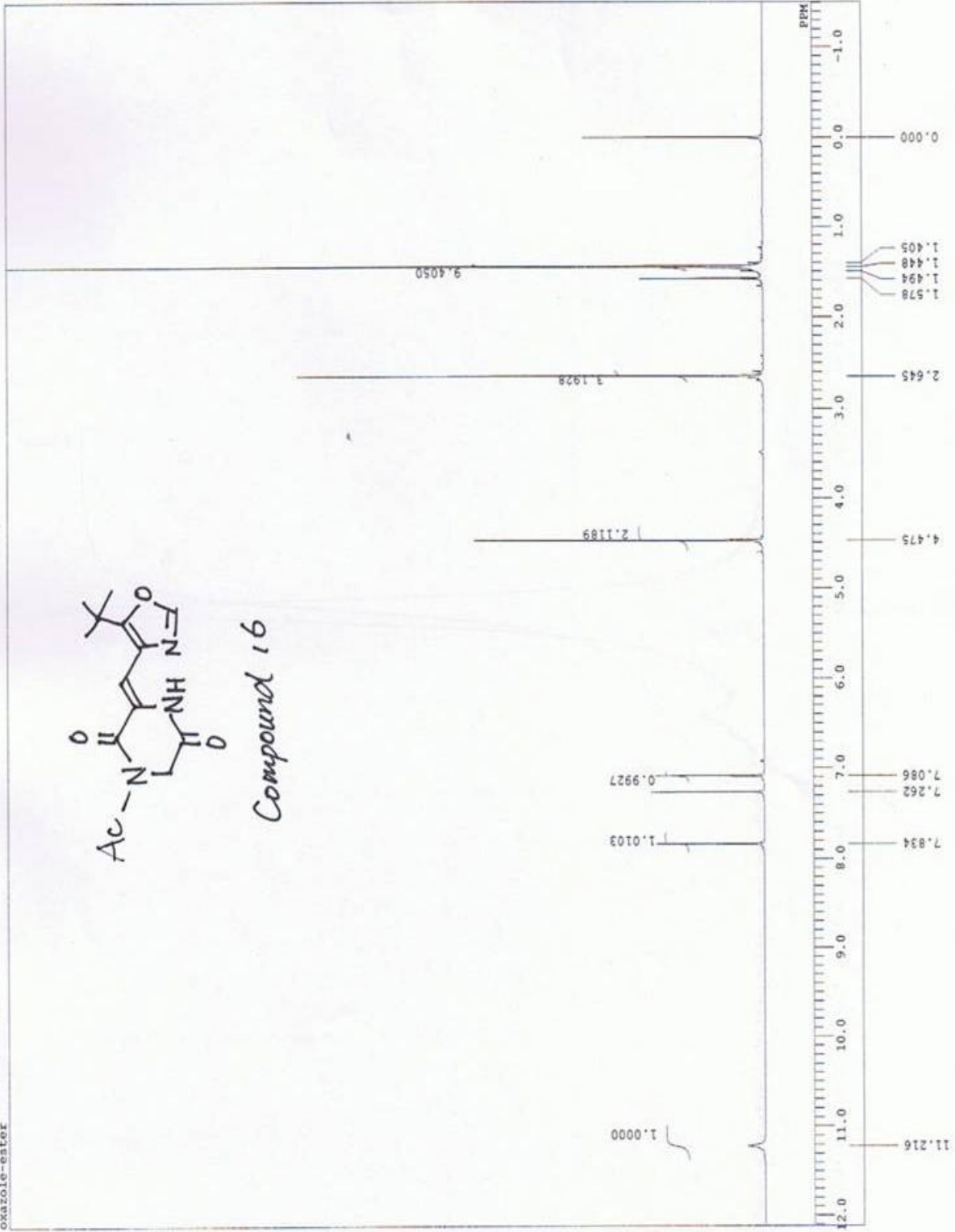
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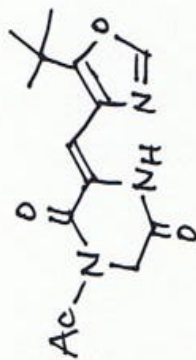
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RGAIN 20



Compound 16



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RGAIN 24



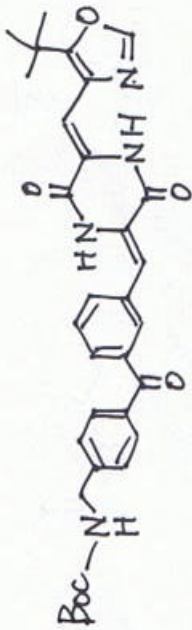
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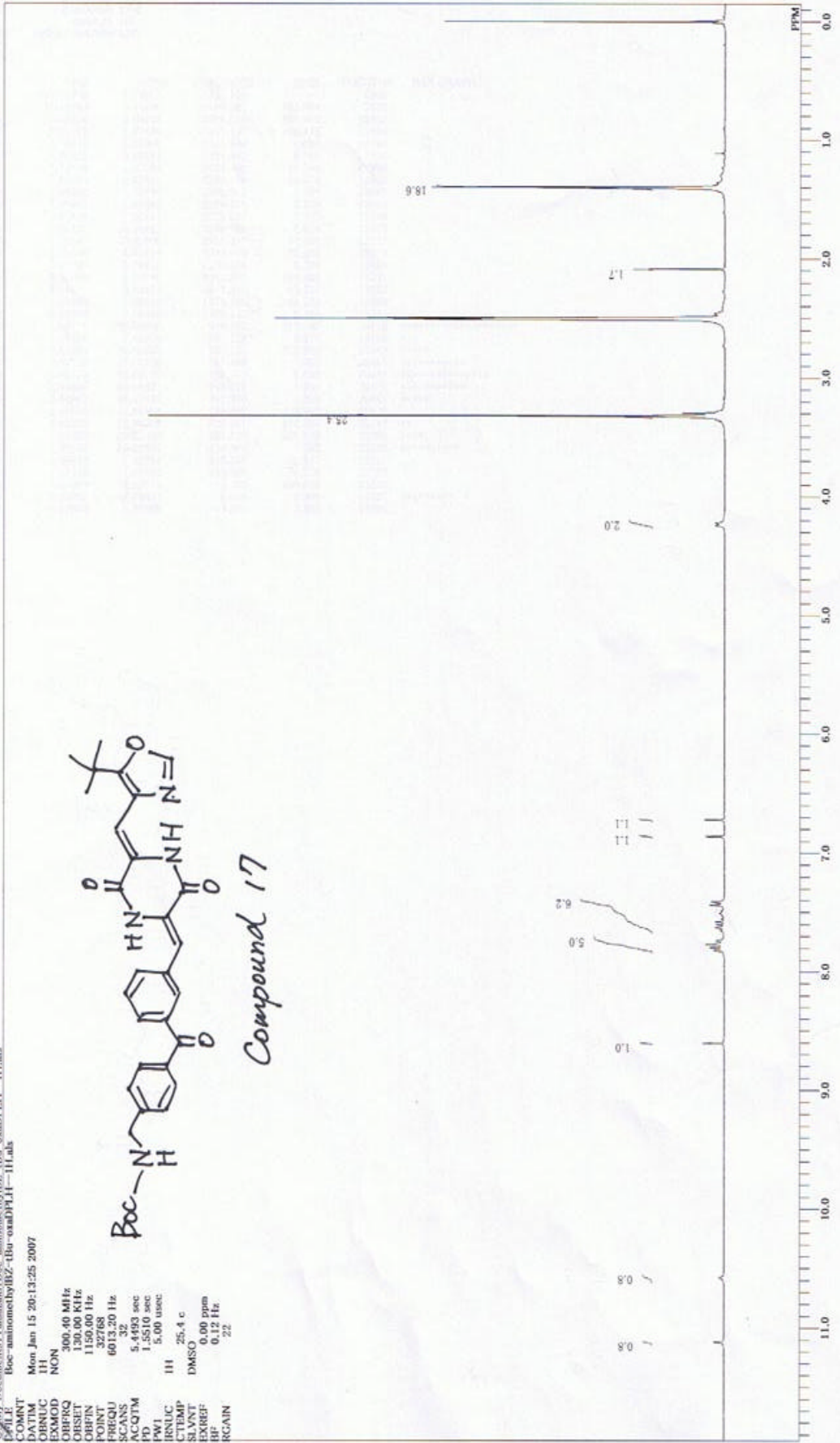
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Mon Jan 15 20:13:25 2007
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 EXREF
 RGAIN

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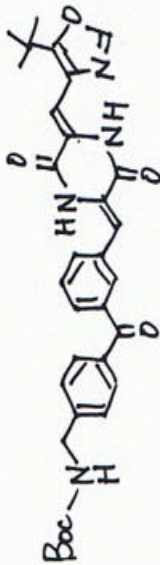
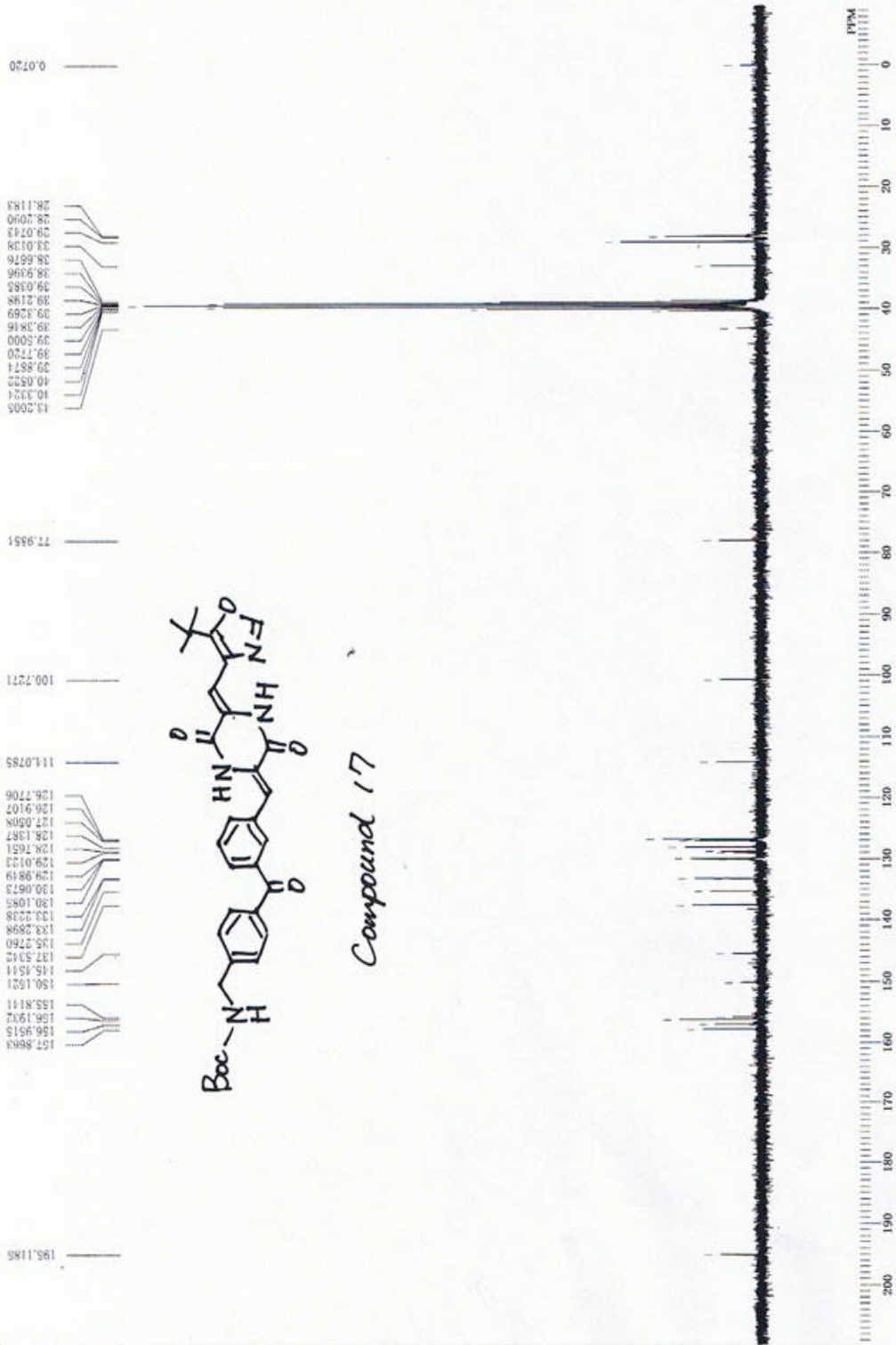


Compound 17



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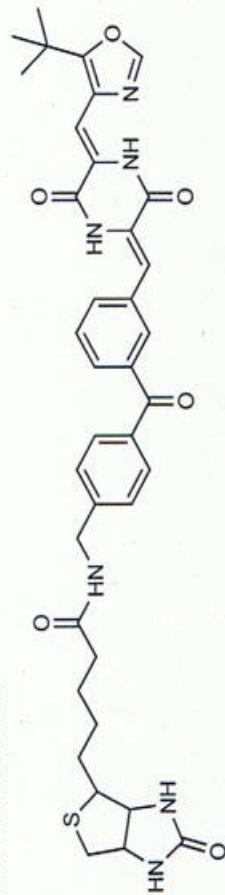
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SLVNT
EXREF
RGAIN



Compound 17

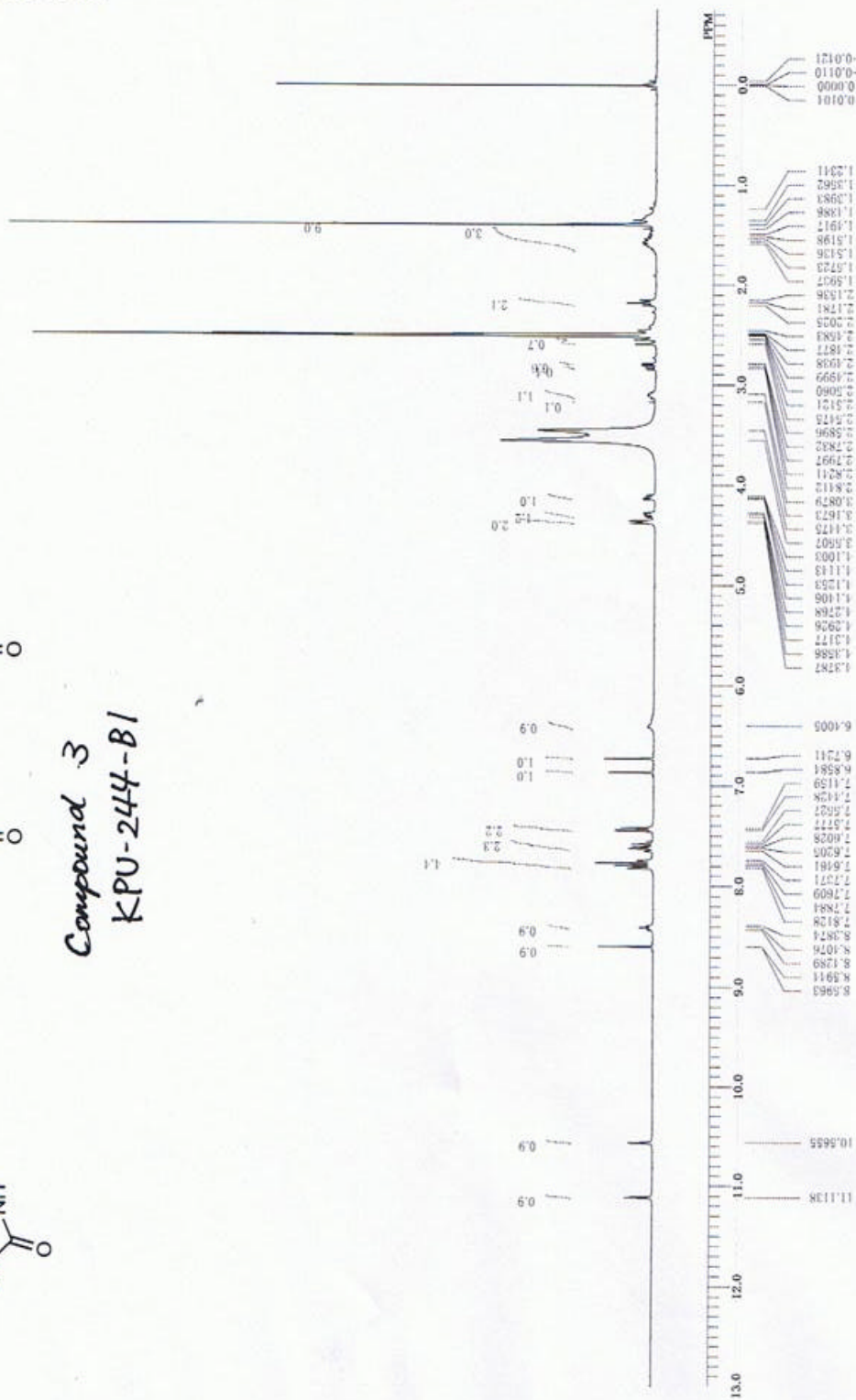
biotin-KPU244

C:\My Documents\Yammaral\Wadler-244T_als

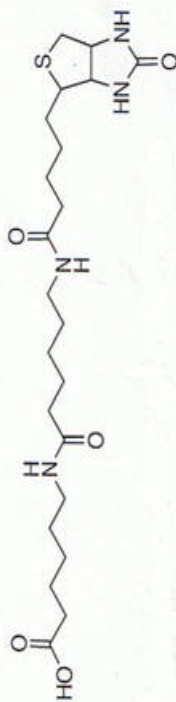


Compound 3
KPU-244-B1

FILE biotin-kpu244T_als
 CONT biotin-KPU244
 DATE Thu Oct 20 23:41:24 2005
 INH NON
 EXMOD 300.40 MHz
 OBSFQ 130.00 KHz
 OBSFN 1150.00 Hz
 POINT 32768
 NAME 1
 SCANS 60/13.20 Hz
 ACQTM 4.00 sec
 PD 1.5510 sec
 PW1 5.00 usec
 IRNUC 1H
 CTEMP 28.2 c
 SLVNT DMSO
 EXREF 0.00 ppm
 RGAIN 0.09 Hz
 21

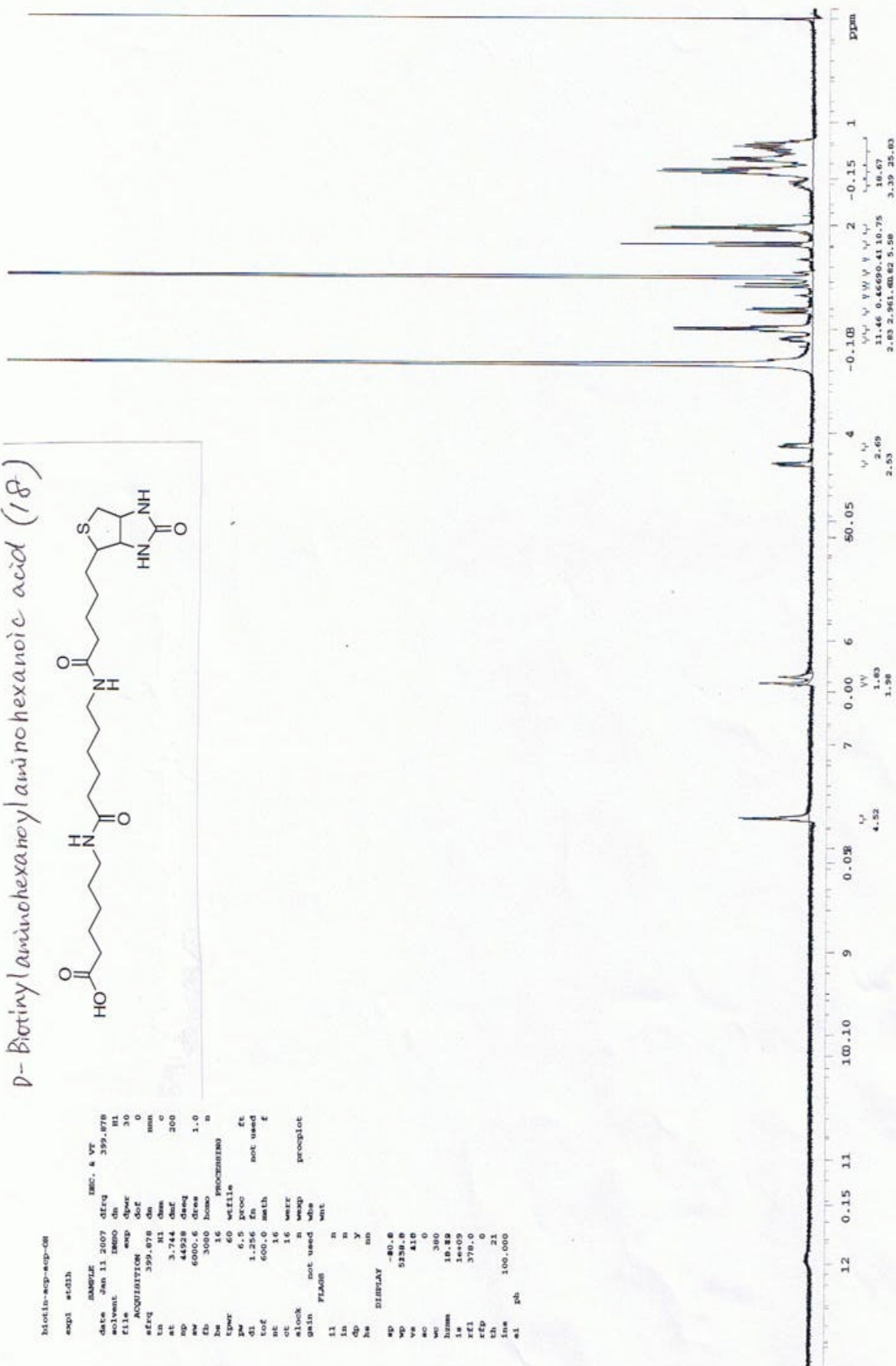


D-Biotinylamino hexanoic acid (18)

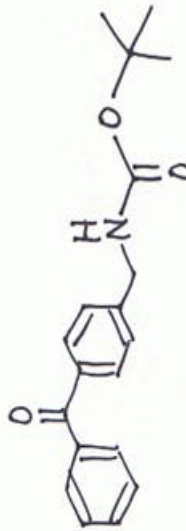


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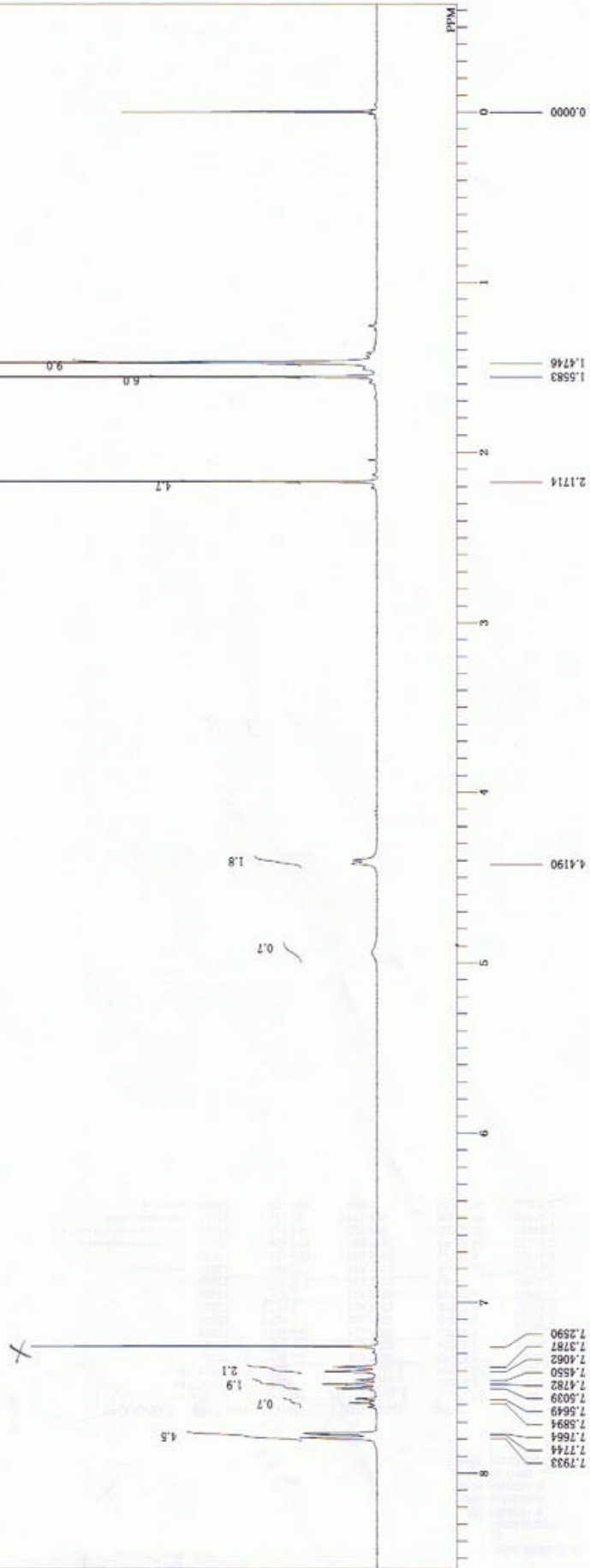
biotin-esp-esp-08
exp01 01033
SAMPLE
date Jan 13 2007 dirg 399.878
solvent dm DMSO-d6
file 30
ACQUISITION
sfrq 399.878 dm
ln H1 dm
at 3.744 dmf
ap 44928 dmf
ar 6000.6 dmf
ds 3000 homo
hs 16
PROCUREMENTS
dpr 60 willis
pr 4.5 p100
ds 1.50 dm
tqc 600.0 dmf
re 16
ce 16 warr
alook n wexp
gain not used wha
procplot
FLAGR
n1 n
ln n
cp y
hs no
DISPLAY
sp -80.6
wp 5238.8
vs 410
ac 0
sc 380
hs mm
ln 18.98
st 2
rel 378.0
ksp 0
th 21
lms 100.000
a1 gb
  
```



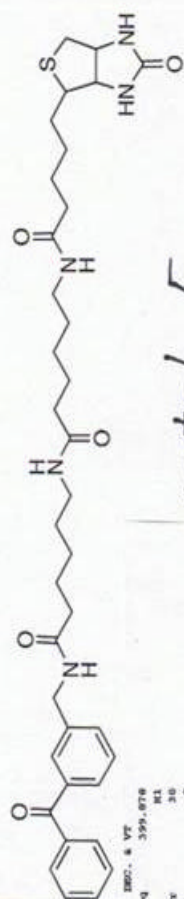
061220-2.xls
 COMNT Wed Dec 20 18:29:09 2006
 DATM 11
 OBNUC 11
 EXMOD NON
 OBRFQ 300.40 MHz
 OBSRT 150.00 KHz
 P1 1.0000000000000000
 P2 32768
 FREQ 6013.20 Hz
 SCANS 64
 ACQTM 5.4453 sec
 PD 1.5510 sec
 5.00 usec
 11 94.8 c
 CDCL3
 SAMP 0.00 ppm
 EXREF 0.09 Hz
 BF 21
 RGAIN



Compound 20

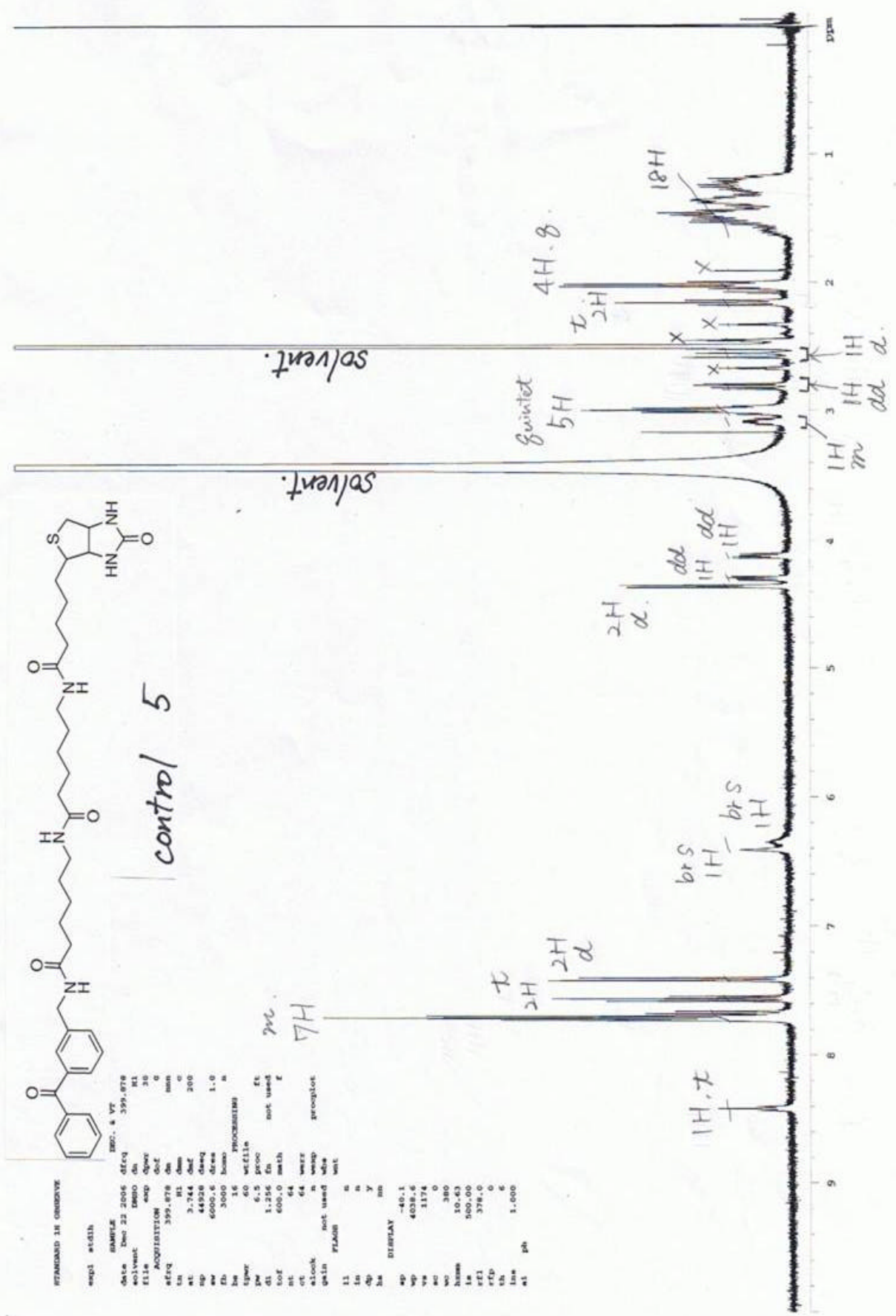


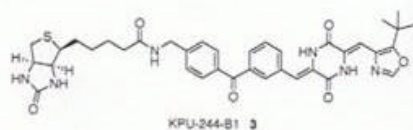
PTERIDINAMID 3H CHROMOLONE



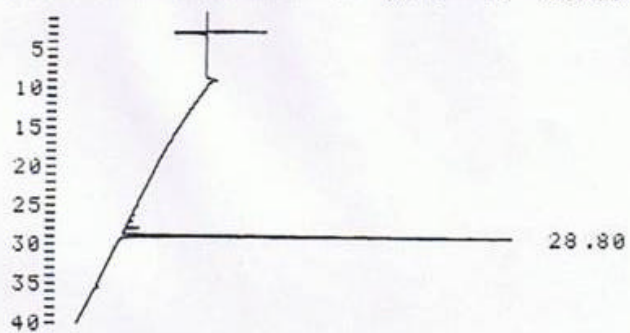
control 5

emp1	std1h
DATE	Dec 23 2006
TIME	08:00
EXP	399.878
ACQ	399.878
IN	31
AT	3.744
SP	44928
AV	6000.5
FS	3000
HS	16
TYPE	60
PR	6.5
DL	1.256
SOX	600.0
HL	64
CT	64
CT	64
Q1	not used
Q2	not used
Q3	not used
Q4	not used
Q5	not used
Q6	not used
Q7	not used
Q8	not used
Q9	not used
Q10	not used
Q11	not used
Q12	not used
Q13	not used
Q14	not used
Q15	not used
Q16	not used
Q17	not used
Q18	not used
Q19	not used
Q20	not used
Q21	not used
Q22	not used
Q23	not used
Q24	not used
Q25	not used
Q26	not used
Q27	not used
Q28	not used
Q29	not used
Q30	not used
Q31	not used
Q32	not used
Q33	not used
Q34	not used
Q35	not used
Q36	not used
Q37	not used
Q38	not used
Q39	not used
Q40	not used
Q41	not used
Q42	not used
Q43	not used
Q44	not used
Q45	not used
Q46	not used
Q47	not used
Q48	not used
Q49	not used
Q50	not used
Q51	not used
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Q53	not used
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Q55	not used
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Q66	not used
Q67	not used
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Q82	not used
Q83	not used
Q84	not used
Q85	not used
Q86	not used
Q87	not used
Q88	not used
Q89	not used
Q90	not used
Q91	not used
Q92	not used
Q93	not used
Q94	not used
Q95	not used
Q96	not used
Q97	not used
Q98	not used
Q99	not used
Q100	not used





CH. 1 C.S 1.25 ATT 7 OFFS 10 00/25/00 04:19



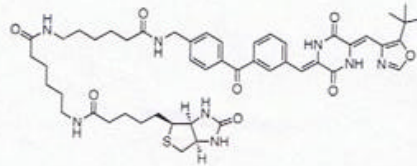
D-2500

00/25/00 04:19

METHOD: TAG: 24 CH: 1

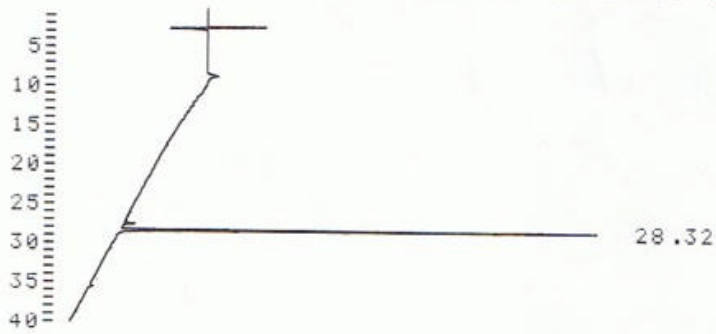
FILE: 1 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	28.80	354198	100.000	BB
TOTAL		354198	100.000	
PEAK REJ :		50000		



KPU-244-B2 4

CH. 1 C.S 1.25 ATT 7 OFFS 10 00/20/00 00:36



D-2500

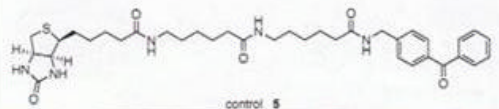
00/20/00 00:36

METHOD:

TAG: 19 CH: 1

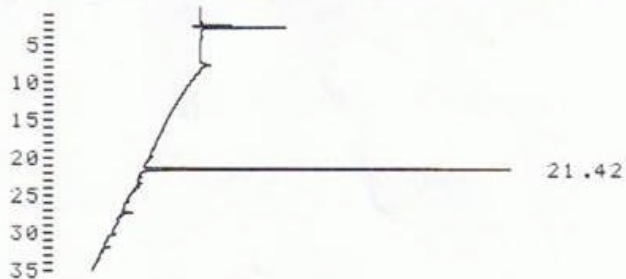
FILE: 1 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	28.32	441569	100.000	BB
TOTAL		441569	100.000	
PEAK REJ :		20000		



0 → 100% (40 min)

CH. 1 C.S 1.25 ATT 7 OFFS 10 01/51/00 01:24



D-2500

01/51/00 01:24

METHOD: TAG: 15 CH: 1

FILE: 1 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	21.42	347736	100.000	BB
TOTAL		347736	100.000	
PEAK REJ :		50000		