Supplementary Information

Highly Specific, Bisubstrate-Competitive Src Inhibitors from DNA-Templated Macrocycles

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Supplementary Results

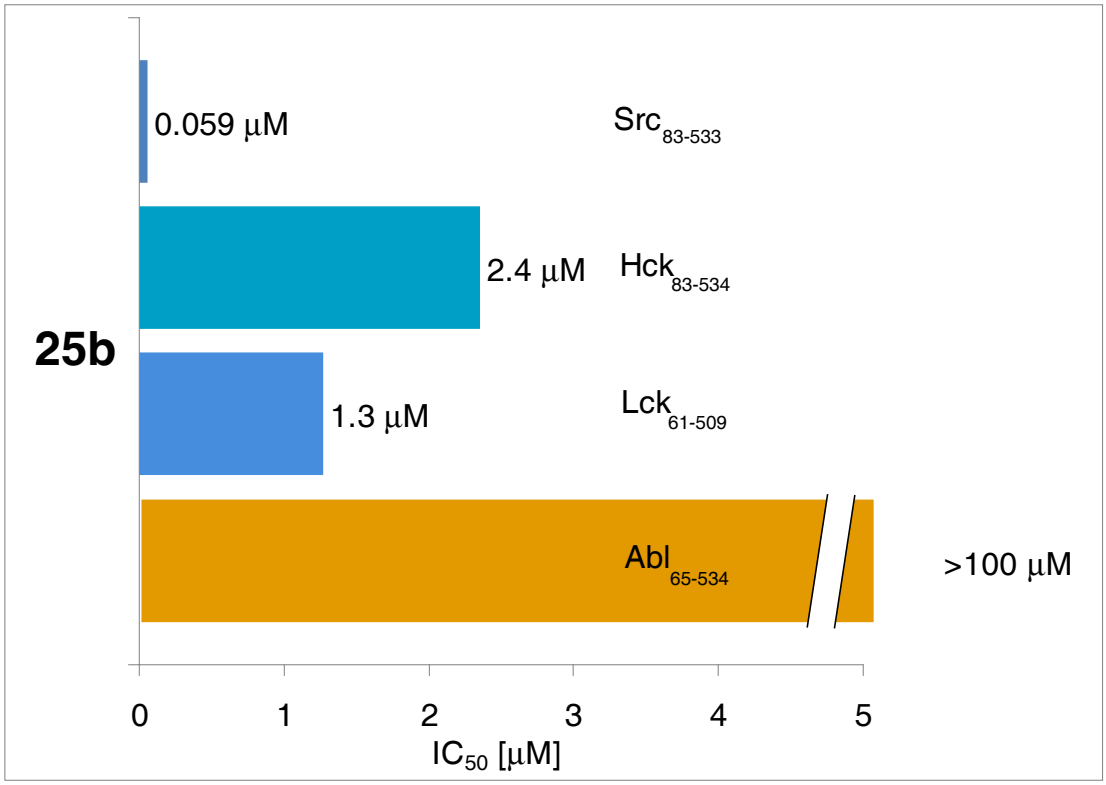
a

IC_{50} [μM]	Src	Hck	Lck	Abl
1	60	> 100	> 100	> 100
2	15	> 100	> 100	> 100
9	6.8	> 100	> 100	> 100

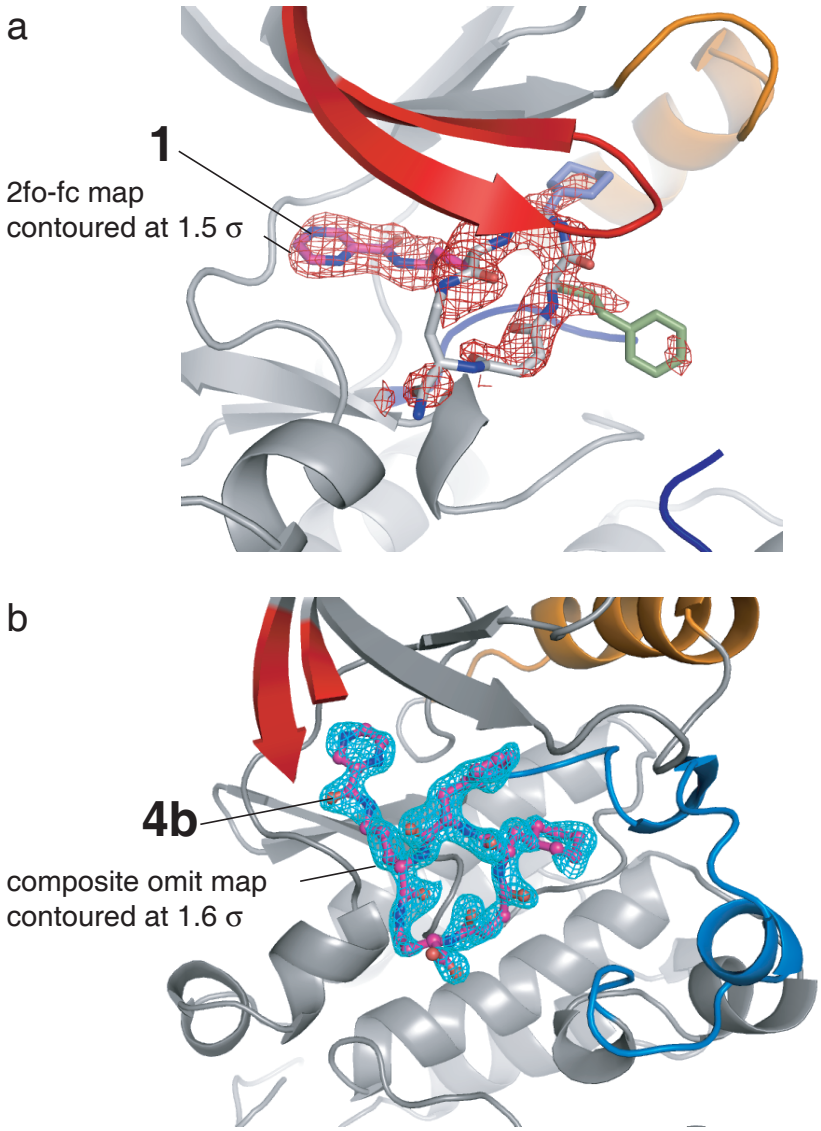
b

IC_{50} [μM]	Src	Hck	Lck	Abl
4b	0.13	0.86	2.4	> 100
25b	0.099	8.4	6.1	> 100

Supplementary Figure 1. Specificity of macrocyclic kinase inhibitors. (a) IC_{50} values of **1**, **2**, and **9** for kinase domains were determined in the presence of 5 μM ATP and 100 μM Src-optimal substrate peptide. (b) IC_{50} values of **4b** and **25b** for kinase domains were determined in the presence of 250 μM ATP and 300 μM Src-optimal substrate peptide.

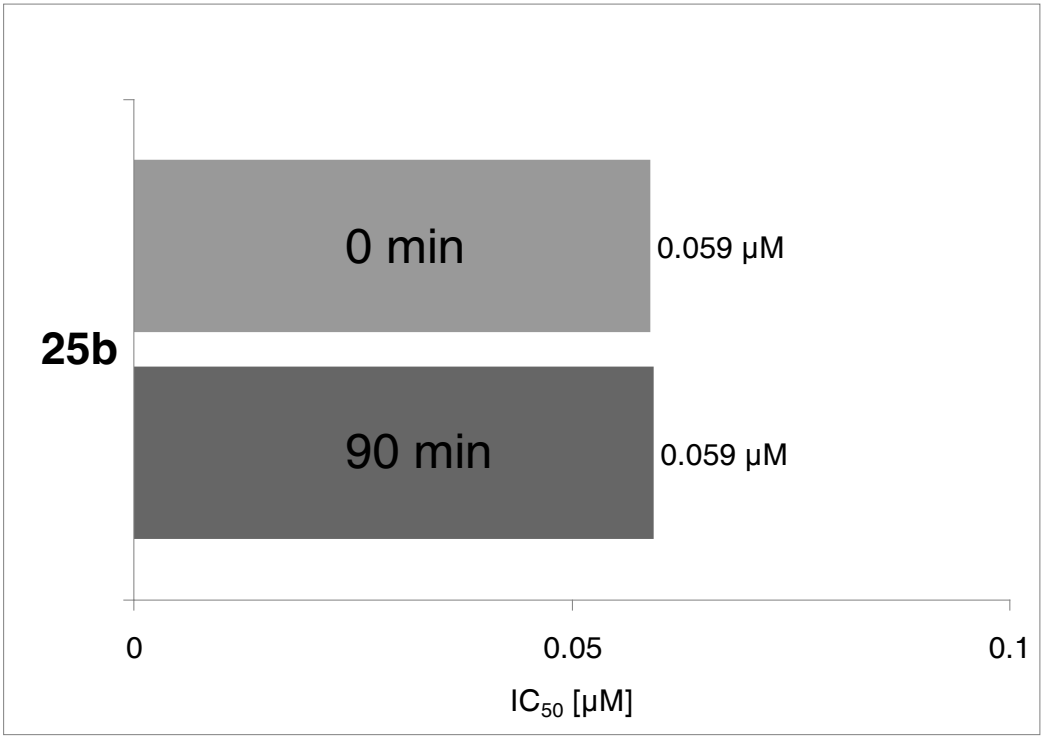


Supplementary Figure 2. In vitro potency of **25b** against three-domain constructs of Src, Hck, Lck, and Abl kinase. The inhibition activity of **25b** was measured using the continuous spectrophotometric kinase assay with 300 µM Src optimal substrate peptide, 250 µM ATP, 100 nM Src, 12.5 nM Hck, 42 nM Lck and 75 nM Abl.

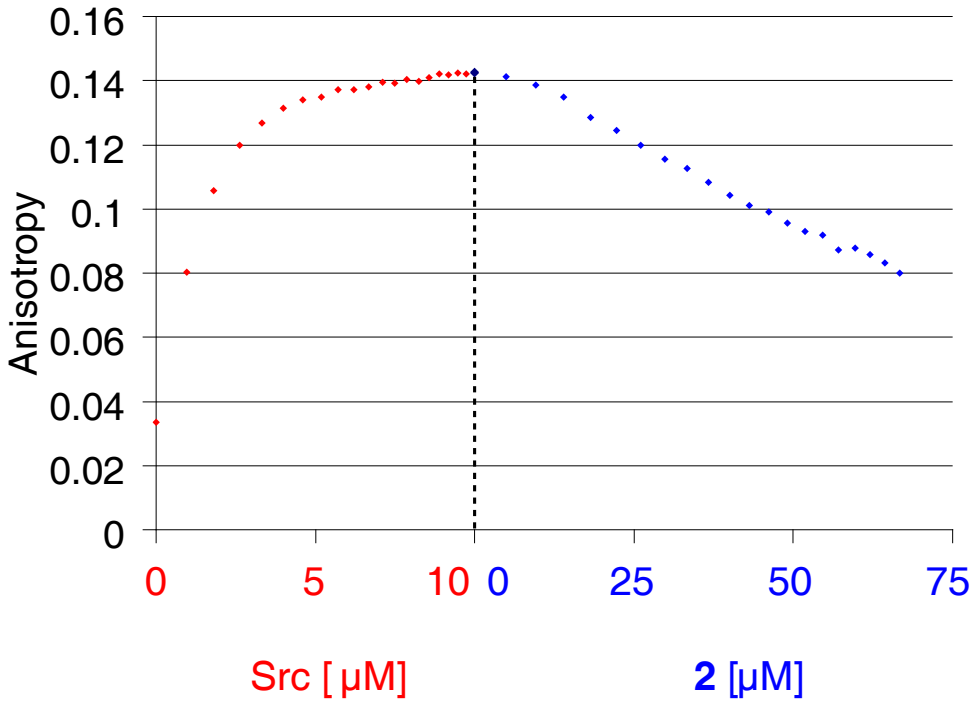


Supplementary Figure 3. Electron density maps and structural models of Src bound to macrocycles. (a) 2fo-fc electron density map around **1** contoured at 1.5 σ (red). (b) Composite omit map (cyan) around **4b**, contoured at 1.6 σ. The starting models for the macrocycle compounds were built in ChemDraw and a semiempirical quantumchemical AM1 geometry optimization was performed in REEL 0.9 (Restraints Editor Exclusively Ligands) (Nigel Moriarty) via the Phenix 1.6.1-336 interface (PHENIX: a comprehensive Python-based system for macromolecular structure solution. P. D. Adams, P. V. Afonine, G. Bunkóczi, V. B. Chen, I. W. Davis, N. Echoo ls, J. J. Headd, L.-W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner), R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger and P. H. Zwart. Acta Cryst. D66, 213-221 (2010)). The optimized models of the macrocycles were built manually into the experimental electron density using COOT (Paul Emsley and Bernhard Lohkamp and William G. Scott and Kevin Cowtan; Features and Development of Coot;Acta Crystallographica Section D - Biological Crystallography).

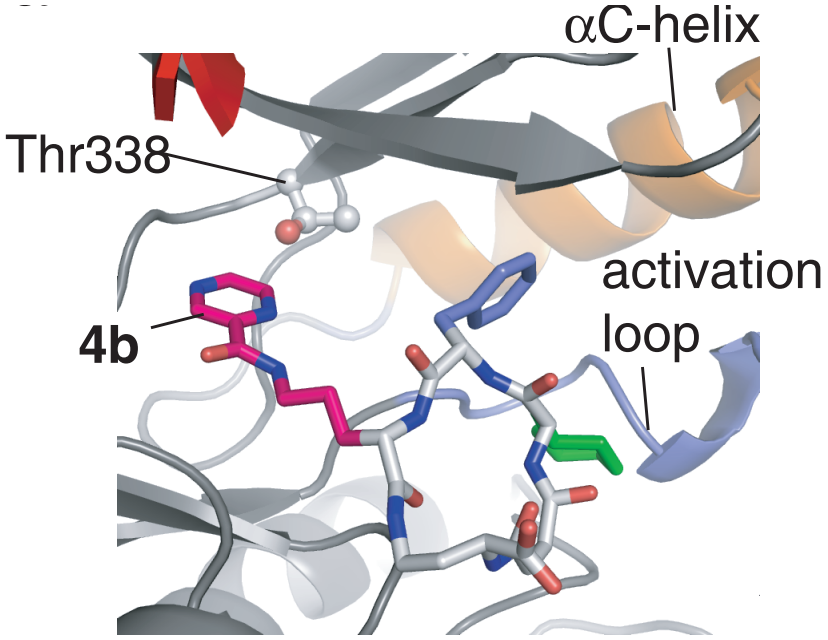
Supplementary Figure 4. Determinants of macrocycle specificity. Sequence alignment of the phosphate-binding loop and β3-αC loop regions of the kinases tested here. Amino acids corresponding to Src residues that are within 5Å of **4b** in the co-crystal structure are highlighted in cyan. Amino acids differing in sequence from Src kinase domain are colored in red. The numbering of residues corresponds to chicken c-Src numbering. The half-maximal inhibitory concentration (IC_{50}) was determined in the presence of 250 μM ATP and 300 μM Src-optimal substrate peptide.



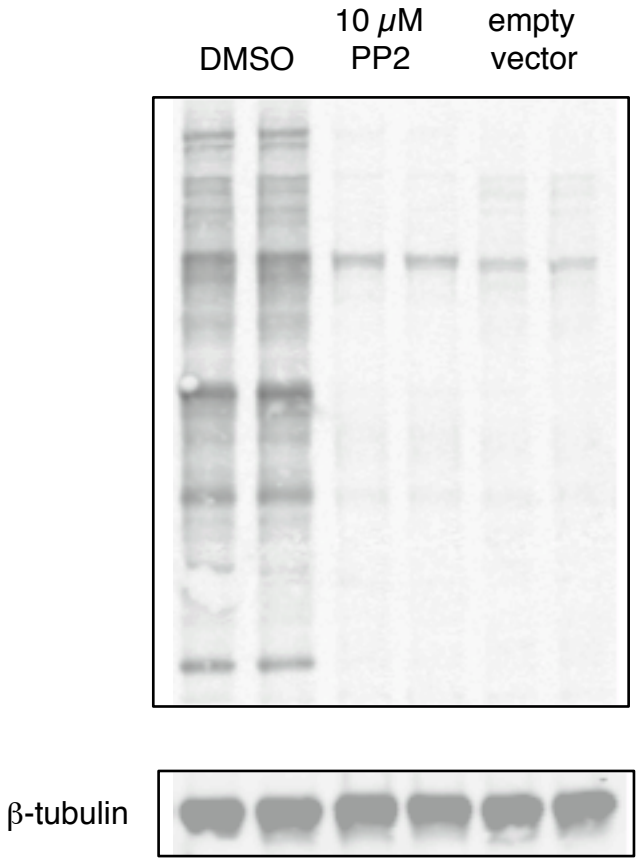
Supplementary Figure 5. Time-dependent inhibition study. Src_{83-533} (100 nM) was incubated with 0-640 nM **25b** and kinase activity was measured either immediately ("0 min") or after 90 min incubation at 30 ºC ("90min") using the spectrophotometric assay with Src optimal peptide at 300 µM and 250 µM ATP.



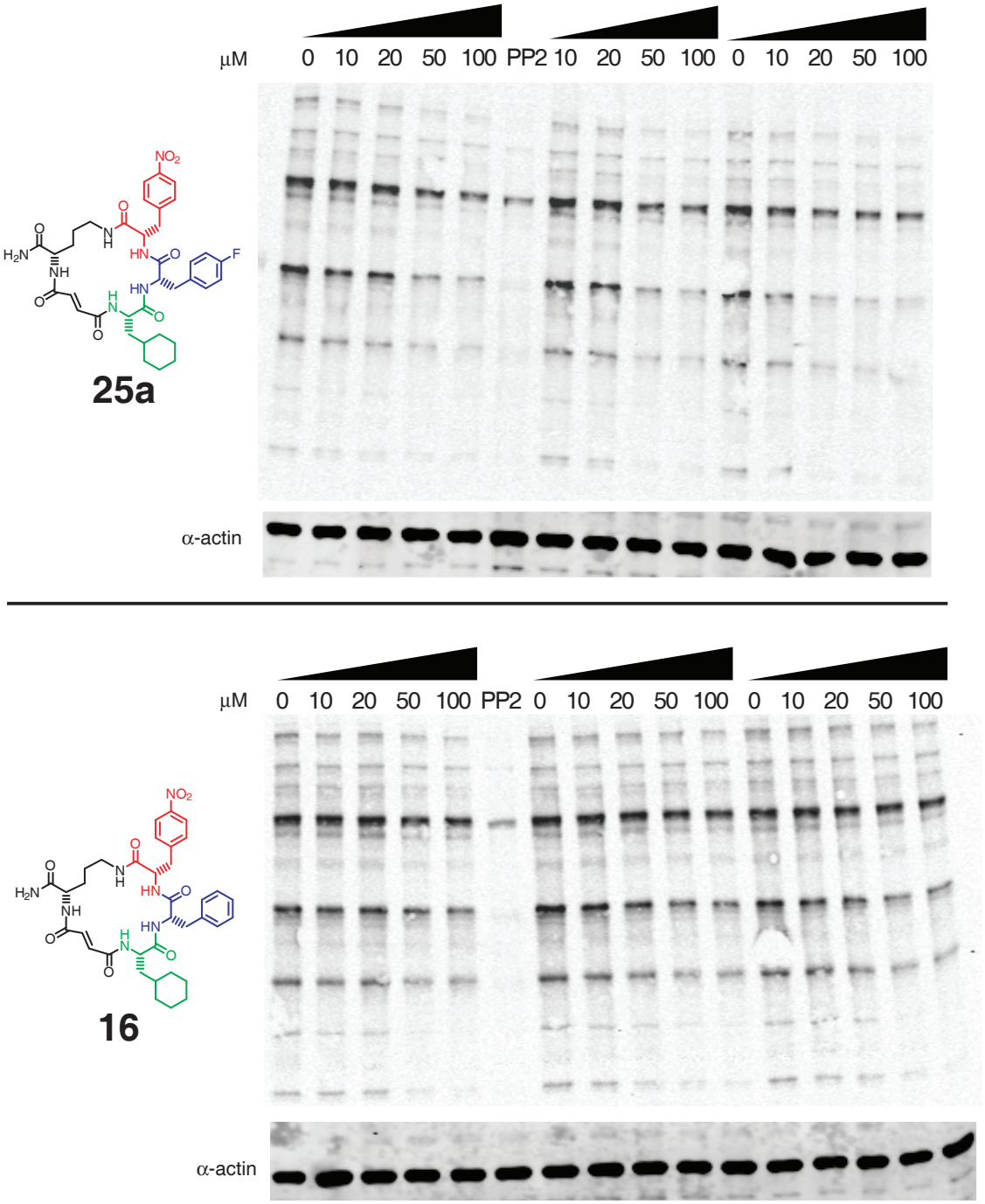
Supplementary Figure 6. Reversibility of inhibitor binding to Src kinase. Src kinase domain was added to 0.5 μM fluorescein-**2**. The anisotropy of fluorescein (left half of the plot, concentration referring to Src kinase domain added to the fluorescently labeled compound) was followed as Src concentrations increased. The resulting increase in anisotropy indicated binding of Src to fluorescein-**2**. Following saturation of fluorescein-**2** anisotropy, we added increasing amounts of unlabeled **2** to the mixture of Src and fluorescein-**2**. The decrease in anisotropy indicates the replacement of fluorescein-**2** with unlabeled **2** and confirms the reversibility of **2**-Src binding.



Supplementary Figure 7. Activity of the macrocycles against the Thr338Ile gatekeeper mutant of Src kinase. (a) Structure of the Src•**4b** complex with residues 272-283 of the P-loop (red) removed for clarity. The gatekeeper residue Thr338 is rendered in spheres and sticks.



Supplementary Figure 8. Phosphotyrosine levels in 3T3 ($src^{-/-}$) cells expressing Src Y529F. Treatment with the Src-family selective kinase inhibitor PP2 (lanes 3 and 4) reduces global phosphotyrosine levels detected by 4G10 antibody to comparable levels as in 3T3 ($src^{-/-}$) transfected with empty vector (lanes 5 and 6).



Supplementary Figure 9. Macrocycles **25a** and **16** inhibit Src kinase activity in cultured murine cells. 3T3 (src-/-) cells transfected with a plasmid encoding Src Y529F were seeded in a 48-well plate and grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Individual wells were then treated with the indicated concentration of macrocycle in serum-free DMEM for six hours. The final concentration of DMSO in each treatment was 2%. After removing the small molecule and washing with phosphate-buffered saline, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and global phosphotyrosine levels were quantified by Western blot using the 4G10 antibody.

Supplementary Table 1 Data collection and refinement statistics (molecular replacement)

	Src•1	Src•4b
Data collection		
Space group	$P2_1$	P321
Cell dimensions		
a, *b*, *c* (Å)	42.2, 117.3, 62.7	143.6, 143.6, 41.5
α, β, γ (°)	90.0, 90.1, 90.0	90.0, 90.0, 120.0
Resolution (Å)	50-2.24	41.5-1.9
R_{sym} or R_{merge}	0.069 (0.30)	0.098 (0.42)
I / σI	14.2 (3)	13.7 (4.1)
Completeness (%)	97.9 (85.7)	100 (99.9)
Redundancy	3.1	6.0
Refinement		
Resolution (Å)	2.24	1.9
No. reflections	29,870	38,763
R_{work} / R_{free}	0.1929 / 0.2496	0.1622 / 0.1849
No. atoms		
Protein	4140	2223
Ligand/ion	106	51
Water	107	324
B-factors		
Protein	Chain A: 48.4 Chain B: 48.9	17.03
Ligand/ion	Chain A: 53.2 Chain B: 53.4	24.08
Water	39.2	27.44
R.m.s. deviations		
Bond lengths (Å)	0.002	0.007
Bond angles (°)	0.687	1.117
PDB accession code	3U51	3U4W

* One crystal was used to solve each structure
*Highest-resolution shell is shown in parentheses.

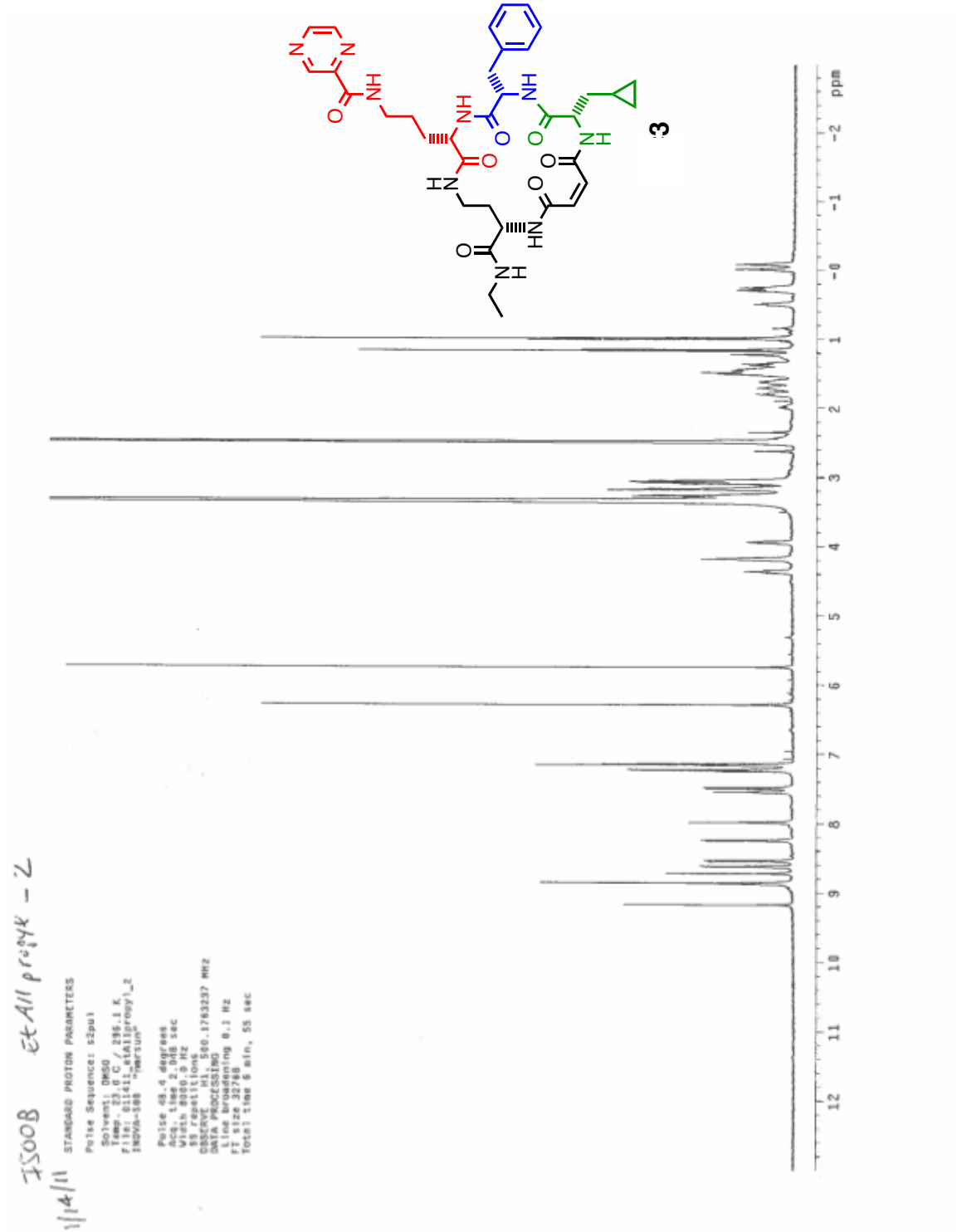
Supplementary Table 2. Mass spectrometry data for macrocycle compounds described in this work.

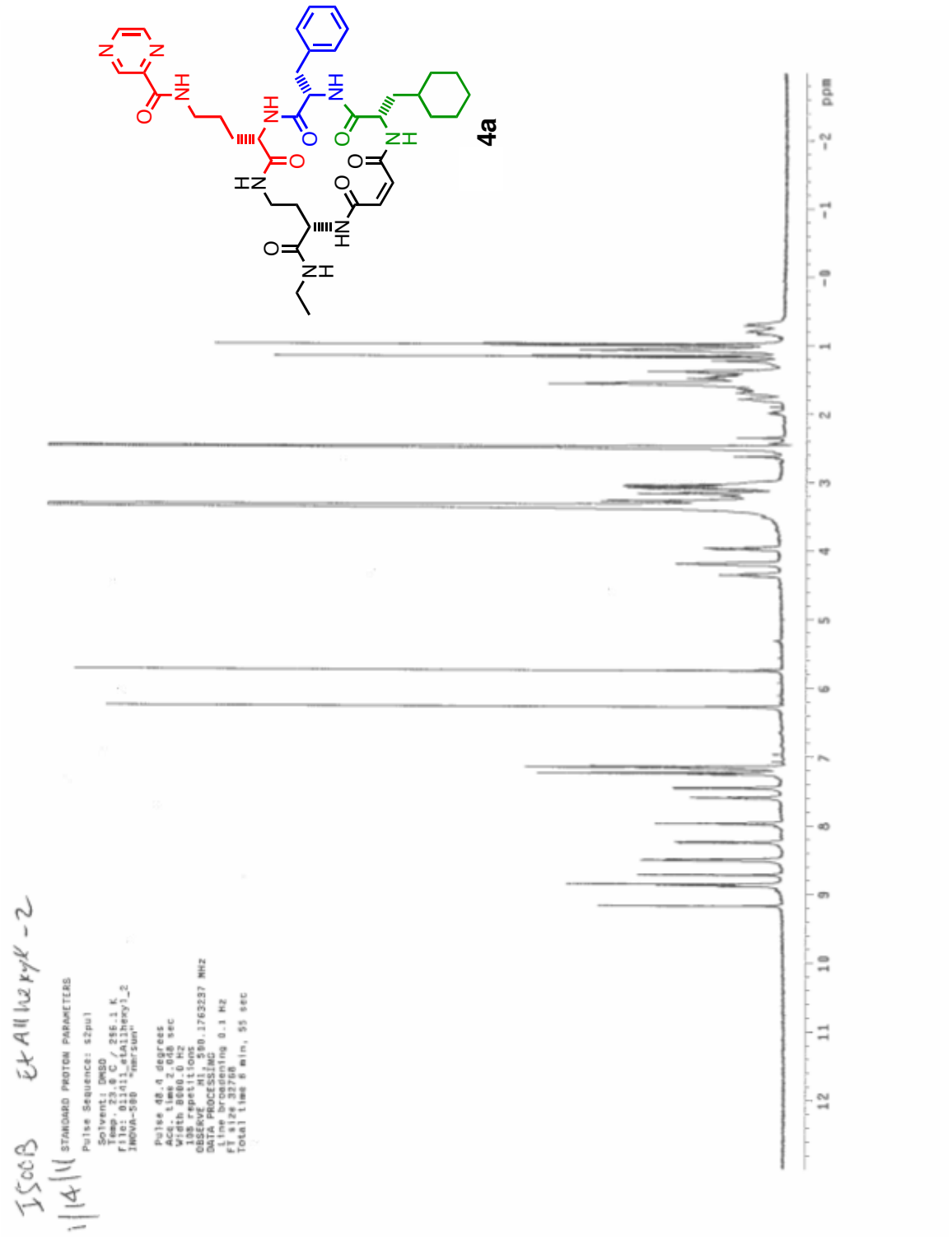
compound	expected $[M+H]^+$	observed $[M+H]^+$
1	744.4	characterized in ref. 1
2	666.3	characterized in ref. 1
3	704.4	704.4
4a	746.4	746.4
4b	719.4	719.4
5	751.4	751.4
6	751.4	751.4
7	751.4	751.4
8	751.4	751.4
9	652.3	characterized in ref. 1
10	662.3	662.3
11	668.3	668.3
12	752.2	752.2
13	698.3	698.3
14	774.3	774.3
15	748.3	748.3
16	704.3	704.3
17	673.4	673.4
18	693.3	693.3
19	737.3	737.3
20	727.3	727.3
21	684.4	684.4
22	702.4	702.4
23	715.4	715.4
24	720.3	720.3
25a	722.3	722.3
25b	723.3	723.3
26	718.4	718.4
27	737.3	737.3
28	737.3	737.3
29	737.3	737.3
30	737.3	737.3
31	737.3	737.3
fluorescein-**1**	1201.5	1201.5
fluorescein-**2**	1123.5	1123.5
fluorescein-**9**	1109.4	1109.4

Supplementary Reference

1) Kleiner, R.E., Dumelin, C.E., Tiu, G.C., Sakurai, K. & Liu, D.R. In vitro selection of a DNA-templated small-molecule library reveals a class of macrocyclic kinase inhibitors. *J Am Chem Soc* **132**, 11779-91 (2010).

^{1}H NMR spectra of compounds described in this work

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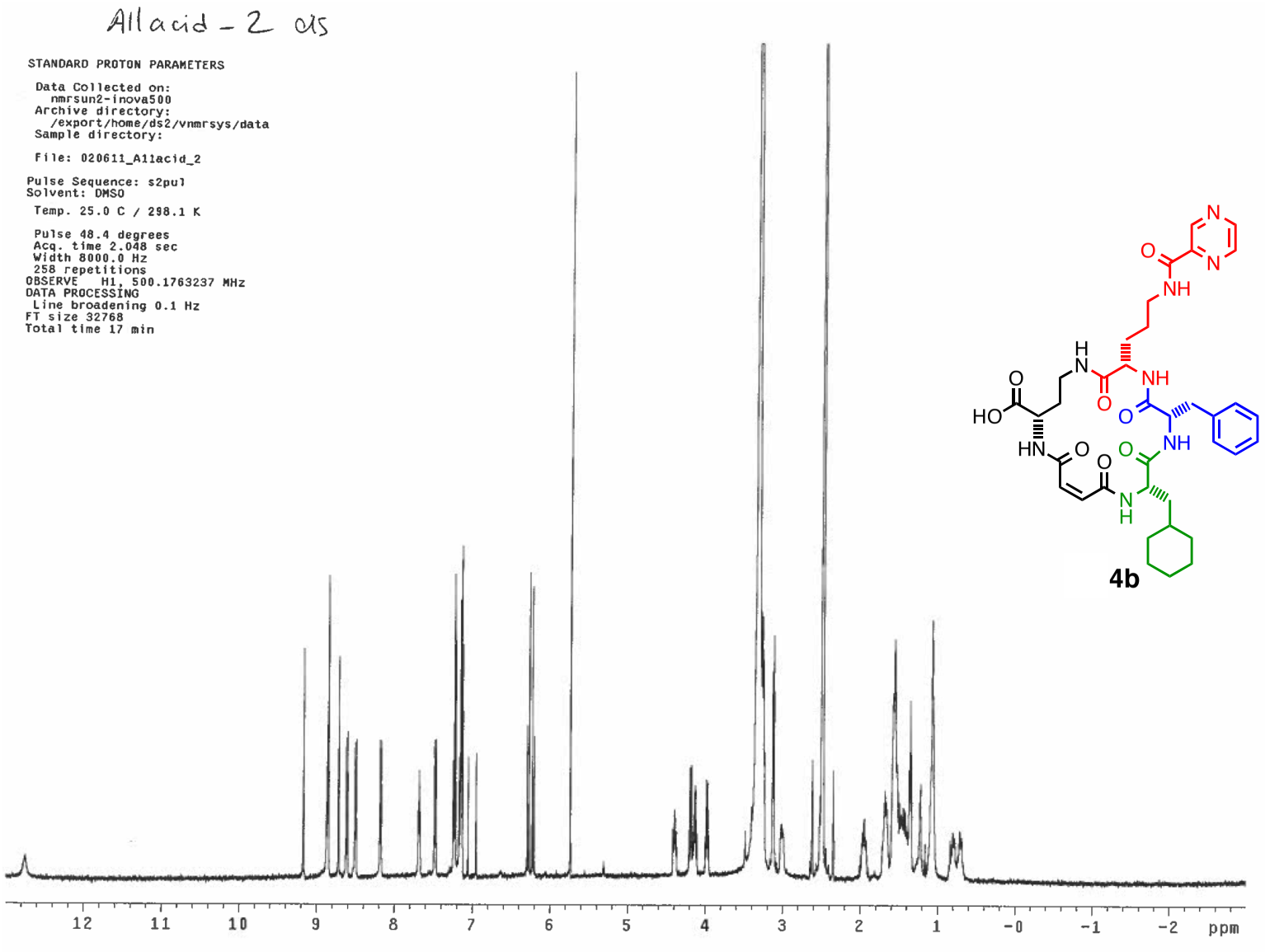
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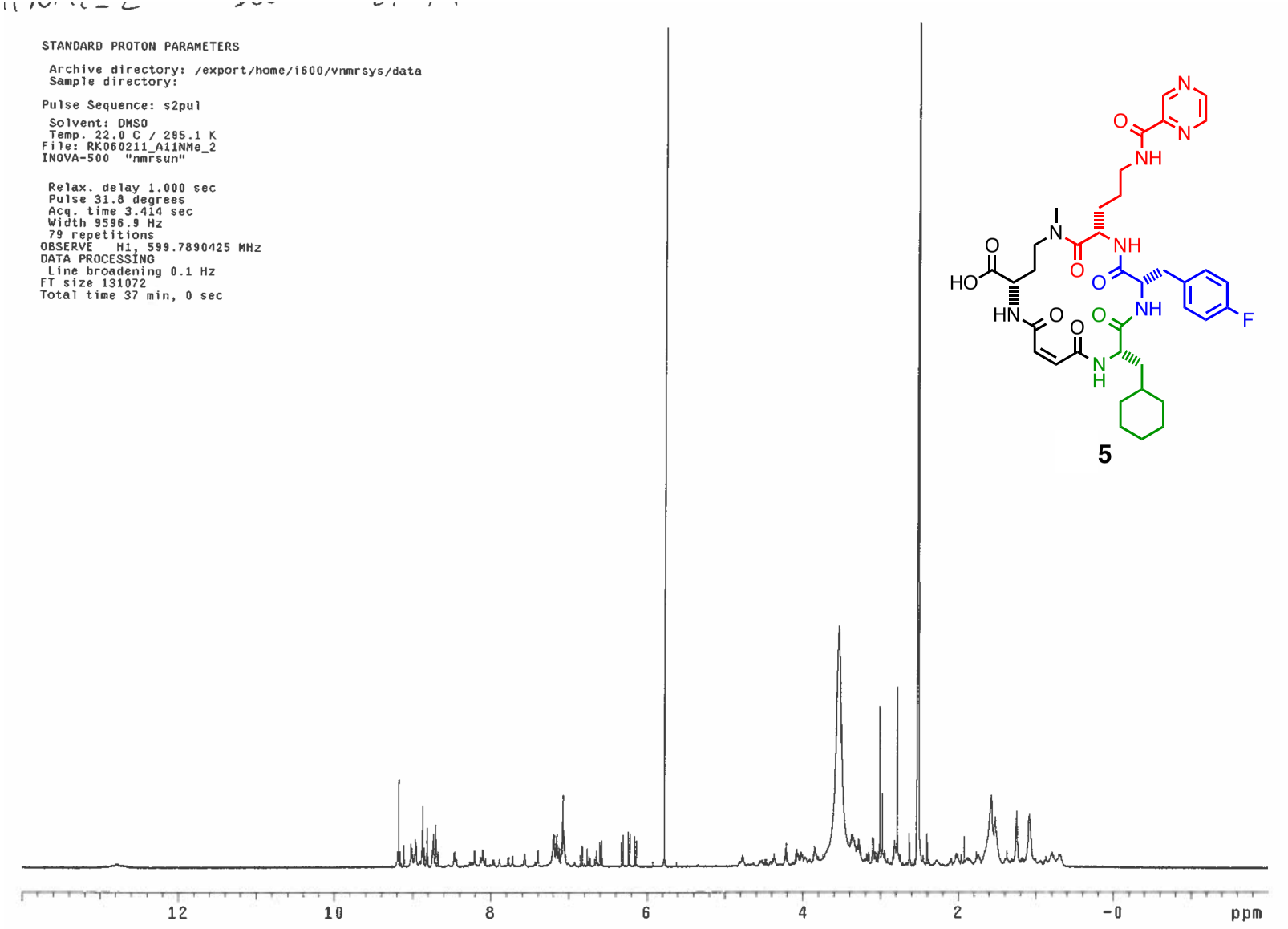
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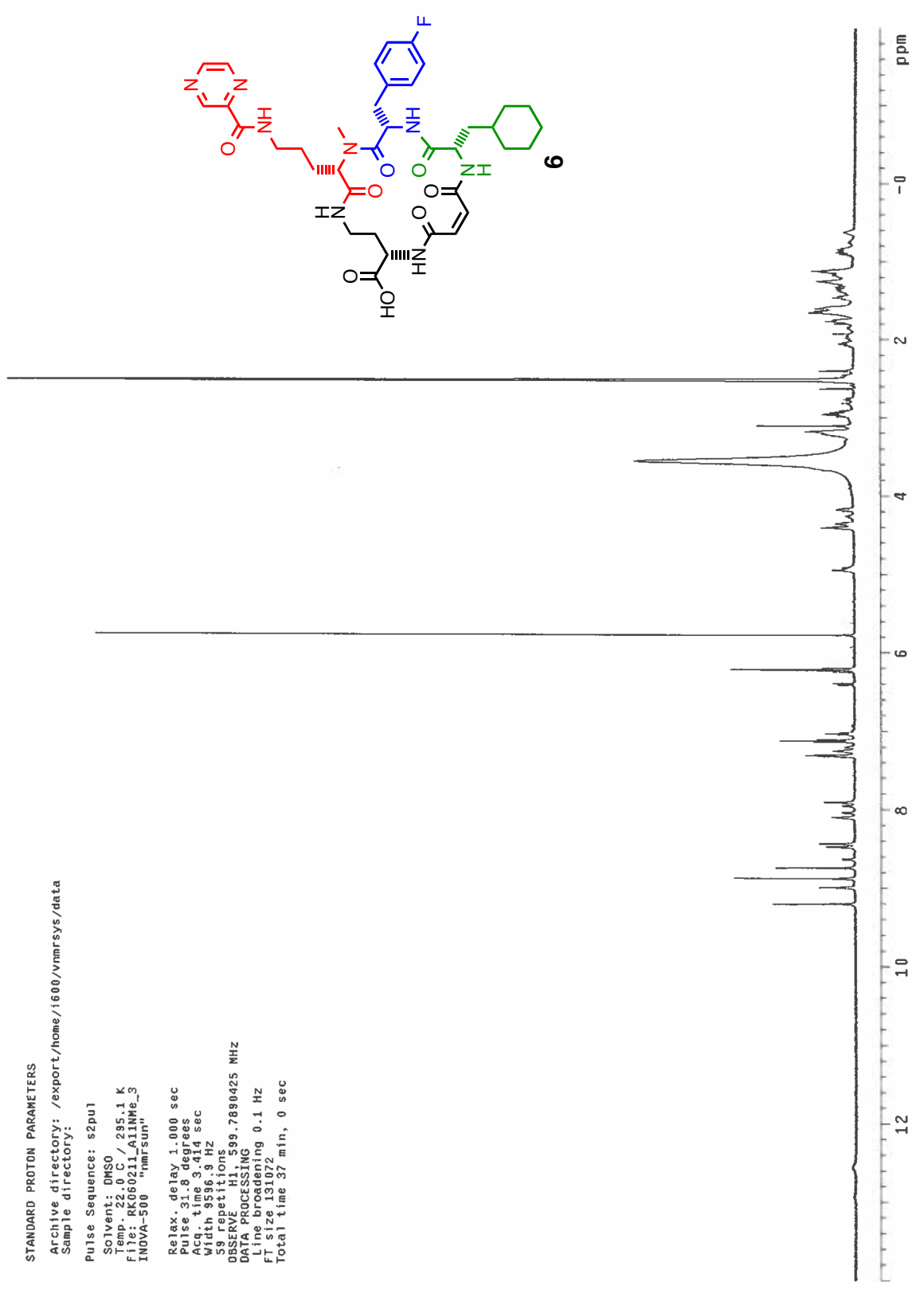
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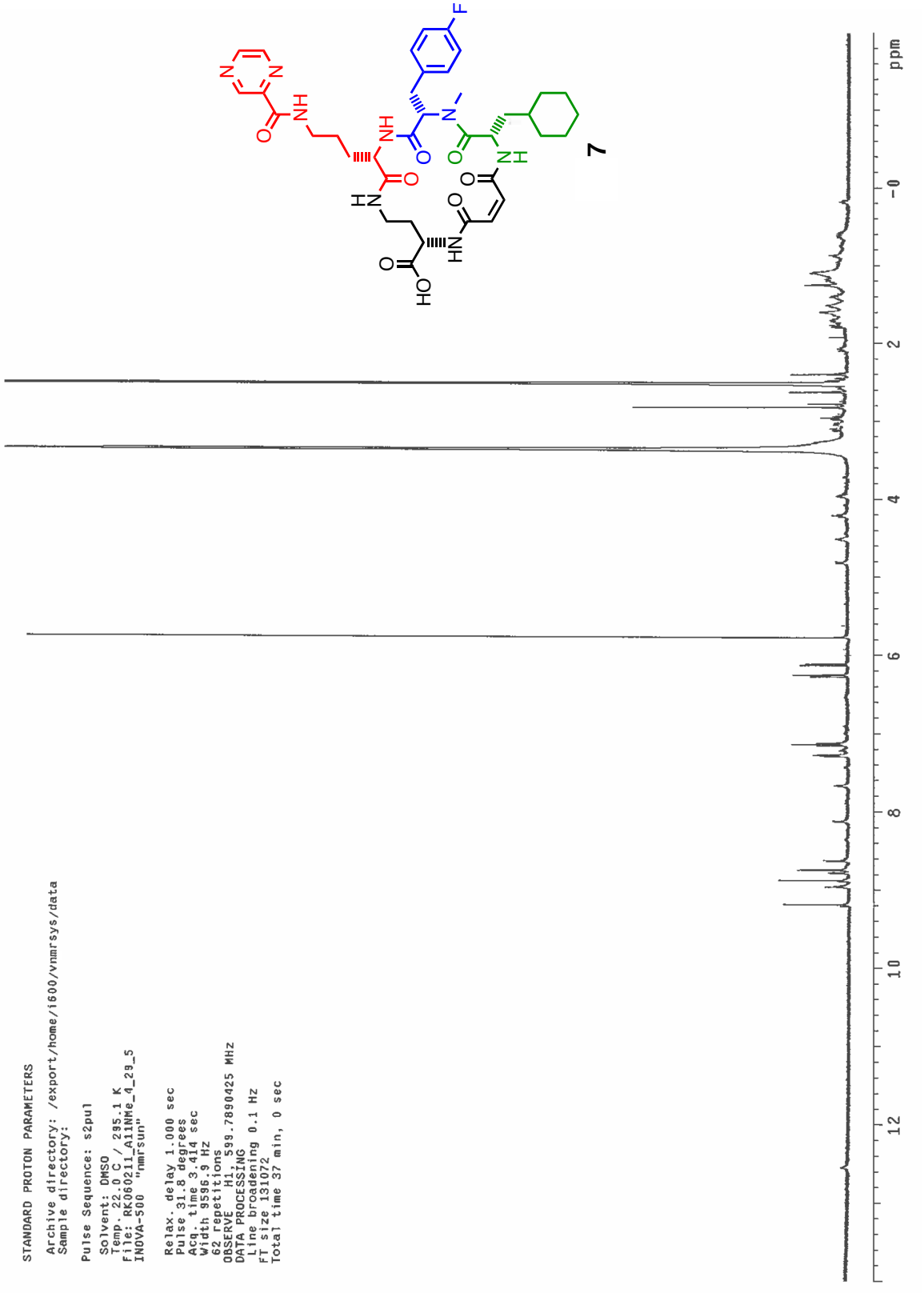
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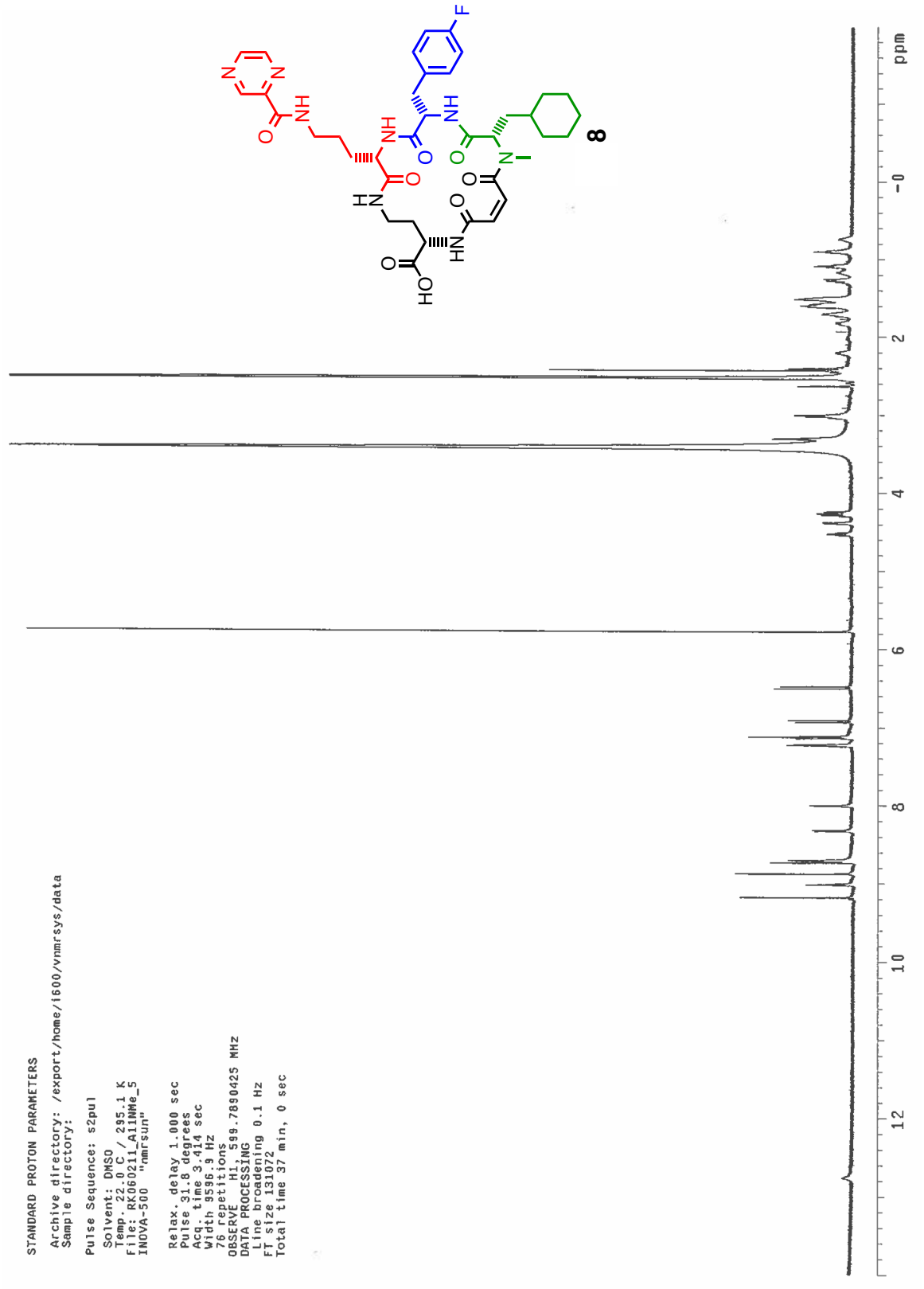
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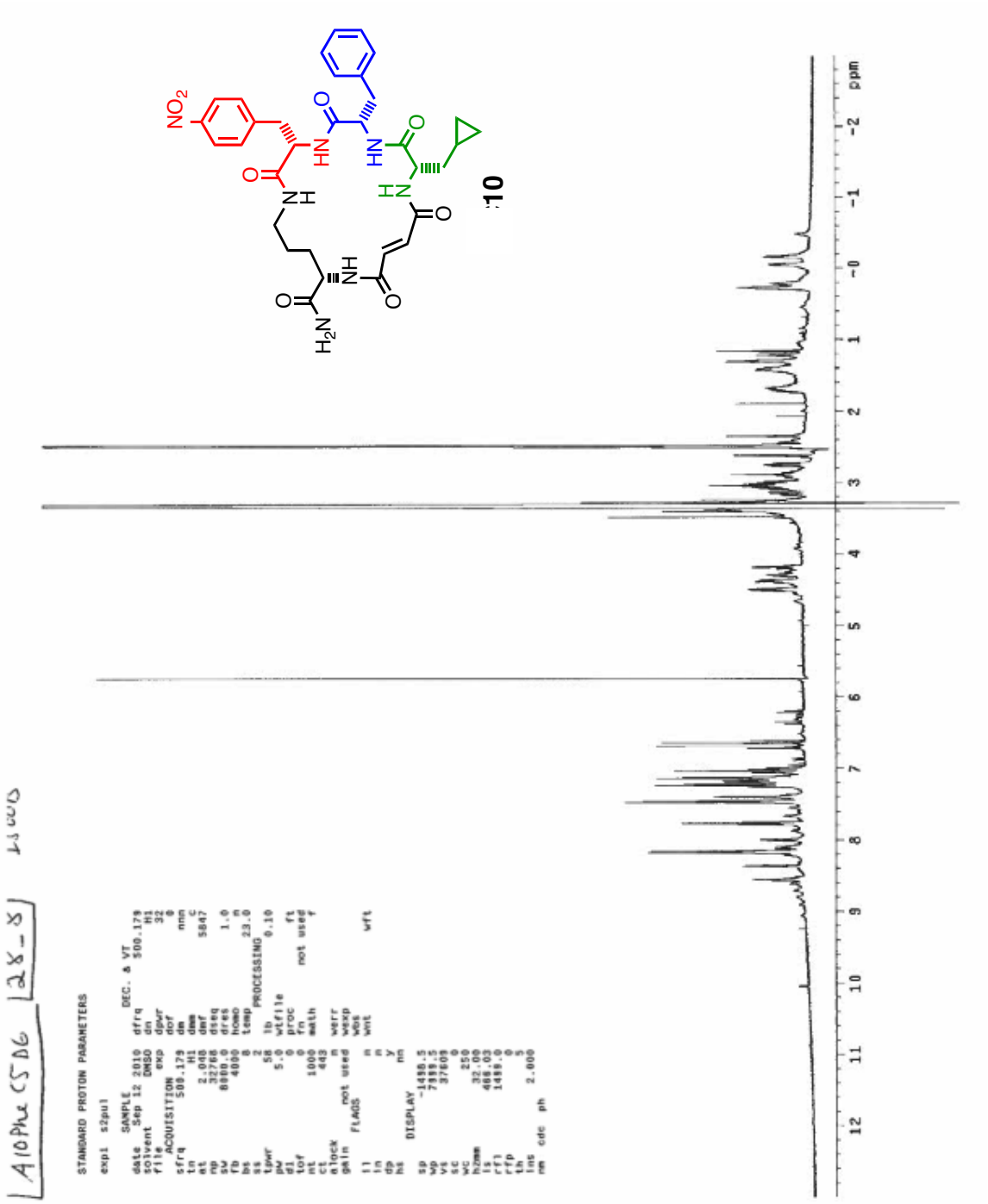
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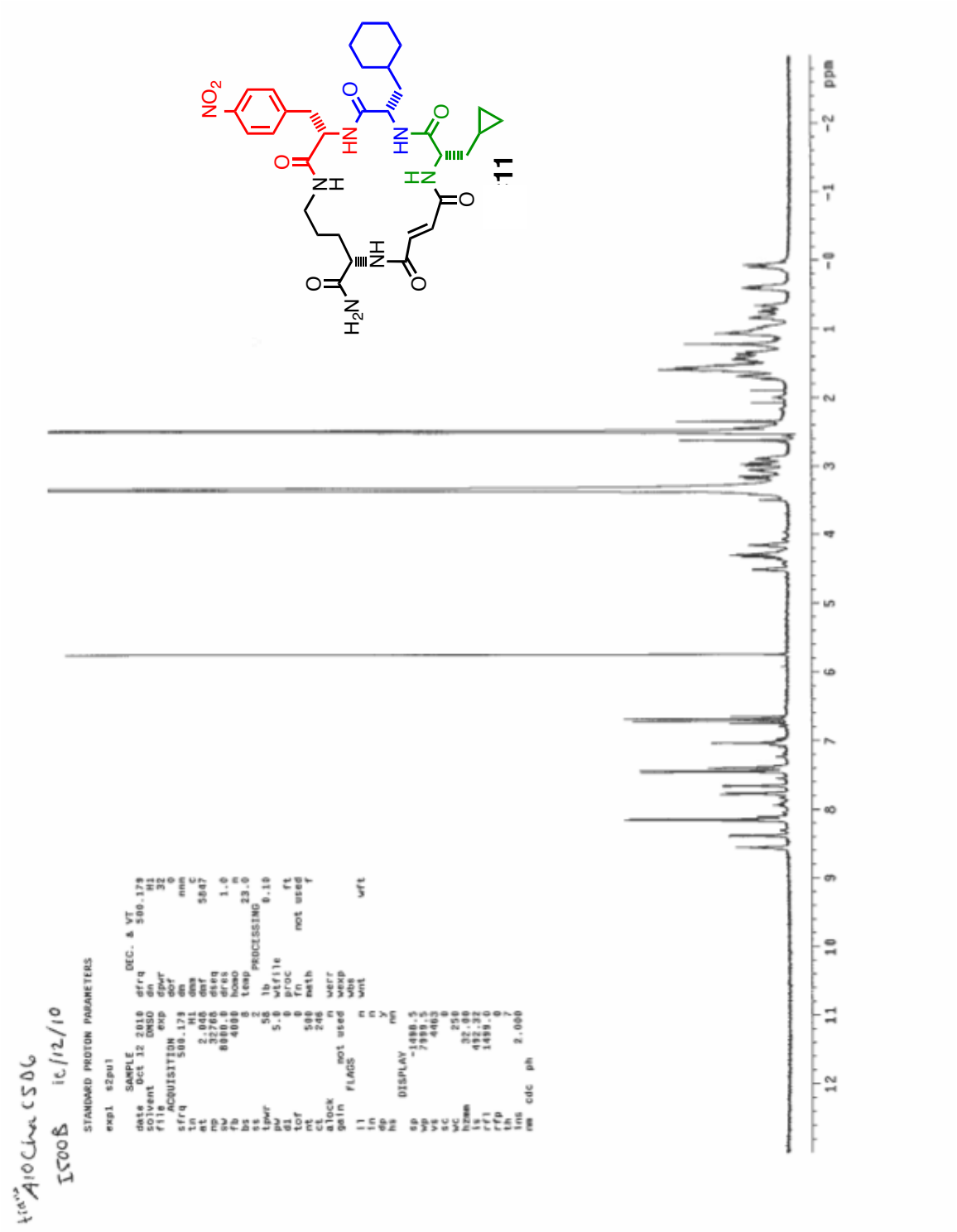
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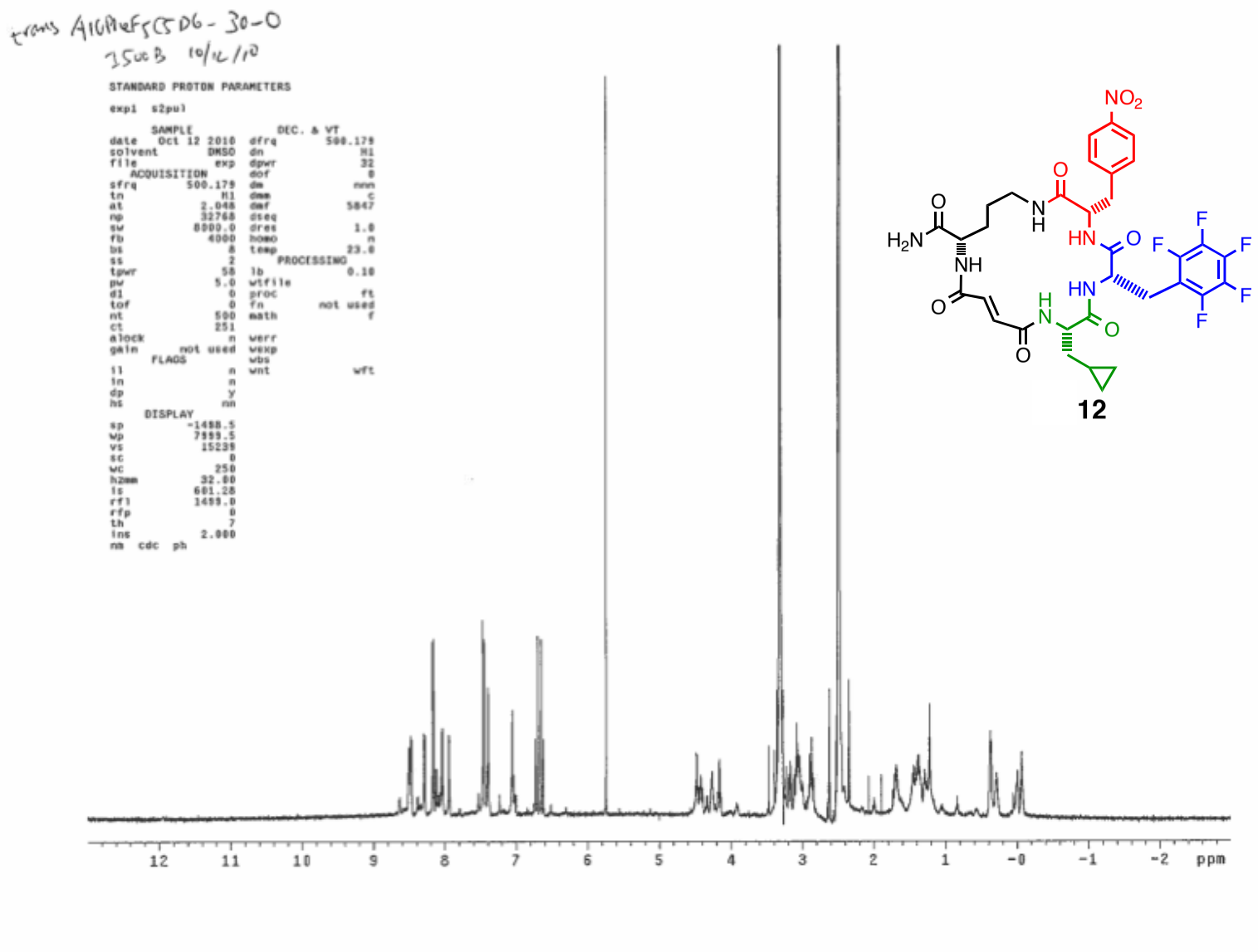
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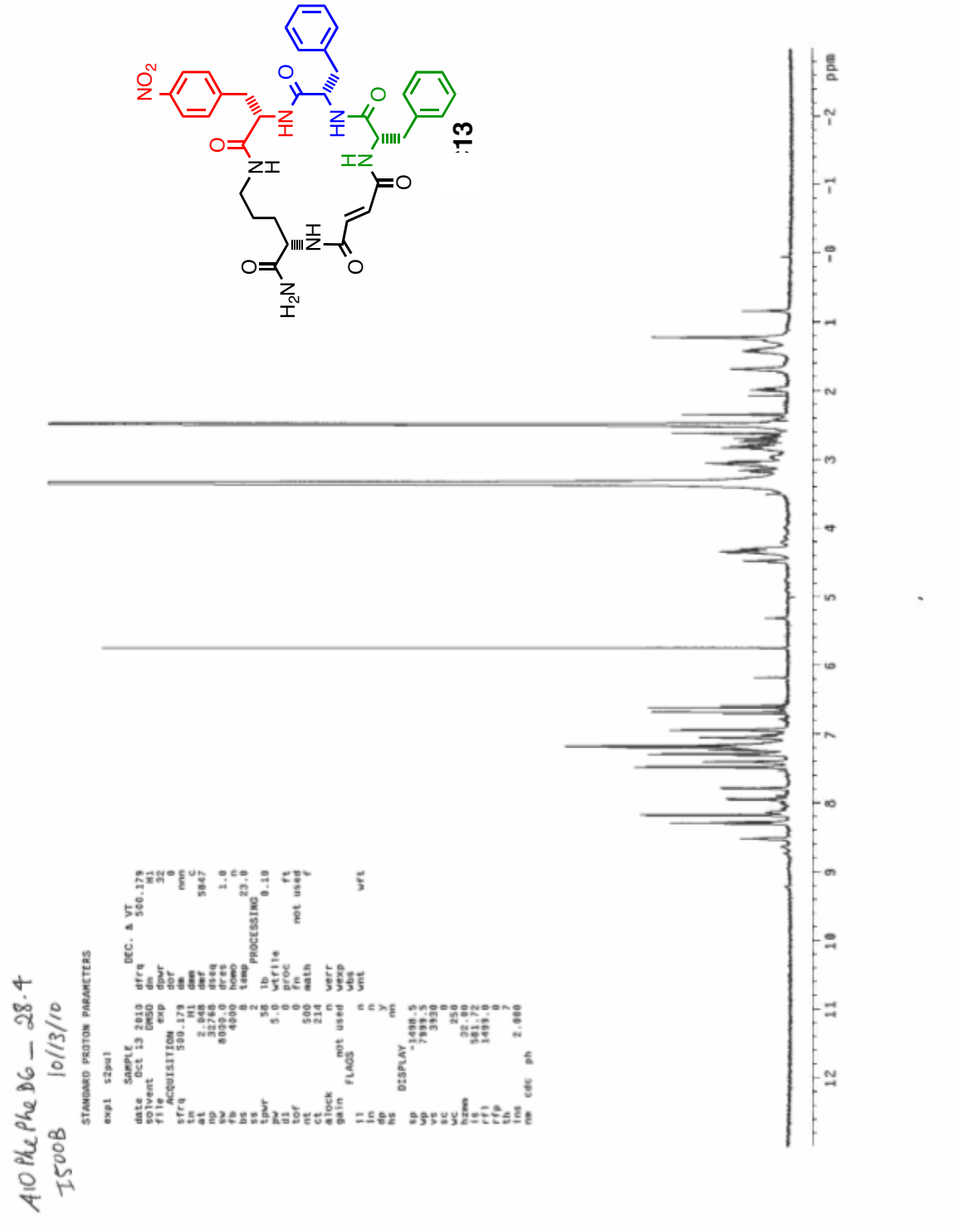
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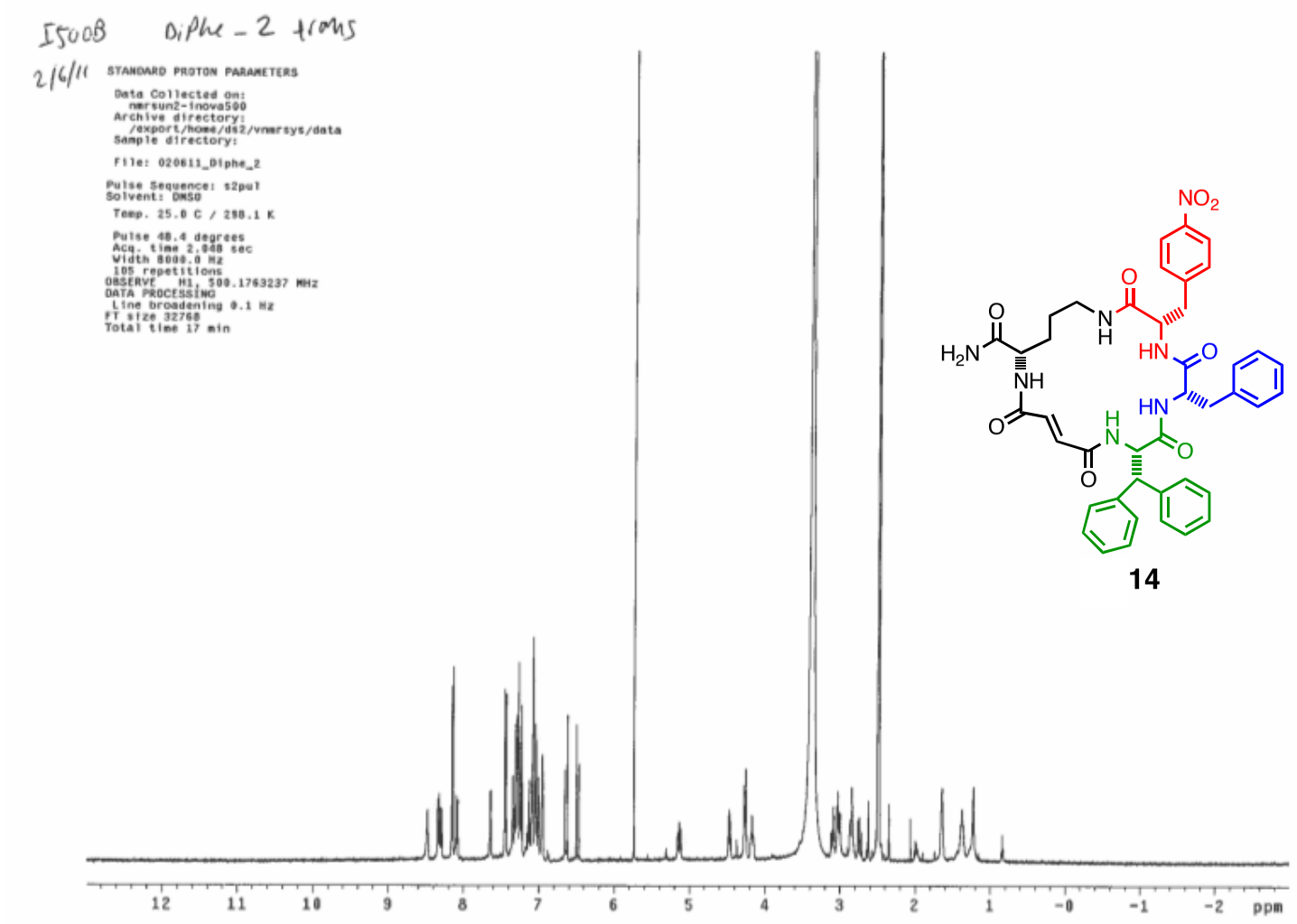
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H
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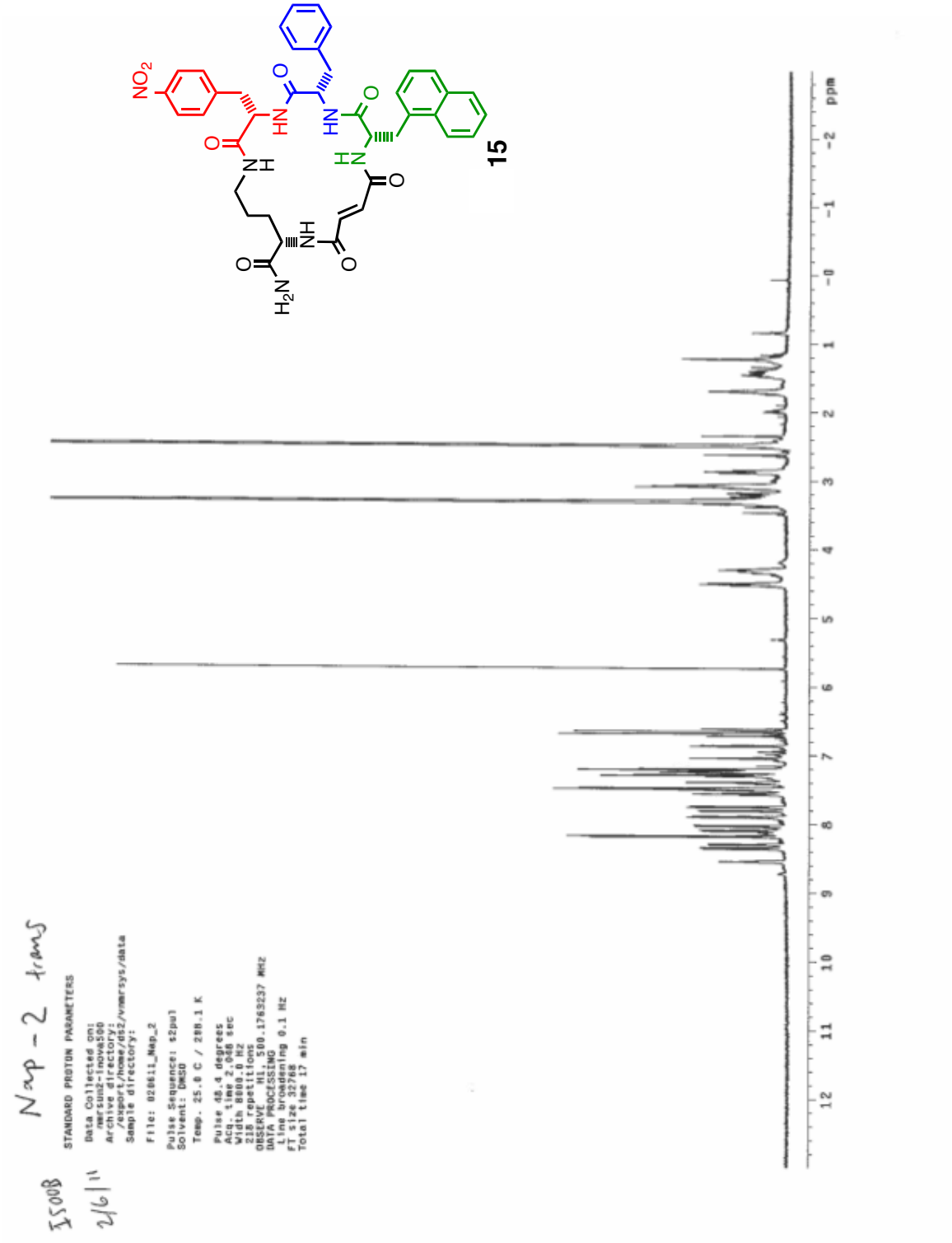
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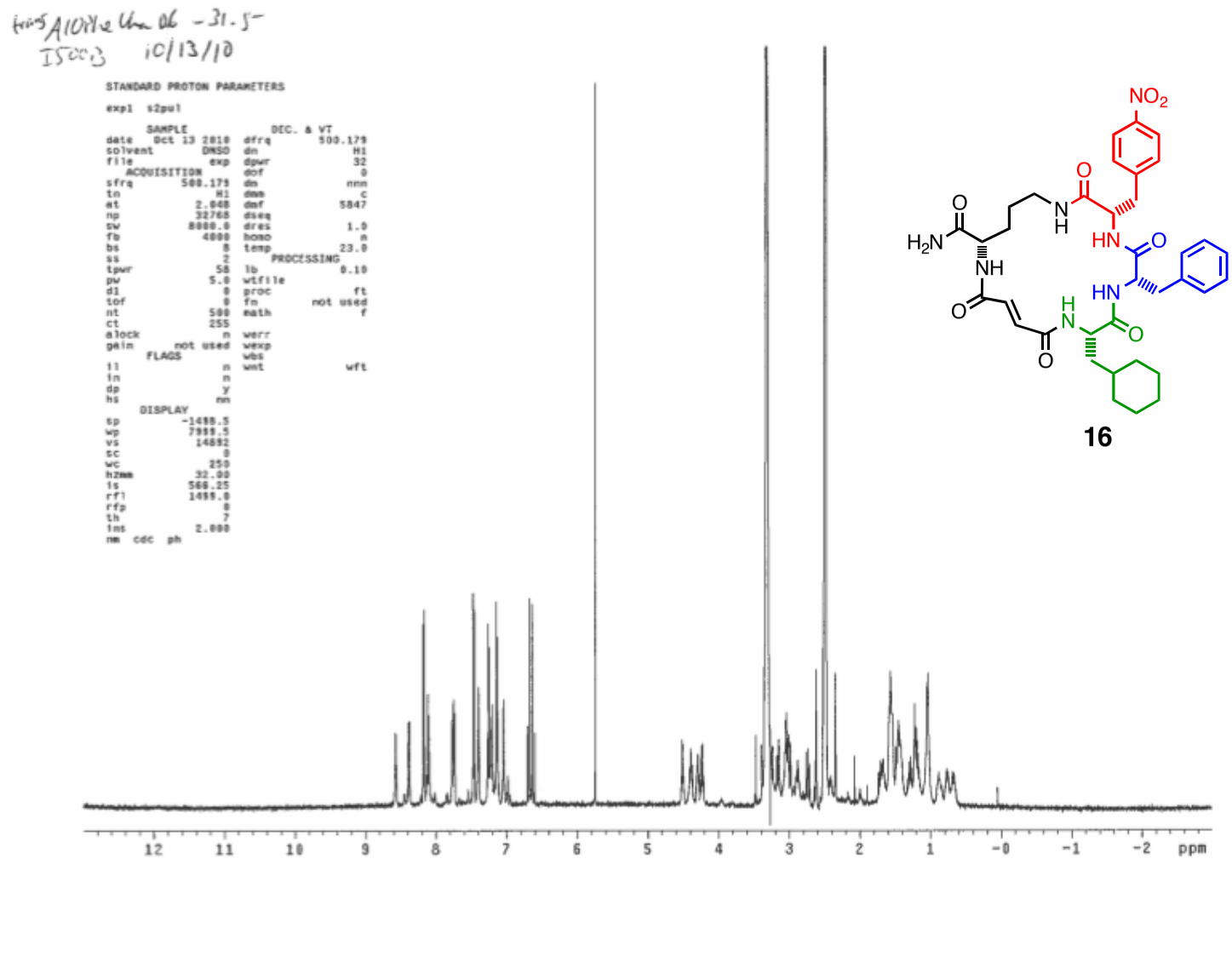
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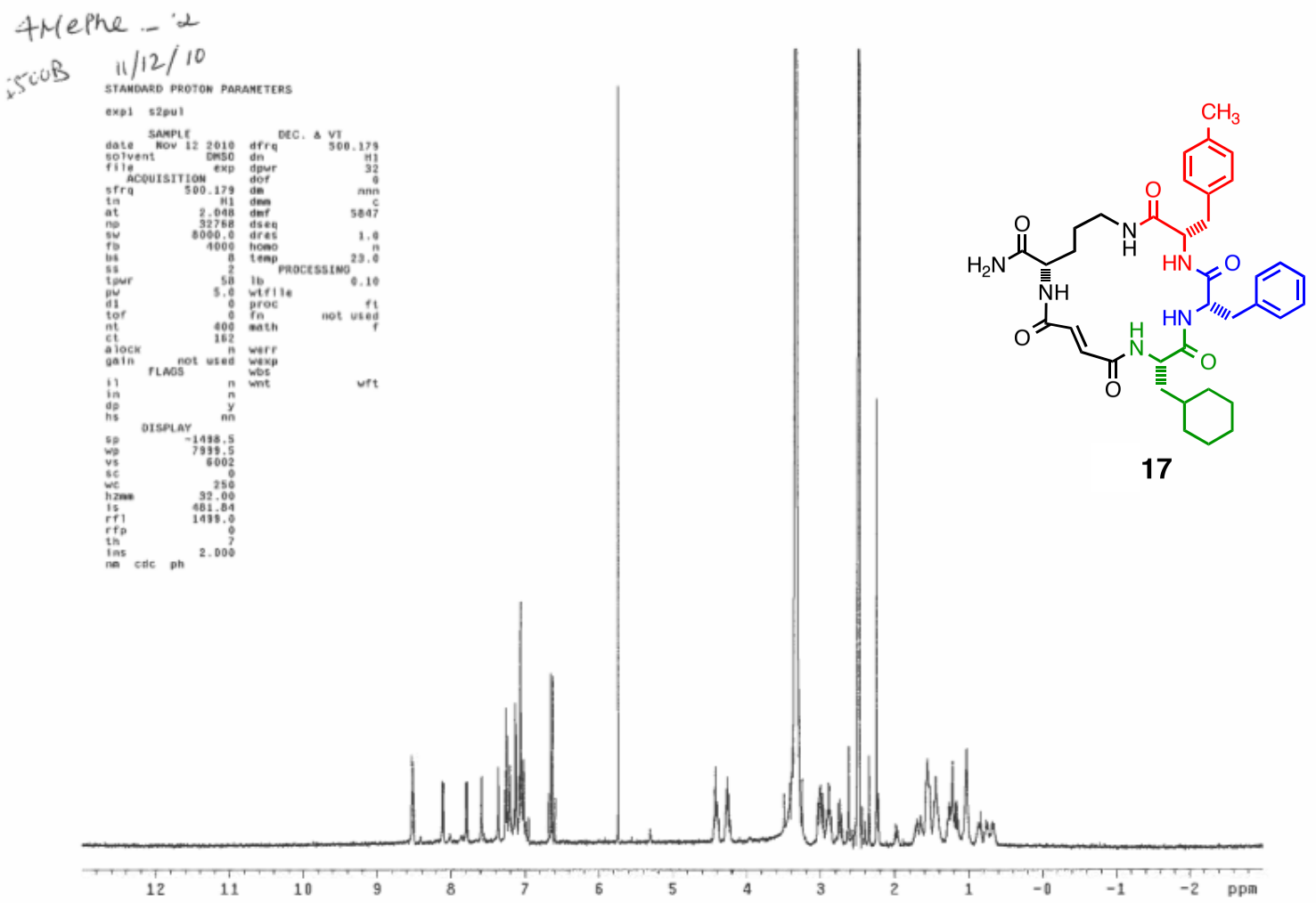
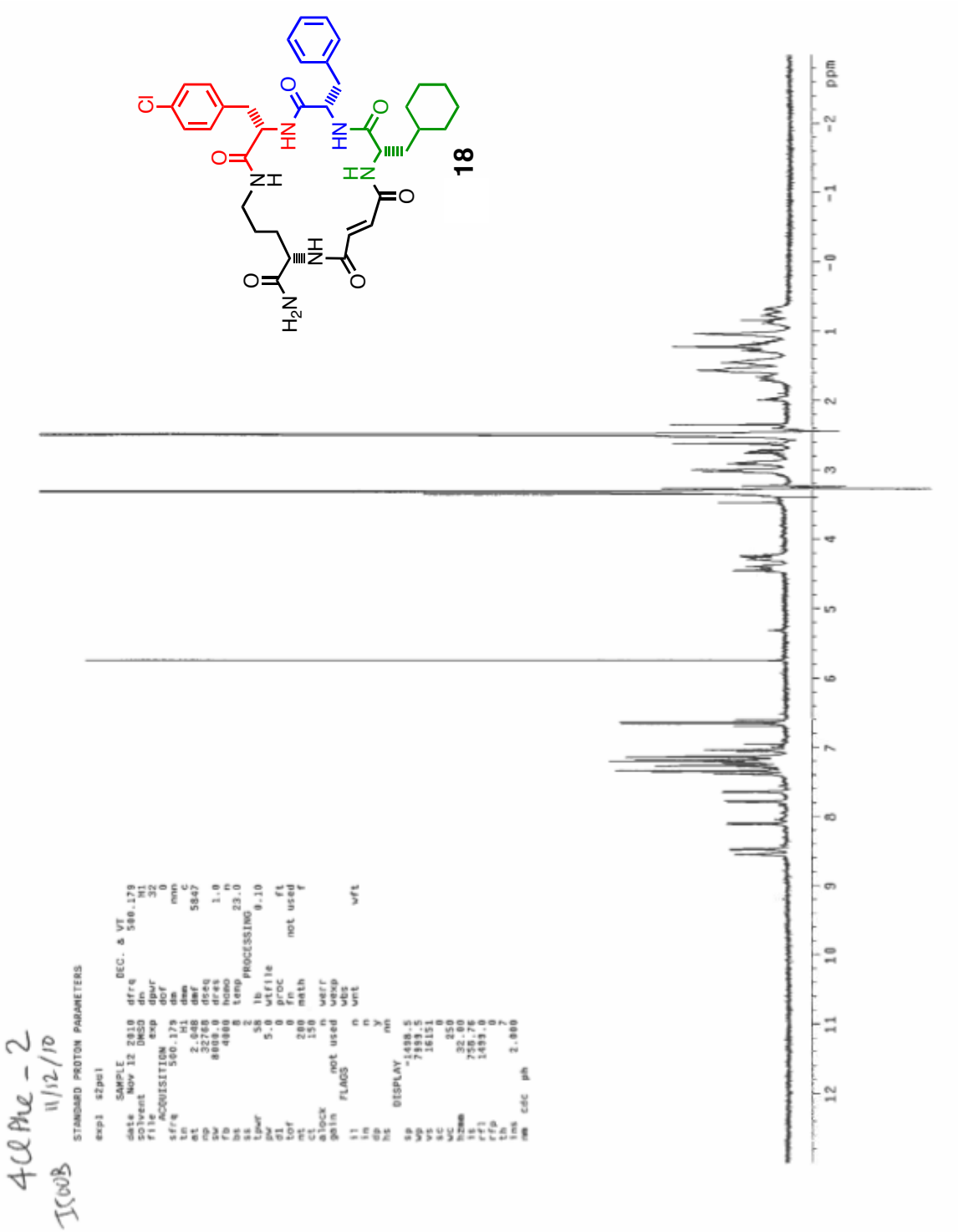
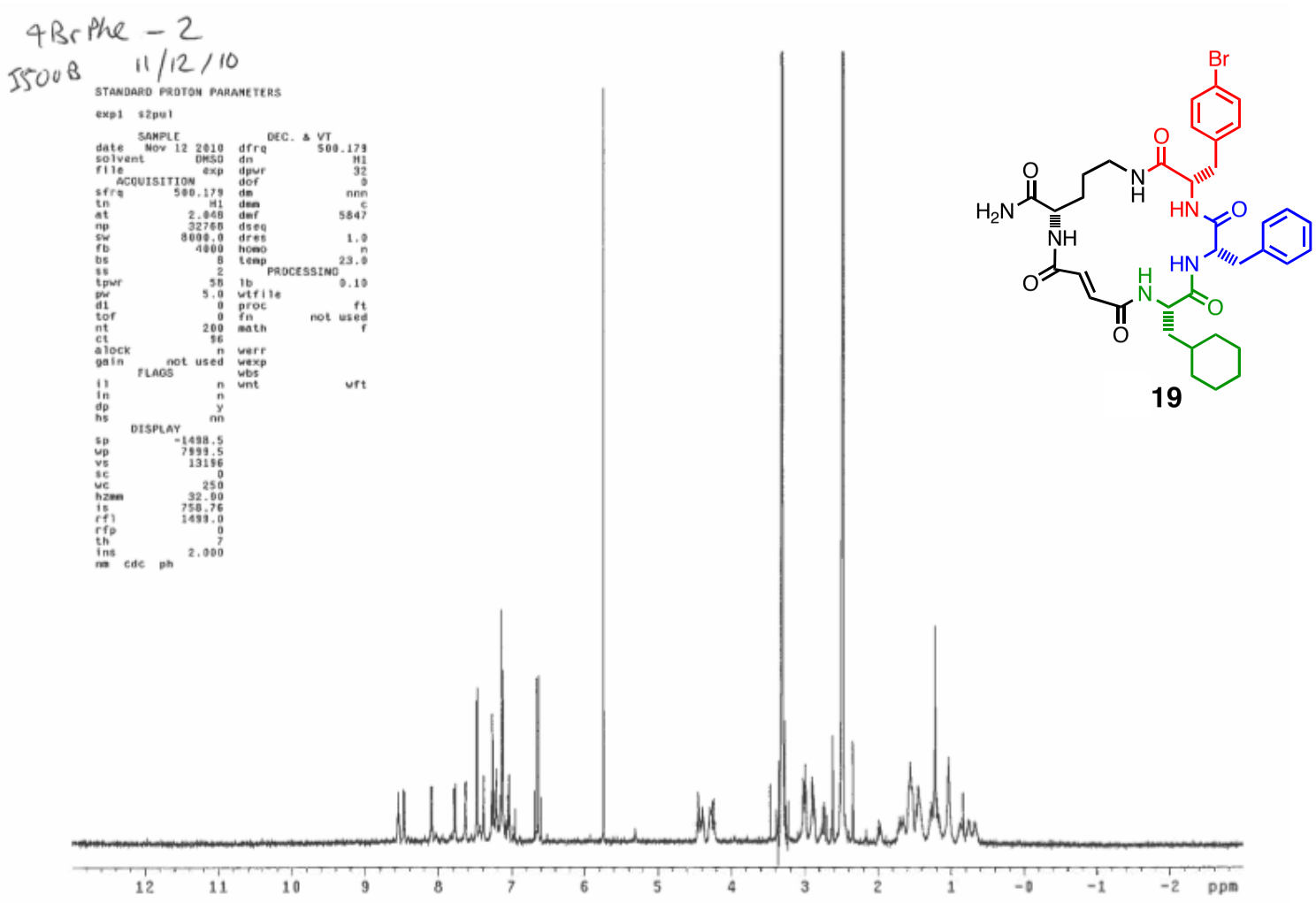
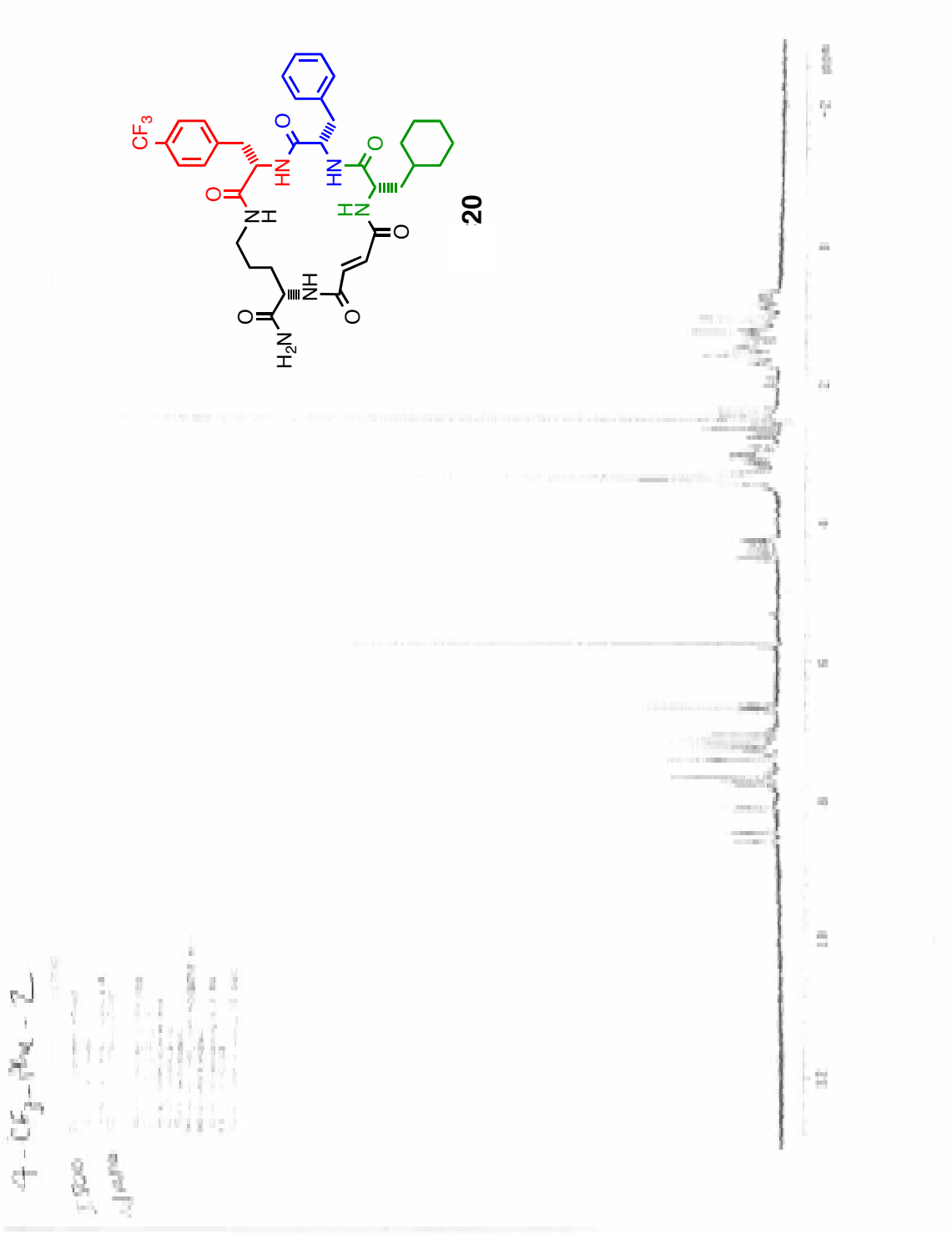
wp 7988.5
vs 14892
sc 0
wc 250
hzmm 32.00
is 566.25
rfl 1498.0
rfp 0
th 7
ins 2.000
nm cdc ph
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
dm nnn
dmm c
dmf 5847
dseq
dres 1.0
homo n
temp 23.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
NO2
O
N
H
HN
O
H2N
NH
O
HN
O
H
N
O
O
16
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm

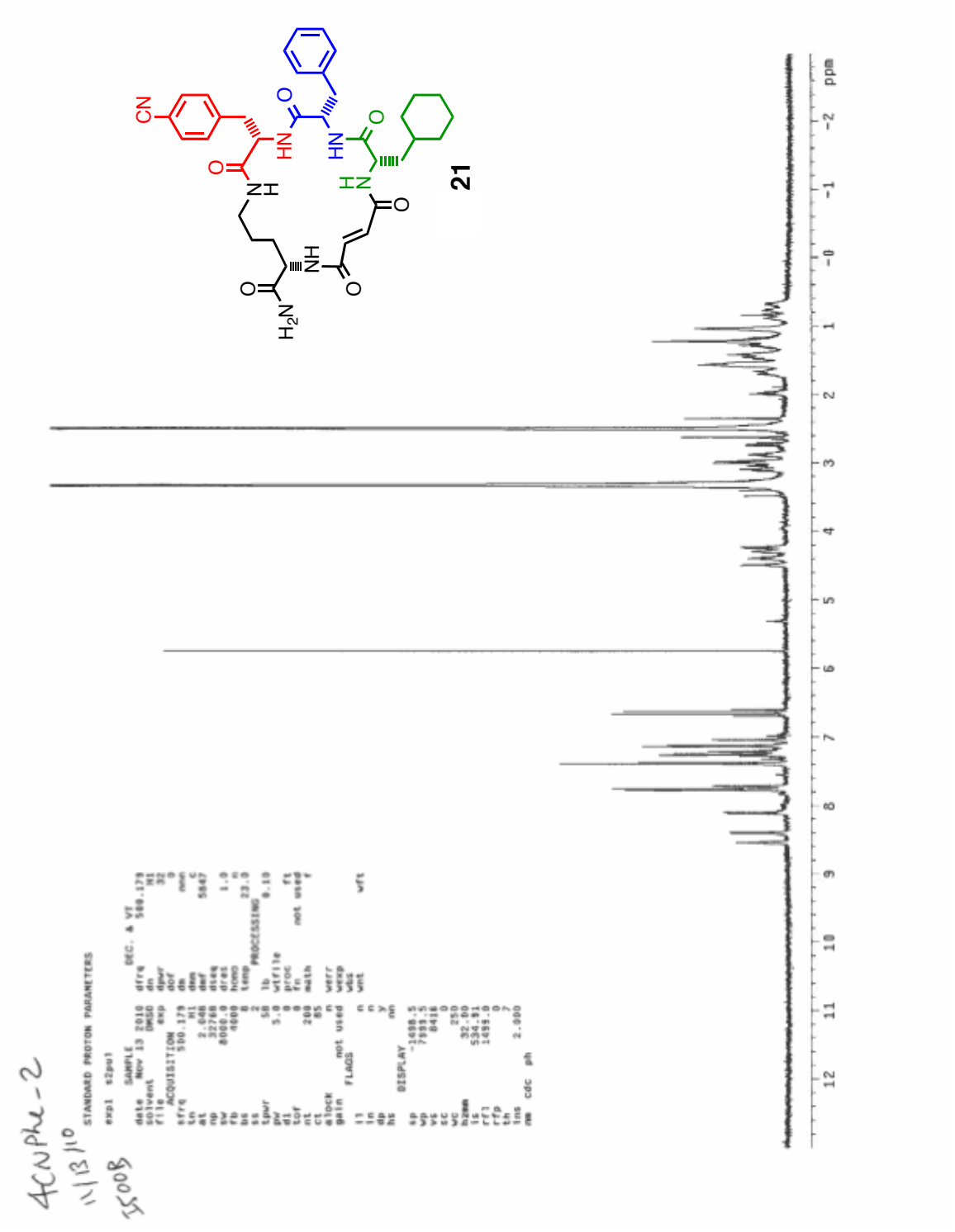

4Mephe - 2
+5COB
11/12/10
STANDARD PROTON PARAMETERS
expl s2pul
SAMPLE
date Nov 12 2010
solvent DMSO
file exp
ACQUISITION
sfrq 500.179
tn H1
at 2.048
np 32768
sw 8000.0
fb 4000
bs 8
ss 2
tpwr 58
pw 5.0
d1 0
tof 0
nt 400
ct 162
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -1498.5
wp 7939.5
vs 6002
sc 0
wc 250
hzmm 32.00
is 481.84
rfl 1499.0
rfp 0
th 7
ins 2.000
nm cdc ph
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
dm nnn
dmm c
dmf 5847
dseq
drst 1.0
homo n
temp 23.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
12 11 10 9 8 7 6 5 4 3 2 1 -0 -1 -2 ppm
CH3
O
N
H
HN
O
H2N
NH
O
HN
O
H
N
O
O
17

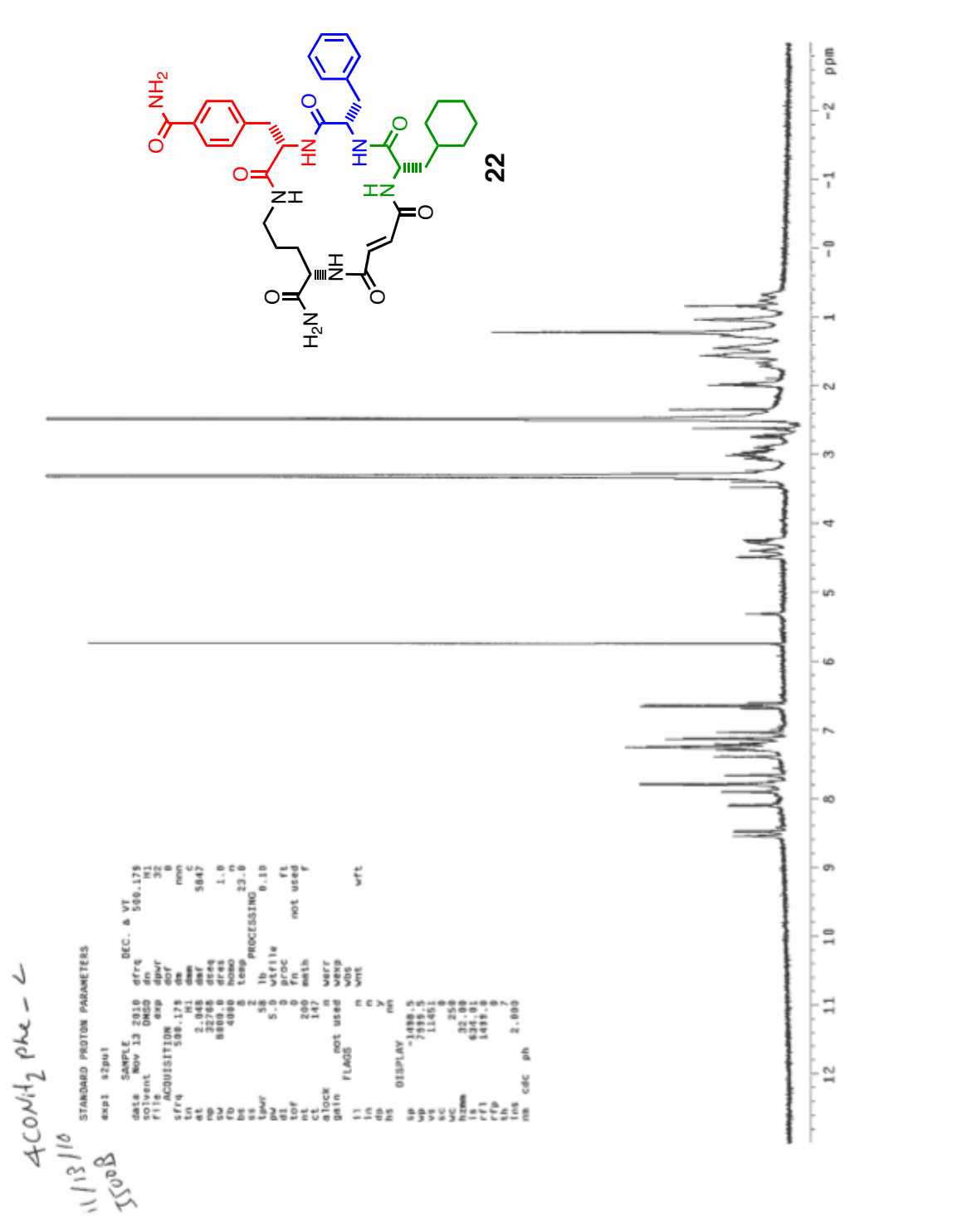

4Cl Phe - 2
11/12/10
ICOOB
STANDARD PROTON PARAMETERS
18
Cl
HN
NH
NH
NH
O
H_2N
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm

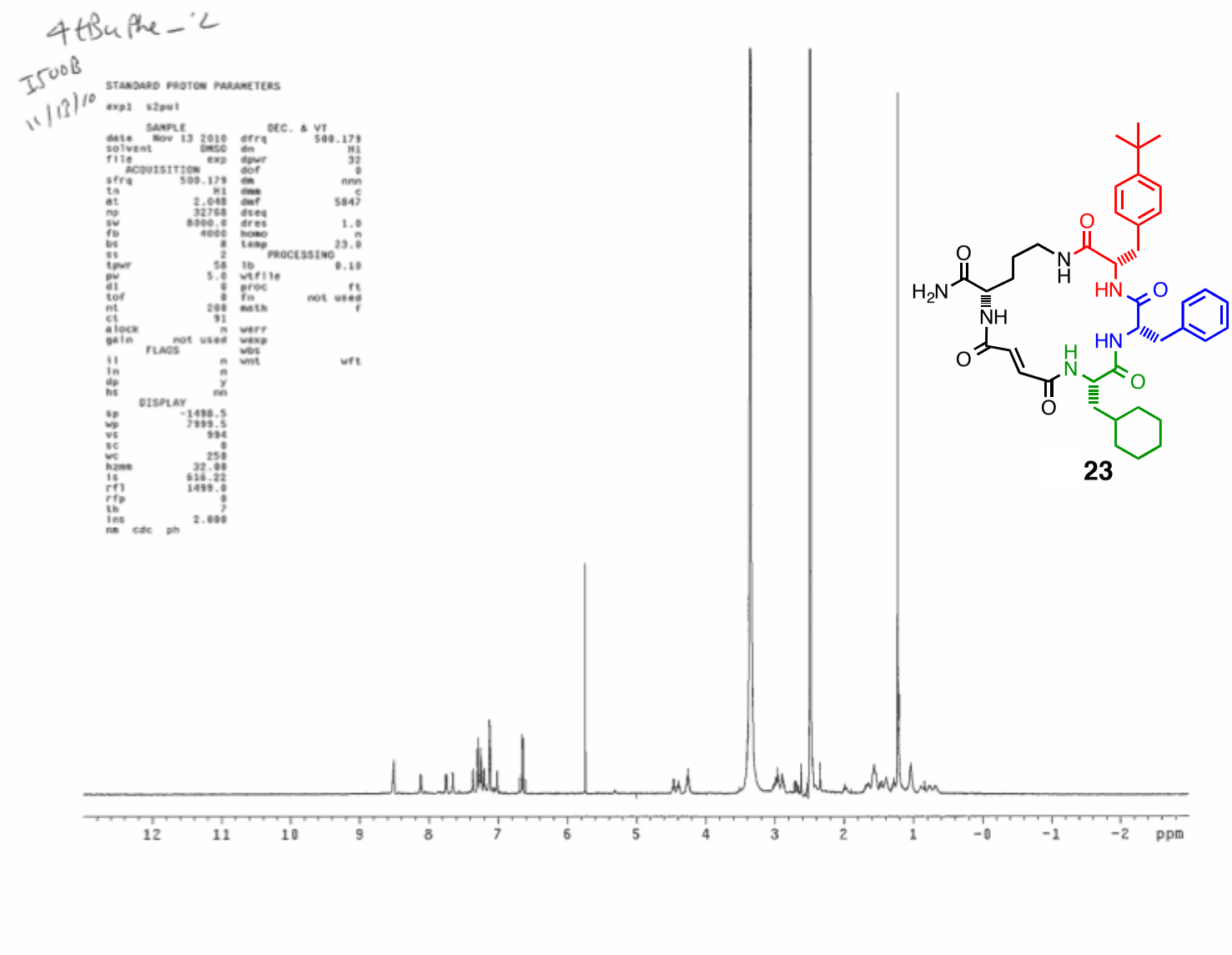

4BrPhe – 2
I500B 11/12/10
STANDARD PROTON PARAMETERS
exp1 s2pul
SAMPLE
date Nov 12 2010
solvent DMSO
file exp
ACQUISITION
sfrq 500.179
tn H1
at 2.048
np 32768
sw 8000.0
fb 4000
bs 8
ss 2
tpwr 55
pw 5.0
d1 0
tof 0
nt 200
ct 96
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -1498.5
wp 7993.5
vs 13196
sc 0
wc 250
hzmm 32.00
is 750.76
rfl 1499.0
rfp 0
th 7
ins 2.000
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
dm nnn
dmm c
dmf 5847
dseq
dres 1.0
homo n
temp 23.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
Br
O
N
H
HN
O
H2N
NH
HN
O
H
N
O
O
19
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm

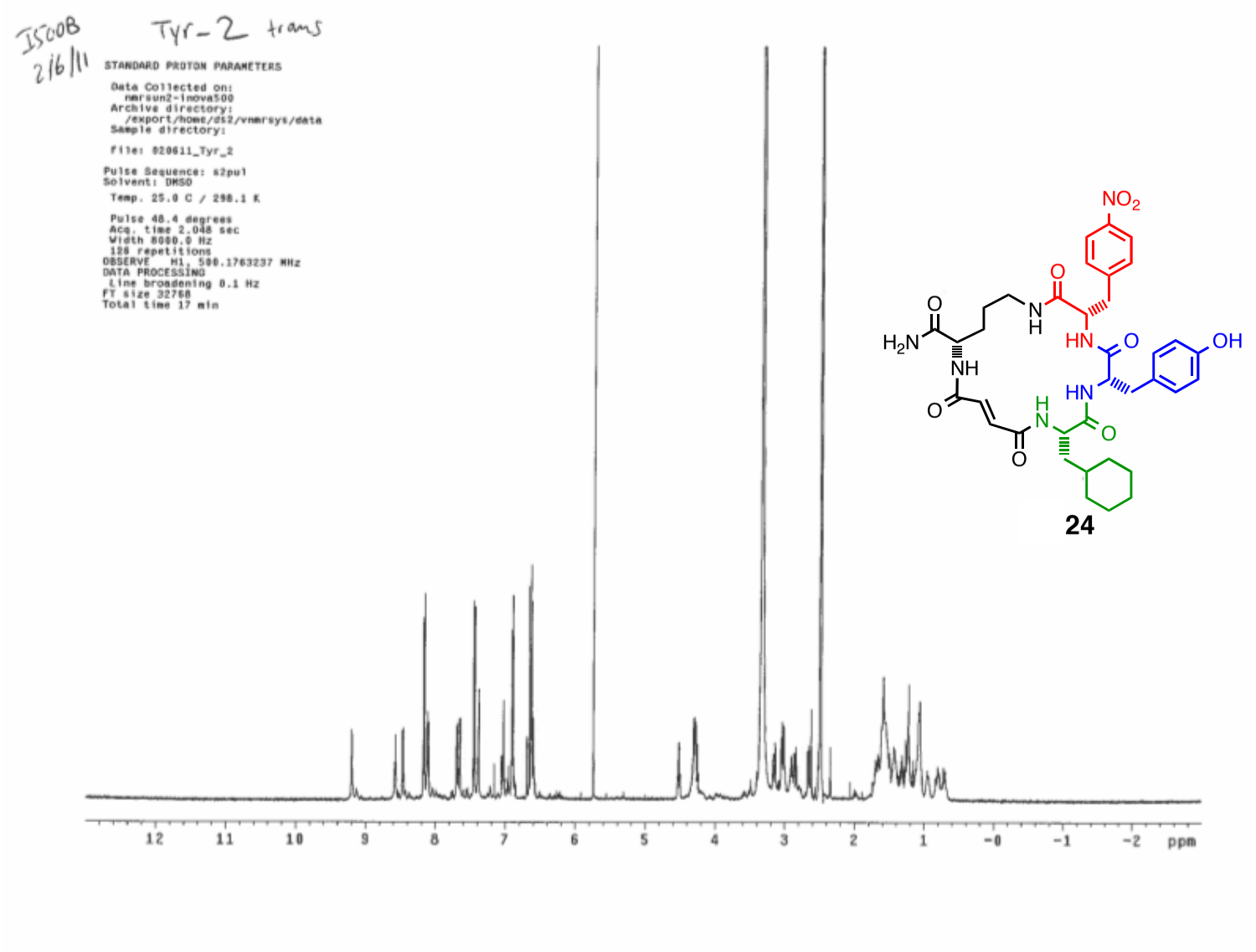


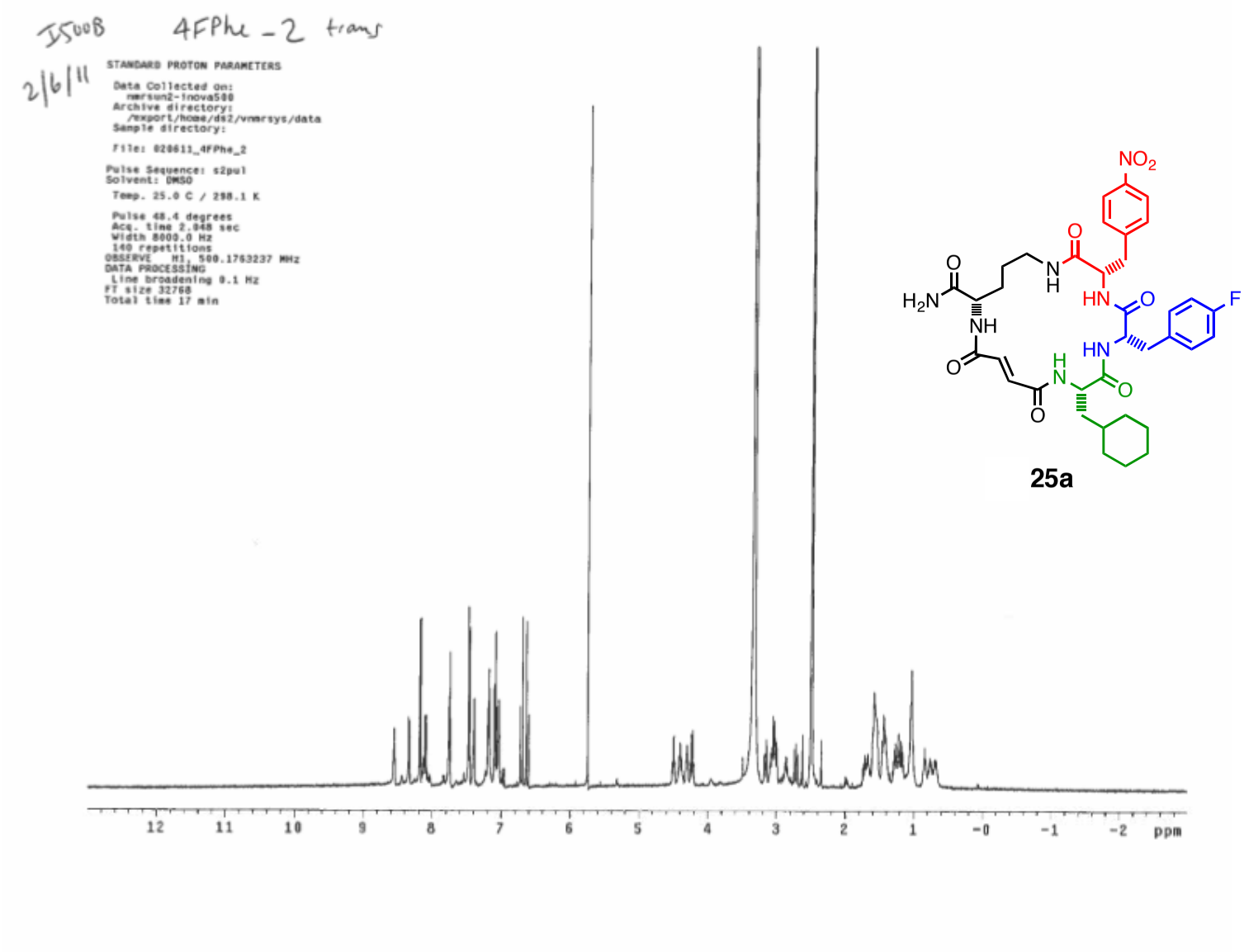
20

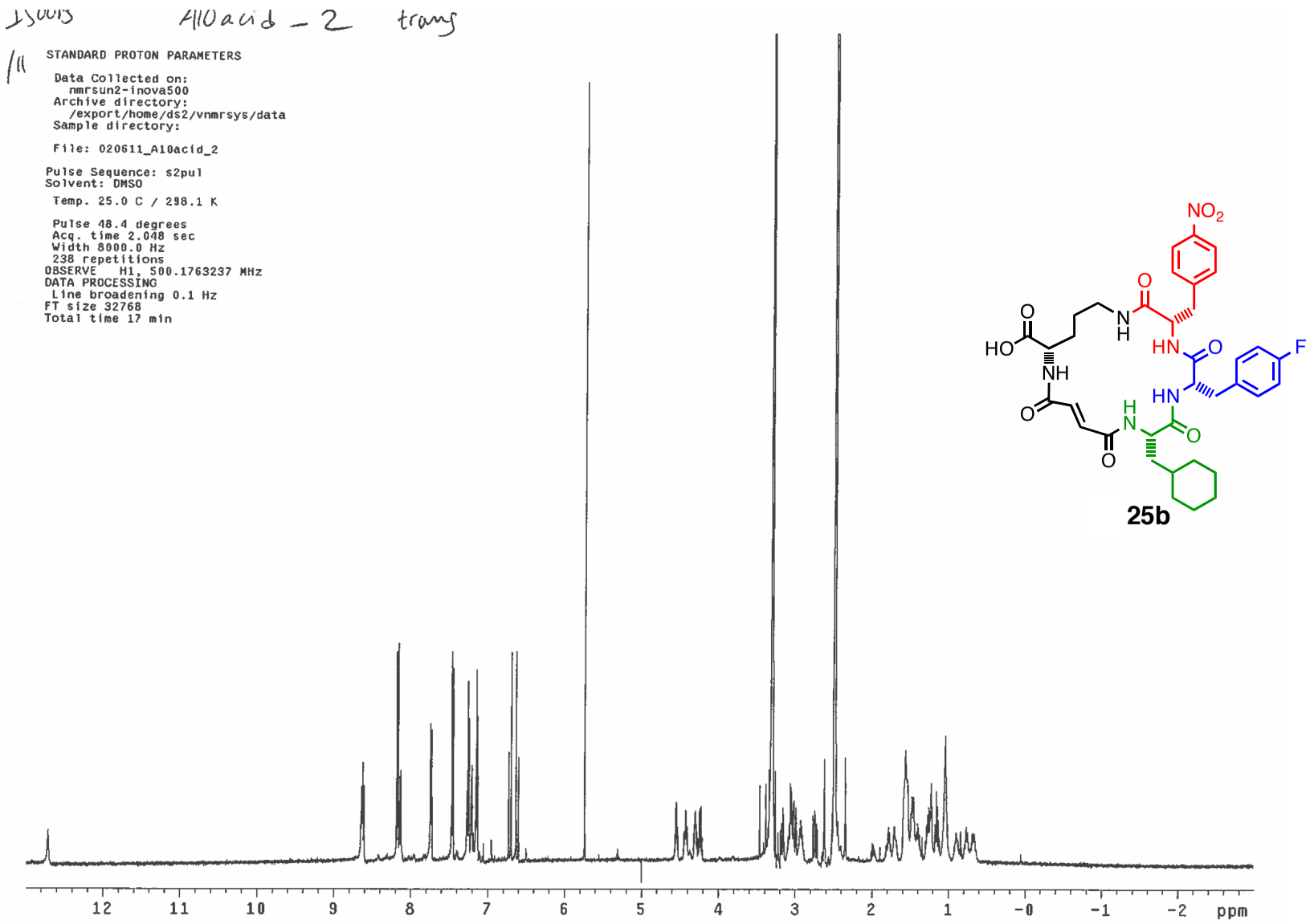

4CNPhe-2
11/13/10
15008
STANDARD PROTON PARAMETERS
21
ppm

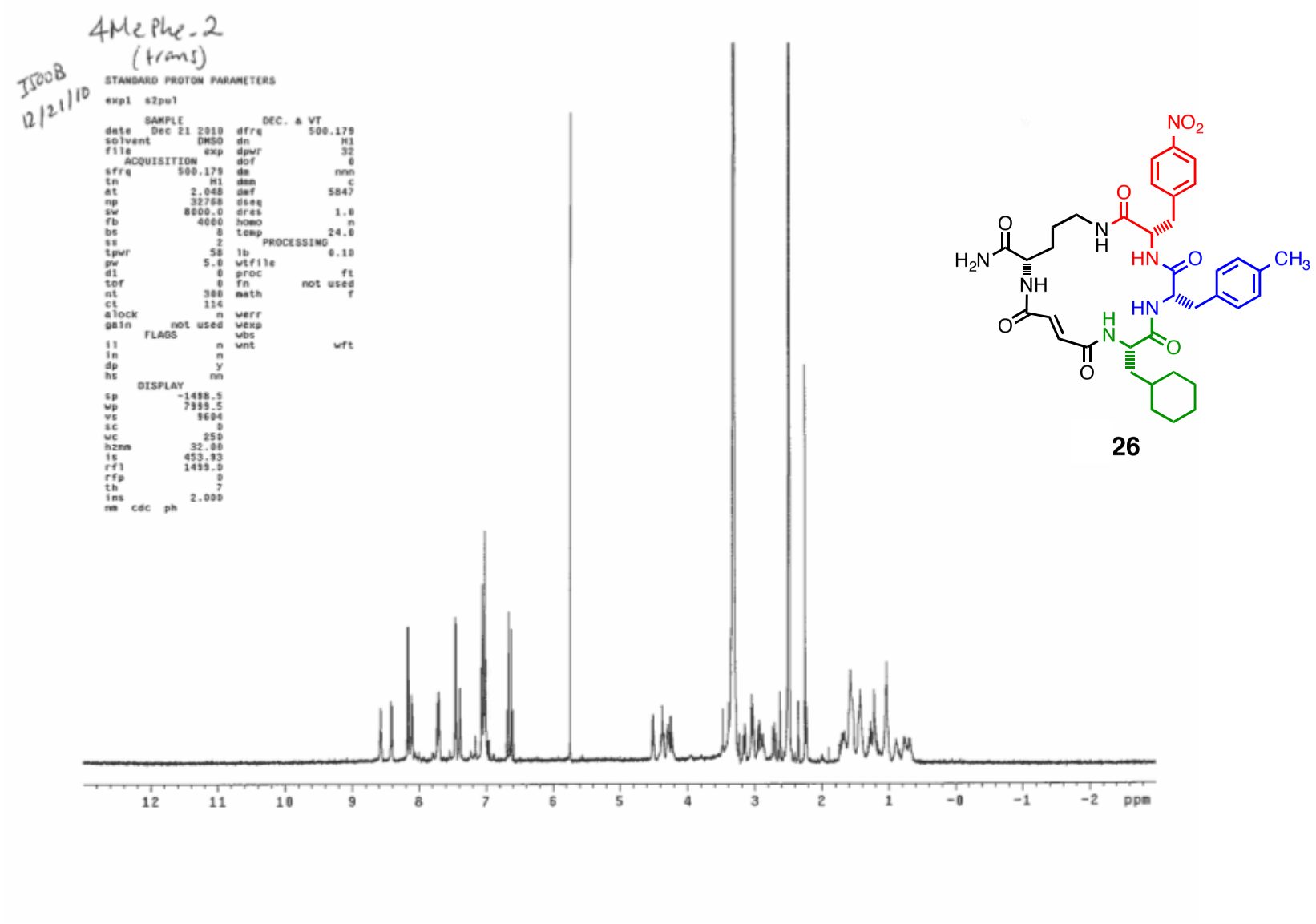

22
12 11 10 9 8 7 6 5 4 3 2 1 -0 -1 -2 ppm

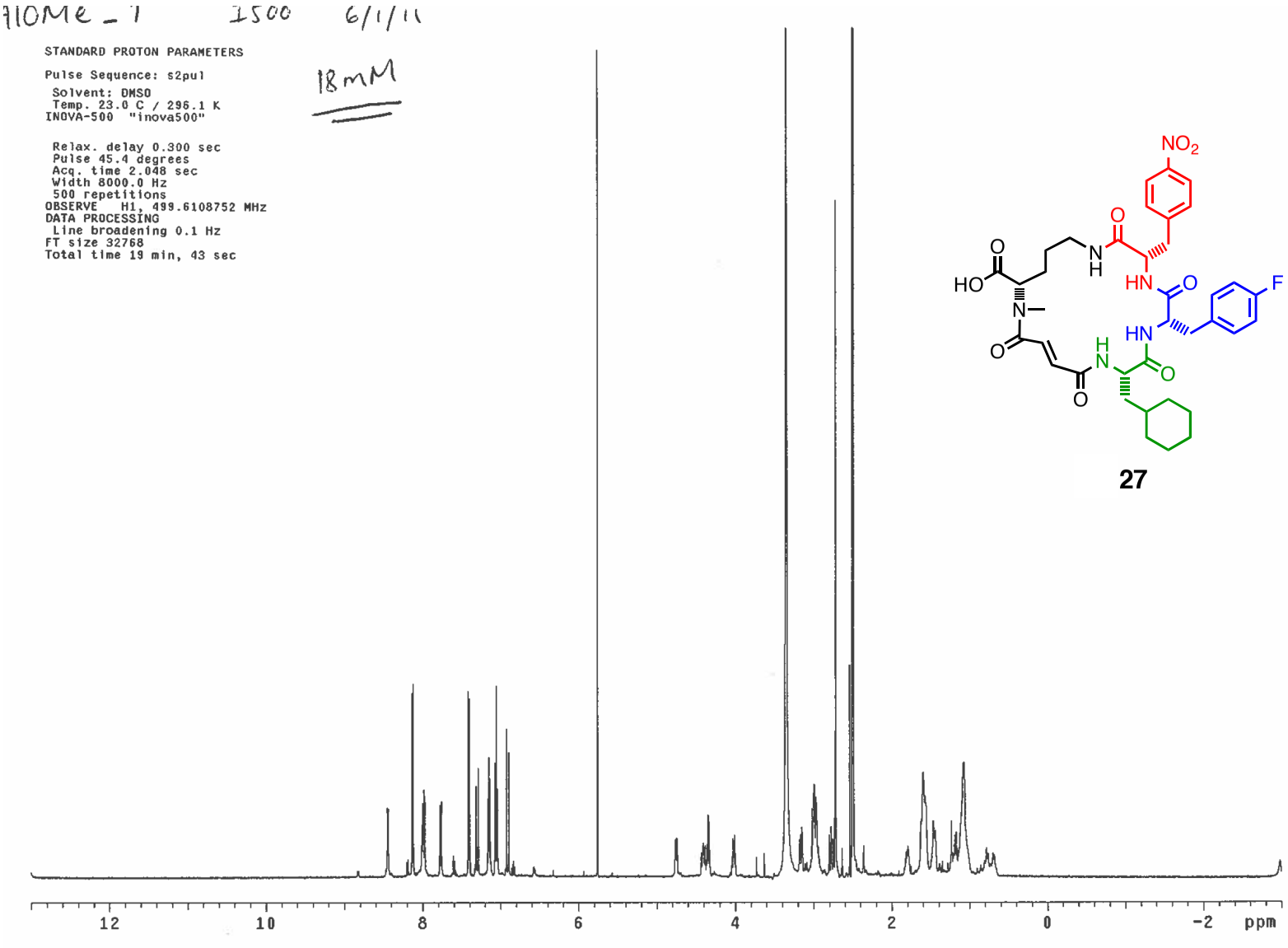

4tBuPhe-2
I500B
11/13/10
STANDARD PROTON PARAMETERS
exp1 s2pul
SAMPLE
date Nov 13 2010
solvent DMSO
file exp
ACQUISITION
sfrq 500.179
tn H1
at 2.048
np 32768
sw 8000.0
fb 4000
bs 8
ss 2
tpwr 58
pw 5.0
dl 0
tof 0
nt 200
ct 91
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -1498.5
wp 7999.5
vs 994
sc 0
wc 250
hzmm 32.00
is 616.22
rfl 1499.0
rfp 0
th 7
ins 2.000
nm cdc ph
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
dm nnn
dmm c
dmf 5847
dseq
dres 1.0
homo n
temp 23.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm
23


I500B
2/6/11
Tyr-2 trans
STANDARD PROTON PARAMETERS
Data Collected on:
nmrsun2-inova500
Archive directory:
/export/home/ds2/vnmrsys/data
Sample directory:
File: 020611_Tyr_2
Pulse Sequence: s2pul
Solvent: DMSO
Temp. 25.0 C / 298.1 K
Pulse 48.4 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
128 repetitions
OBSERVE H1, 500.1763237 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 17 min
NO2
OH
HN
NH
H2N
24
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm


I500B 4FPhe-2 trans
2/6/11
STANDARD PROTON PARAMETERS
Data Collected on:
nmrsun2-inova500
Archive directory:
/export/home/ds2/vnmrsys/data
Sample directory:
File: 020611_4FPhe_2
Pulse Sequence: s2pul
Solvent: DMSO
Temp. 25.0 C / 298.1 K
Pulse 48.4 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
140 repetitions
OBSERVE H1, 500.1763237 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 17 min
NO2
F
25a
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm

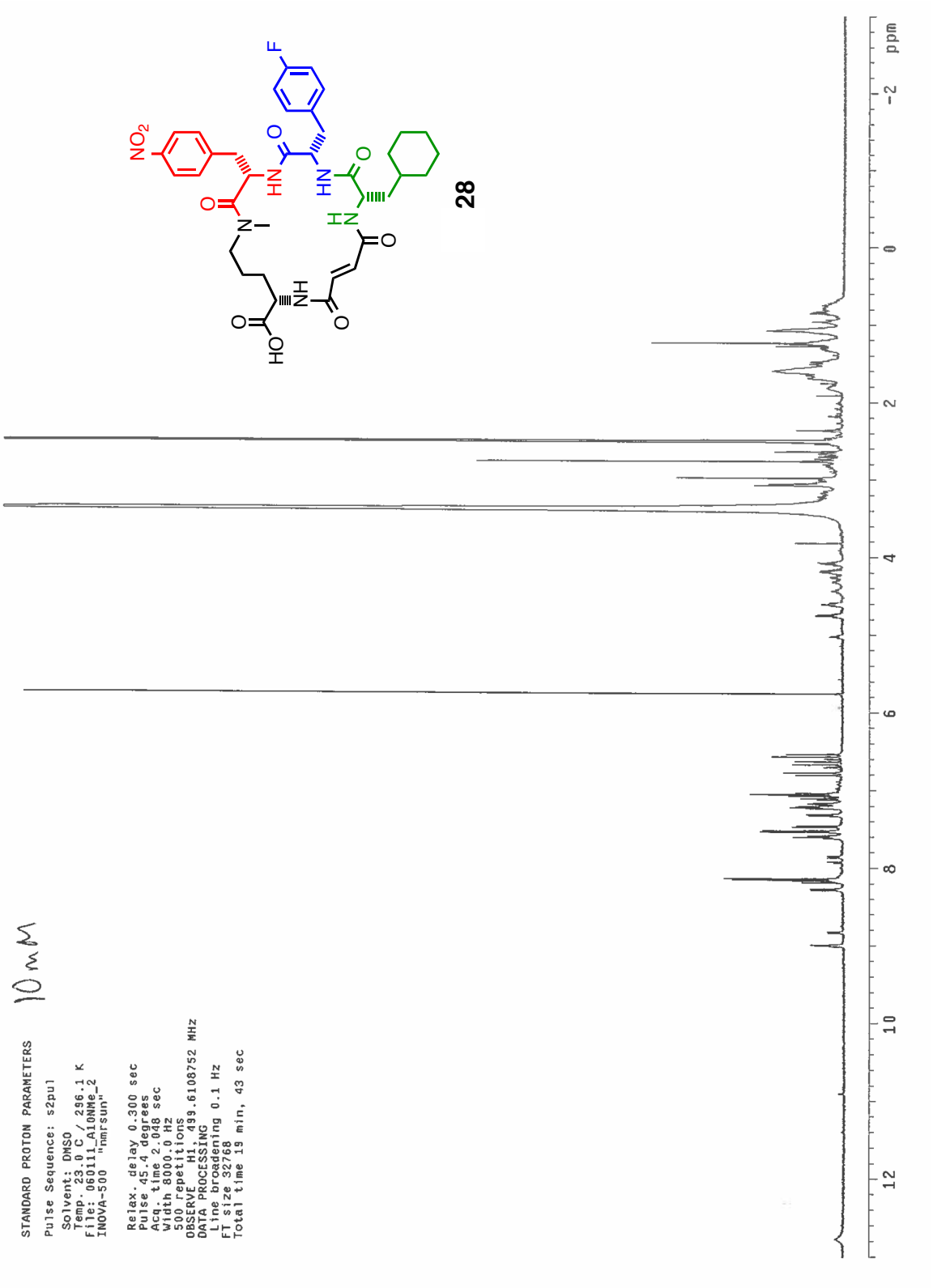

A10acid – 2 trans
STANDARD PROTON PARAMETERS
Data Collected on:
nmrsun2-inova500
Archive directory:
/export/home/ds2/vnmrsys/data
Sample directory:
File: 020611_A10acid_2
Pulse Sequence: s2pul
Solvent: DMSO
Temp. 25.0 C / 298.1 K
Pulse 48.4 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
238 repetitions
OBSERVE H1, 500.1763237 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 17 min
12 11 10 9 8 7 6 5 4 3 2 1 -0 -1 -2 ppm
NO2
F
HO
NH
HN
HN
H
N
25b

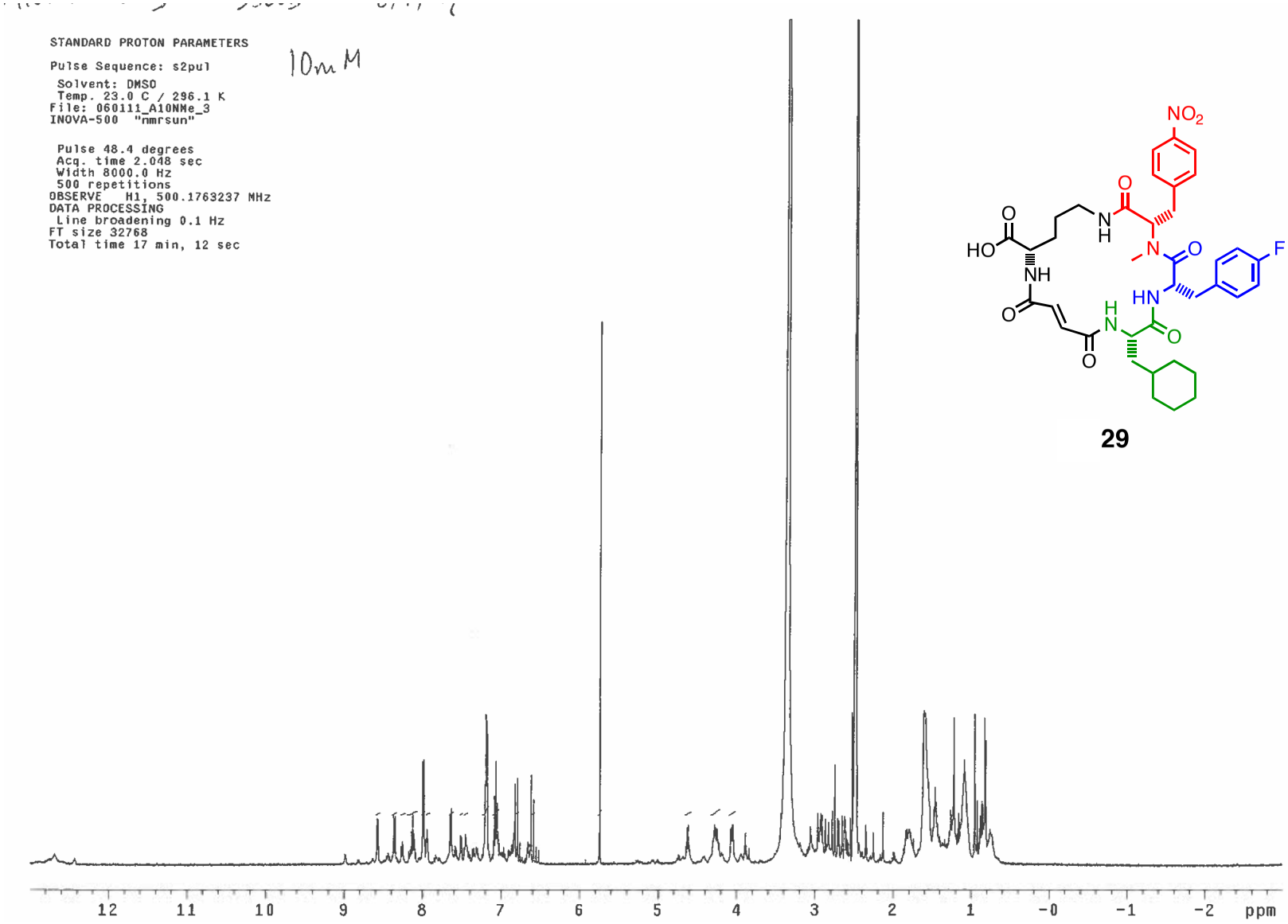

4Me Phe-2
(trans)
I500B
12/21/10
STANDARD PROTON PARAMETERS
expl s2pul
SAMPLE
date Dec 21 2010
solvent DMSO
file exp
ACQUISITION
sfrq 500.179
tn H1
at 2.048
np 32768
sw 8000.0
fb 4000
bs 8
ss 2
tpwr 58
pw 5.0
d1 0
tof 0
nt 300
ct 114
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -1498.5
wp 7999.5
vs 9604
sc 0
wc 250
hzmm 32.00
is 453.93
rfl 1499.0
rfp 0
th 7
ins 2.000
nm cdc ph
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
da nnn
dmm c
dmf 5847
dseq
dres 1.0
homo n
temp 24.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
12 11 10 9 8 7 6 5 4 3 2 1 -0 -1 -2 ppm
NO2
CH3
H2N
NH
HN
HN
N
H
O
26


HOMe_1 1500 6/1/11
STANDARD PROTON PARAMETERS
Pulse Sequence: s2pul
Solvent: DMSO
Temp. 23.0 C / 296.1 K
INOVA-500 "inova500"
Relax. delay 0.300 sec
Pulse 45.4 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
500 repetitions
OBSERVE H1, 499.6108752 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 19 min, 43 sec
18mM
12
10
8
6
4
2
0
-2
ppm

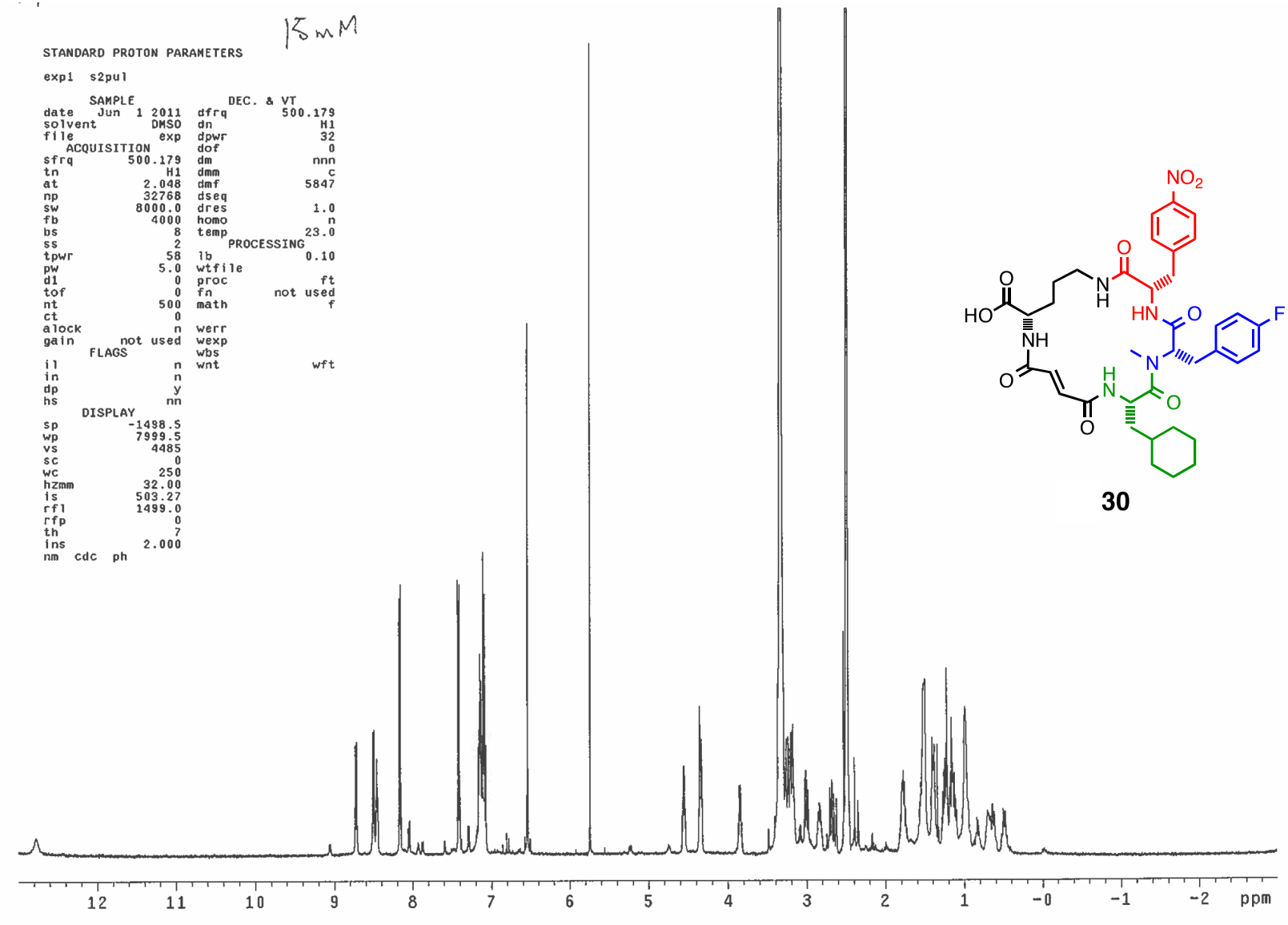
NO2
HO
N
H
HN
HN
F
H
N

27

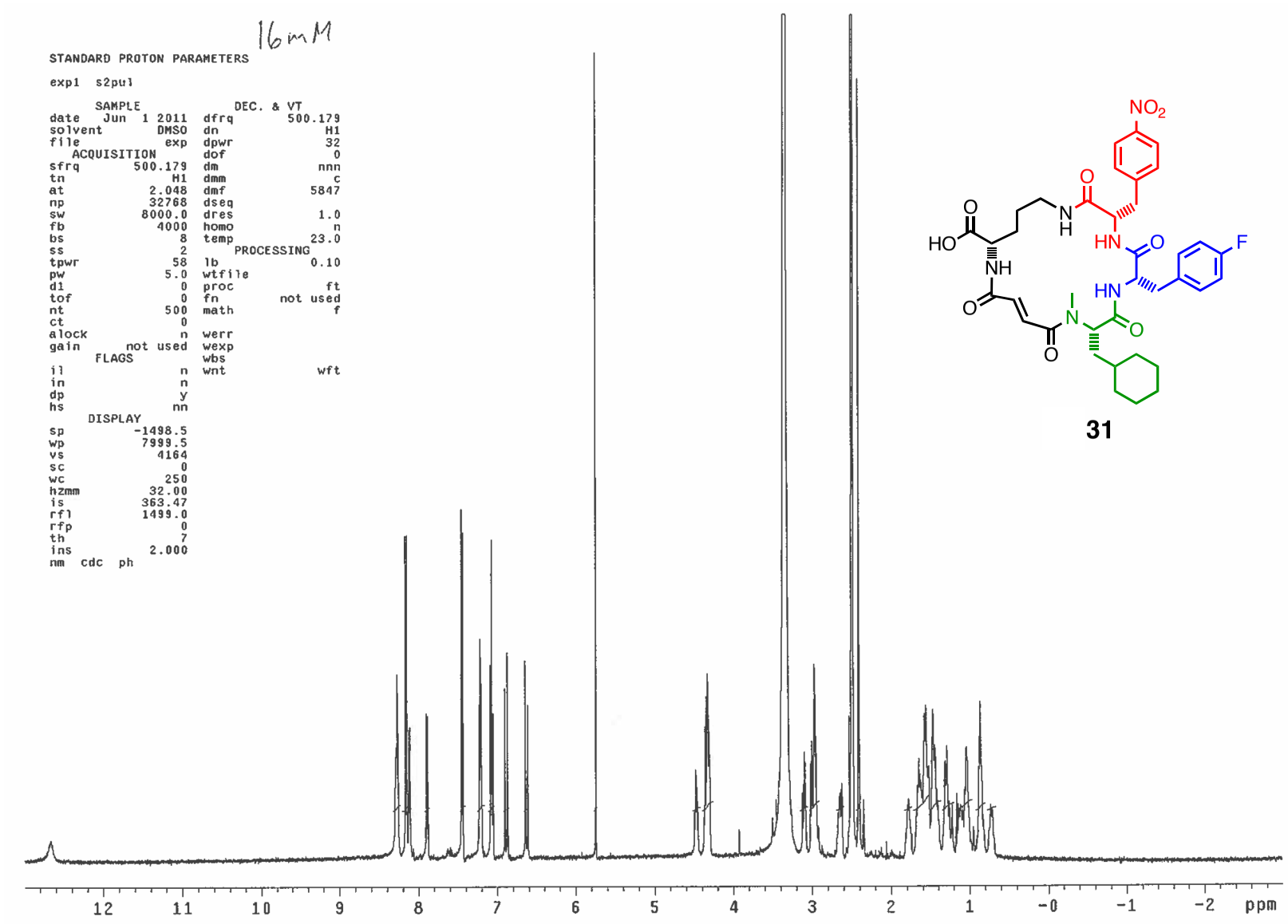

STANDARD PROTON PARAMETERS
Pulse Sequence: s2pul
Solvent: DMSO
Temp. 23.0 C / 296.1 K
File: 060111_A10NMe_2
INOVA-500 "nmrsun"
Relax. delay 0.300 sec
Pulse 45.4 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
500 repetitions
OBSERVE H1, 499.6108752 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 19 min, 43 sec
10 mM
28
ppm

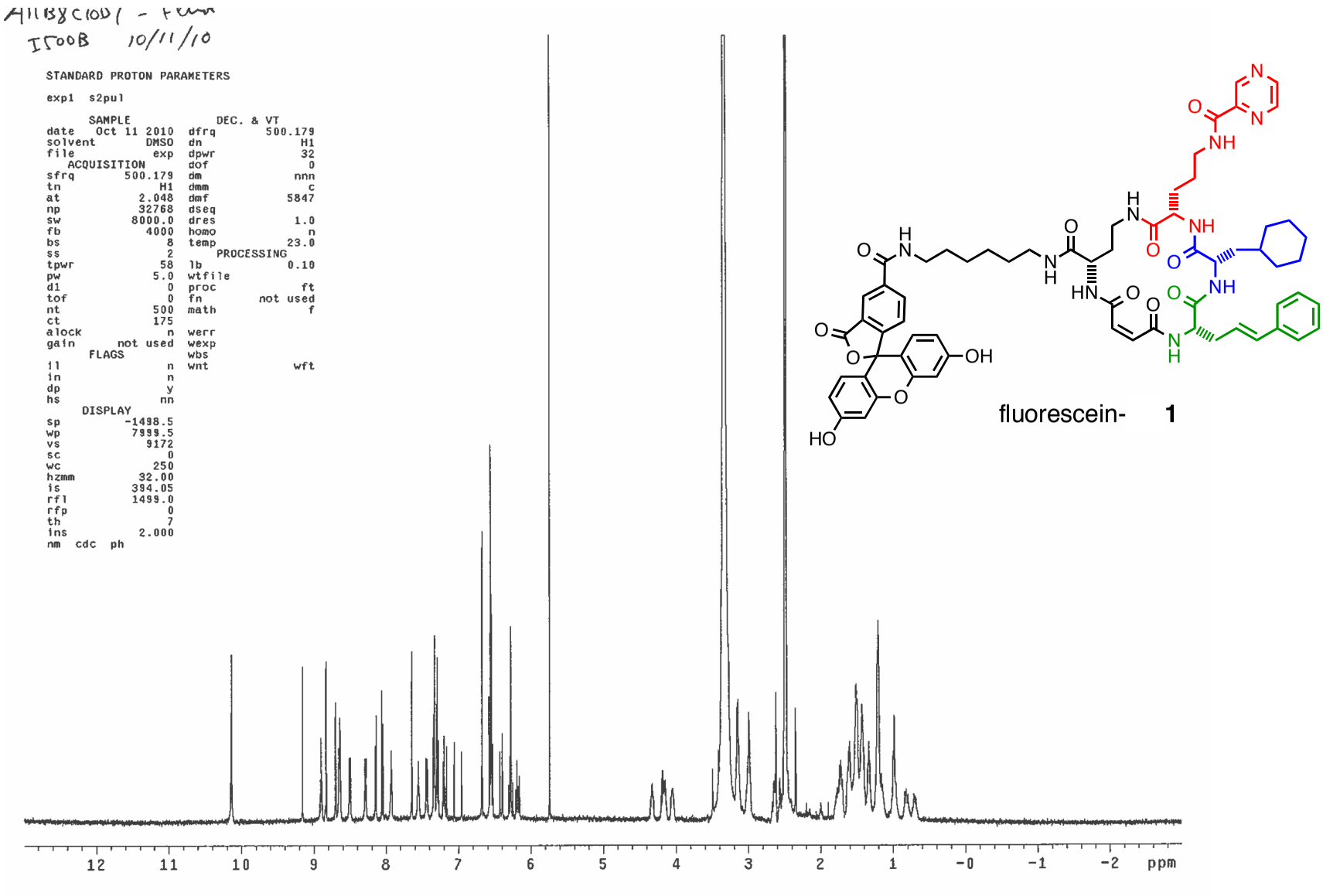

STANDARD PROTON PARAMETERS
Pulse Sequence: s2pul
10mM
Solvent: DMSO
Temp. 23.0 C / 296.1 K
File: 060111_A10NMe_3
INOVA-500 "nmrsun"
Pulse 48.4 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
500 repetitions
OBSERVE H1, 500.1763237 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 17 min, 12 sec
NO2
F
HO
NH
HN
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm

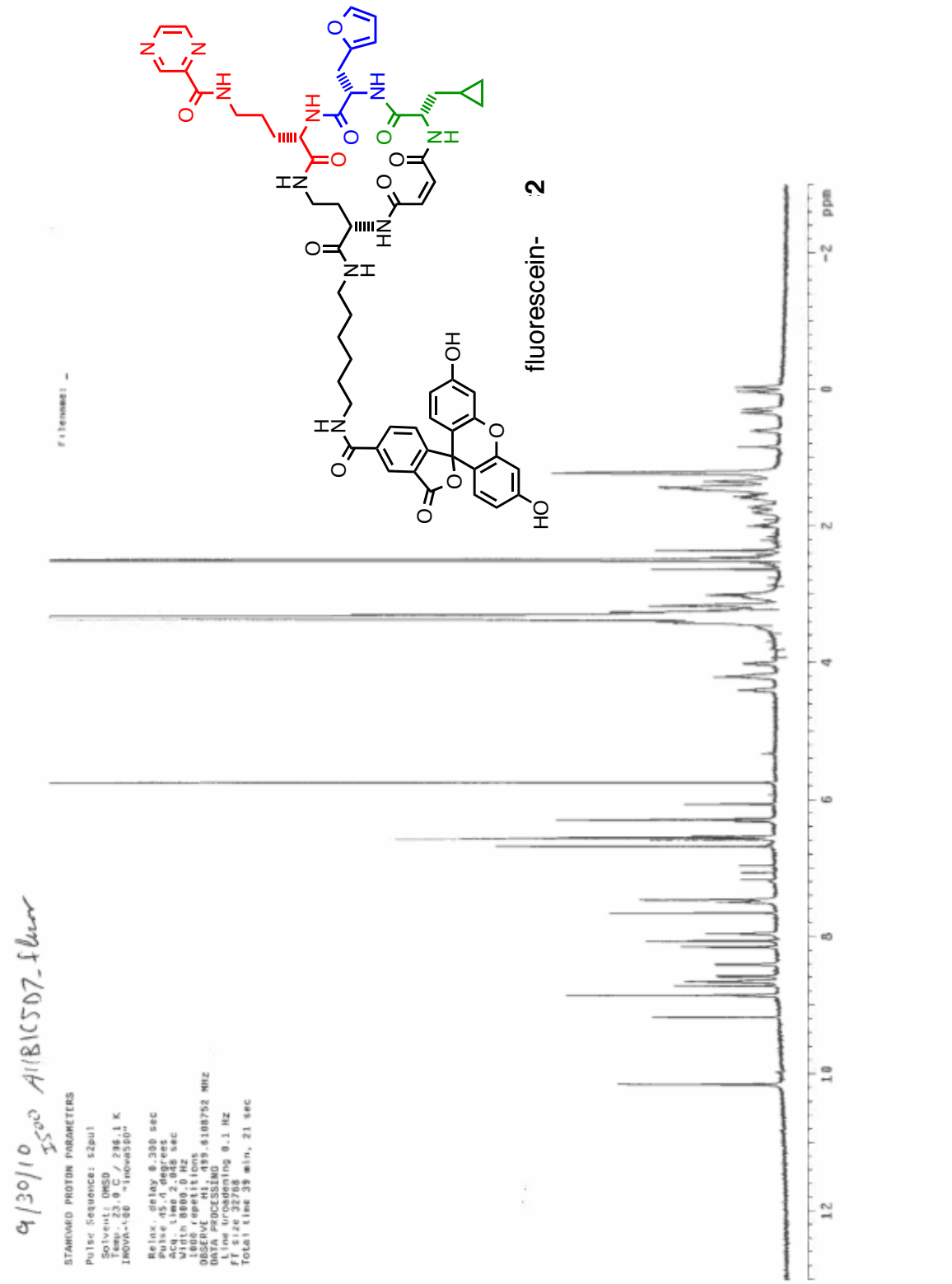
29


15 mM
STANDARD PROTON PARAMETERS
expi s2pul
SAMPLE
date Jun 1 2011
solvent DMSO
file exp
ACQUISITION
sfrq 500.179
tn H1
at 2.048
np 32768
sw 8000.0
fb 4000
bs 8
ss 2
tpwr 58
pw 5.0
d1 0
tof 0
nt 500
ct 0
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -1488.5
wp 7999.5
vs 4485
sc 0
wc 250
hzmm 32.00
is 503.27
rfl 1499.0
rfp 0
th 7
ins 2.000
nm cdc ph
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
dm nnn
dmm c
dmf 5847
dseq
dres 1.0
homo n
temp 23.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
12 11 10 9 8 7 6 5 4 3 2 1 -0 -1 -2 ppm

NO2
HO
O
NH
N
H
HN
O
F
30


16mM
STANDARD PROTON PARAMETERS
exp1 s2pul
SAMPLE
date Jun 1 2011
solvent DMSO
file exp
ACQUISITION
sfrq 500.179
tn H1
at 2.048
np 32768
sw 8000.0
fb 4000
bs 8
ss 2
tpwr 58
pw 5.0
d1 0
tof 0
nt 500
ct 0
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -1498.5
wp 7999.5
vs 4164
sc 0
wc 250
hzmm 32.00
is 363.47
rfl 1499.0
rfp 0
th 7
ins 2.000
nm cdc ph
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
dm nnn
dmm c
dmf 5847
dseq
dres 1.0
homo n
temp 23.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
NO2
HO
NH
HN
HN
N
F
31
12
11
10
9
8
7
6
5
4
3
2
1
-0
-1
-2
ppm


AllBzC10D1 - Fluor
I500B 10/11/10
STANDARD PROTON PARAMETERS
exp1 s2pul
SAMPLE
date Oct 11 2010
solvent DMSO
file exp
ACQUISITION
sfrq 500.179
tn H1
at 2.048
np 32768
sw 8000.0
fb 4000
bs 8
ss 2
tpwr 58
pw 5.0
d1 0
tof 0
nt 500
ct 175
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -1498.5
wp 7999.5
vs 9172
sc 0
wc 250
hzmm 32.00
is 394.05
rfl 1499.0
rfp 0
th 7
ins 2.000
nm cdc ph
DEC. & VT
dfrq 500.179
dn H1
dpwr 32
dof 0
dm nnn
dmm c
dmf 5847
dseq
dres 1.0
homo n
temp 23.0
PROCESSING
lb 0.10
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt wft
fluorescein- 1
12 11 10 9 8 7 6 5 4 3 2 1 -0 -1 -2 ppm

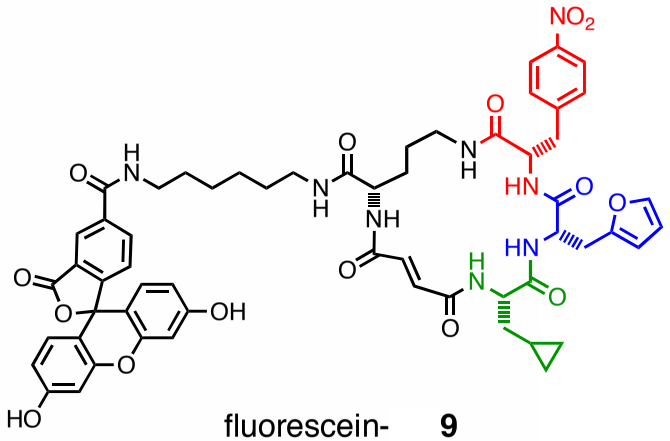

fluorescein- 2
9/30/10 AllBICSD7_fluor
STANDARD PROTON PARAMETERS
Pulse Sequence: s2pul
Solvent: DMSO
Temp: 23.0 C / 296.1 K
INOVA-600 "inova510"
Relax. delay 0.300 sec
Pulse 45.1 degrees
Acq. time 2.662 sec
Width 8800.0 Hz
1000 repetitions
OBSERVE H1, 499.6108752 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 39 min, 21 sec
12
10
8
6
4
2
0
-2
ppm

00B 10/11/10

STANDARD PROTON PARAMETERS

expl s2pul

SAMPLE		DEC. & VT	
date	Oct 11 2010	dfrq	500.179
solvent	DMSO	dn	H1
file	exp	dpwr	32
ACQUISITION		dof	0
sfrq	500.179	dm	nnn
tn	H1	dmm	c
at	2.048	dmf	5847
np	32768	dseq	
sw	8000.0	dres	1.0
fb	4000	homo	n
bs	8	temp	23.0
ss	2	PROCESSING	
tpwr	58	lb	0.10
pw	5.0	wtfile	
d1	0	proc	ft
tof	0	fn	not used
nt	1000	math	f
ct	203		
alock	n	werr	
gain	not used	wexp	
FLAGS		wbs	
il	n	wnt	wft
in	n		
dp	y		
hs	nn		
DISPLAY			
sp	-1498.5		
wp	7999.5		
vs	5586		
sc	0		
wc	250		
hzmm	32.00		
is	406.01		
rfl	1499.0		
rfp	0		
th	7		
ins	2.000		
nm cdc ph			



fluorescein- **9**