Supporting Information

Extracellularly oxidative activation and inactivation of matured prodrug for cryptic self-resistance in naphthyridinomycin biosynthesis

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Supplementary Tables

Table S1. Strains and plasmids used in this study

Strain/Plasmid	Characteristics*	Reference
Strains		
E. coli DH5α	Host for general cloning	Invitrogen
E. coli Rosetta^{TM}2 (DE3)	Host for gene expression	Novagen
E. coli BL21 (DE3)	Host for gene expression	Invitrogen
S.lividans 1326	Host for protein expression	1
S. lusitanus NRRL 8034	Wild type, NDM producing strain	NRRL
S. lusitanus TG3020	Δ*napU* gene replacement mutant	This work
S. lusitanus TG3021	Δ*napU* gene complementation mutant with pTG3030	This work
*S. lividans*TG3026	NapU protein expression with pTG3034	This work
Plasmids		
pMD19-T	Ap^R, *E. coli* subcloning vector	Takara
pLY10	Am^R, Heterologous expression vector in *Streptomyces*, pHZ1358 derivative with *PermE** inserted	Gift from Dr. Wenqing Chen
pKC1139	Am^R, *E. coli-Streptomyces* shuttle vector for gene inactivation	1
pSET152	Am^R, *E. coli-Streptomyces* shuttle vector for gene complementation	1
pET37b	Protein expression in *E. coli*	
pET28a	Protein expression in *E. coli*	
pTG3030	pSET152 derivative for gene complementation of *napU*	This work
pTG3031	pTG3001 derivative for gene replacement of *napU*	This work
pTG3033	pET37b derivative containing *napU* gene for protein expression in *E. coli*	This work
pTG3034	pLY10 derivative containing *napU*gene for protein expression in *S.lividans*	This work
pTG3035	pET37b derivative containing truncated *napU* gene for expression of signal peptide-removed NapU in *E. coli*	This work
pTG3036	pET37b derivative containing mutated *napU* (C170A) gene for protein expression	This work
pTG3037	pET37b derivative containing mutated *napU* (H110A) gene for protein	This work

	expression	
pTG3038	pET37b derivative containing mutated *napU* (C170A/H110A) gene for protein expression	This work
pTG3039	pET37b derivative containing mutated *napU* (Y184F) gene for protein expression	This work
pTG3040	pET37b derivative containing mutated *napU* (Y187F) gene for protein expression	This work
pTG3041	pET37b derivative containing mutated *napU* (Y441F) gene for protein expression	This work
pTG3042	pET37b derivative containing mutated *napU* (Y486F) gene for protein expression	This work
pTG3043	pET37b derivative containing mutated *napU* (Y489F) gene for protein expression	This work

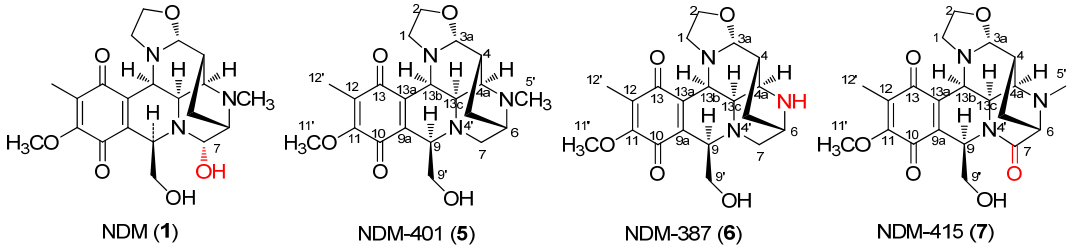
Abbreviations: Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Am^R, apramycin resistance. Sp^R, spectinomycin resistance.

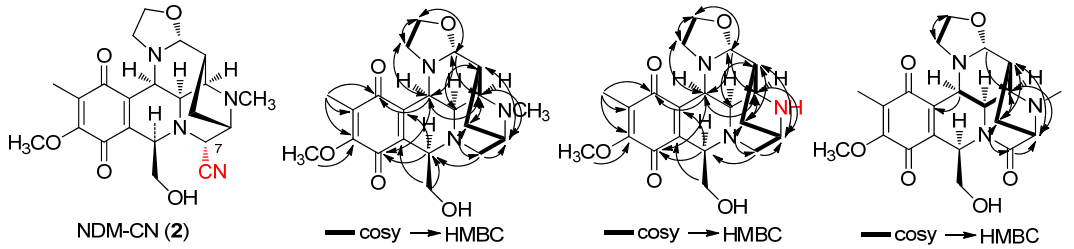
1. Kieser, T.; Bibb, M.; Butter, M.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; The John Innes Foundation, Norwich., 2001.

Table S2. PCR primers used in this study

Primers	Sequence	Enzyme	Application
napU-Red-for	CAGGAGGTCGCCGTCCGTTCCGGCGGTCACTGCCTCGAGATTCCGGGGATCCGTCGACC		Gene replacement
napU-Red-rev	GACGCGCAGGATCGAGTCCCGGTGGACGTAGGCGGTCGCTGTAGGCTGGAGCTGCTTC		
napU-p-for	CATATGTCTGAACTGAACCGCCG	*Nde*I	Protein expression
napU-p-rev	AAGCTTGGCGGGCCGGATCGACAGC	*Hin*dIII	
napU-cut-for	CATATGGCCGGGAAGAACACCGCGAAG	*Nde*I	
napU-C-for	AAGCTTTCCGCTTGTGAAAGGAGACC	*Hin*dIII	Complement-ation
napU-C-rev	TCTAGAGCTTTCTGTGACGCTTGGAC	*Xba*I	
napU-H110A-for	GTCGCCGTCCGTTCCGGCGGTGCCTGCCTCGAGAAC		Site-directed mutation
napU-H110A-rev	GGCACCGCCGGAACGGACGGCGACCTCCTGGC		
napU-C170A-for	GTGACGATACCCTCGGCGGGCGCCTCGGAAGTGGGC		Site-directed mutation
napU-C170A-rev	GGCGCCCGCCGAGGGTATCGTCACGCCCCAGCC		
napU-Y184F-for	GCGGGCACATACTCGGCGGCGGCTTCAACTTCTACTCC		Site-directed mutation
napU-Y184F-rev	GAAGCCGCCGCCGAGTATGTGCCCGCCGAGGC		
napU-Y187F-for	CTCGGCGGCGGCTACAACTTCTTCTCCCGCATCCACGG		Site-directed mutation
napU-Y187F-rev	GAAGAAGTTGTAGCCGCCGCCGAGTATGTGCC		
napU-Y442F-for	GGGACTCGATCCTGCGCGTCTTCTTCACGCCCGGCGTG		Site-directed mutation
napU-Y442F-rev	GAAGACGCGCAGGATCGAGTCCCGGTGGACGTAG		
napU-Y486F-for	GACGCCGCCAACTCGGGGGCGTTCATCAACTACCCGG		Site-directed mutation
napU-Y486F-rev	GAACGCCCCCGAGTTGGCGGCGTCGGGCGCC		
napU-Y489F-for	AACTCGGGGGCGTACATCAACTTCCCGGACGTCGACC		Site-directed mutation
napU-Y489F-rev	GAAGTTGATGTACGCCCCCGAGTTGGCGGCGTC		

Table S3. ^{1}H (500 MHz) and ^{13}C (125 MHz) data of compound **5, 6, 7**[a]



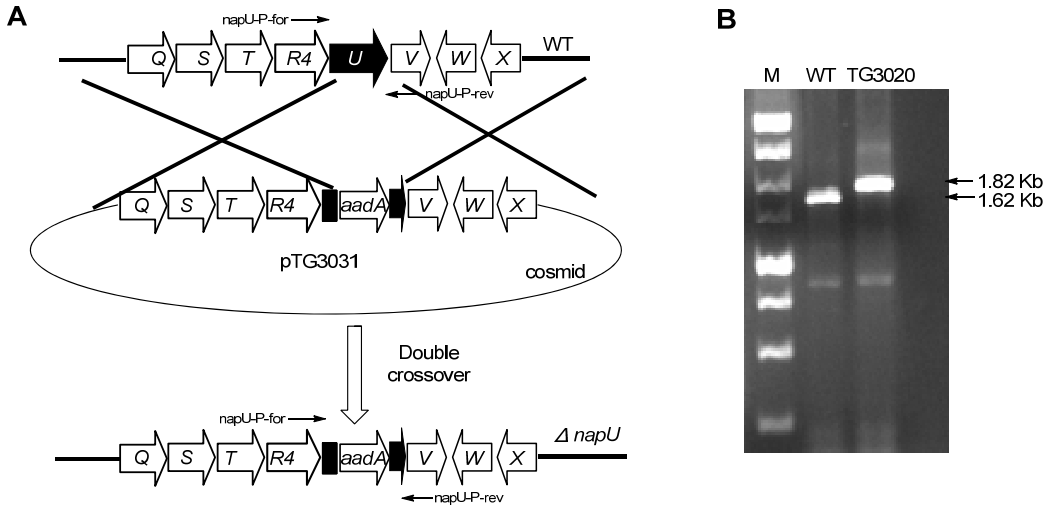


Compound	NDM-401 (**5**)		NDM-387 (**6**)		NDM-415 (**7**)	
No. of C	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	50.50,CH_2	3.05(ddd,1H), 2.97(m,1H)	50.14,CH_2	3.05(ddd,1H), 2.96 (m,1H)	51.42,CH_2	3.01(m,2H),
2	61.32,CH_2	3.69(m,1H), 3.98(dd,1H)	61.34,CH_2	3.69 (m,1H), 3.99 (m,1H)	63.30,CH_2	3.79 (td,1H), 4.09 (dt,1H)
3a	93.99,CH	4.72(s,1H)	93. 67,CH	4.68(s,1H)	94. 64,CH	4.68(s,1H)
4	35.97,CH	2.87(m,1H)	35.65,CH	2.60(m,1H)	35.65,CH	2.98(m,1H)
4’	30.04,CH_2	1.73(dd,1H), 2.32(td,1H)	32.09,CH_2	1.73(dd,1H), 2.16(td,1H)	35.28,CH_2	1.79(dd,1H), 2.65(td,1H)
4a	60.37,CH	3.12(br,1H)	53.68,CH	3.38(br,1H)	59.41,CH	3.48(m,1H)
5’	39.86,CH_3	2.39(s,3H)	-----	-----	36.79,CH_3	2.53(s,3H)
6	60.52,CH	3.22(br,1H)	53.81,CH	3.59(br,1H)	66.75,CH	3.38(d,1H)
7	52.23,CH_2	2.76(d,1H), 2.90(m,1H)	54.15,CH_2	2.58(m,1H), 2.99(m,1H)	174.99,C	-----
9	59.02,CH	3.92(br,1H)	58.81,CH	3.91(br,1H)	53.99,CH	5.23(t,1H)
9’	60.87,CH_2	3.52(d,1H), 3.95(dd,1H)	60.72,CH_2	3.53(d,1H), 3.93(dd,1H)	61.69,CH_2	3.52(dd,1H), 4.53(dd,1H)
9a	142.61,C	------	142.18,C	------	142.09,C	------
10	182.06,C		181.82,C		182.32,C	
11	156.02,C	------	155.75,C	------	157.49,C	------
11’	61.21,CH_3	4.01(s,3H)	69.97,CH_3	4.01(s,3H)	61.69,CH_3	4.07(s,3H)
12	128.48,C	------	128.28,C	------	129.62,C	------
12’	9.07,CH_3	1.95(s,3H)	8.84,CH_3	1.96(s,3H)	9.03,CH_3	1.98(s,3H)
13	187.00,C	------	186.77,C	------	187.52,C	------
13a	143.85,C	------	143.66,C	------	143.12,C	------
13b	48.53,CH	3.67(s,1H)	48.14,CH	3.71(s,1H)	47.73,CH	3.94(s,1H)
13c	54.27,CH	2.68(s,1H)	55.41,CH	2.56(s,1H)	54.32,CH	3.67(d,1H)

[a]The NMR analysis of **5** and **6** is performed in $CDCl_3$; and **7** in CD_3OD.

Supplementary Figures

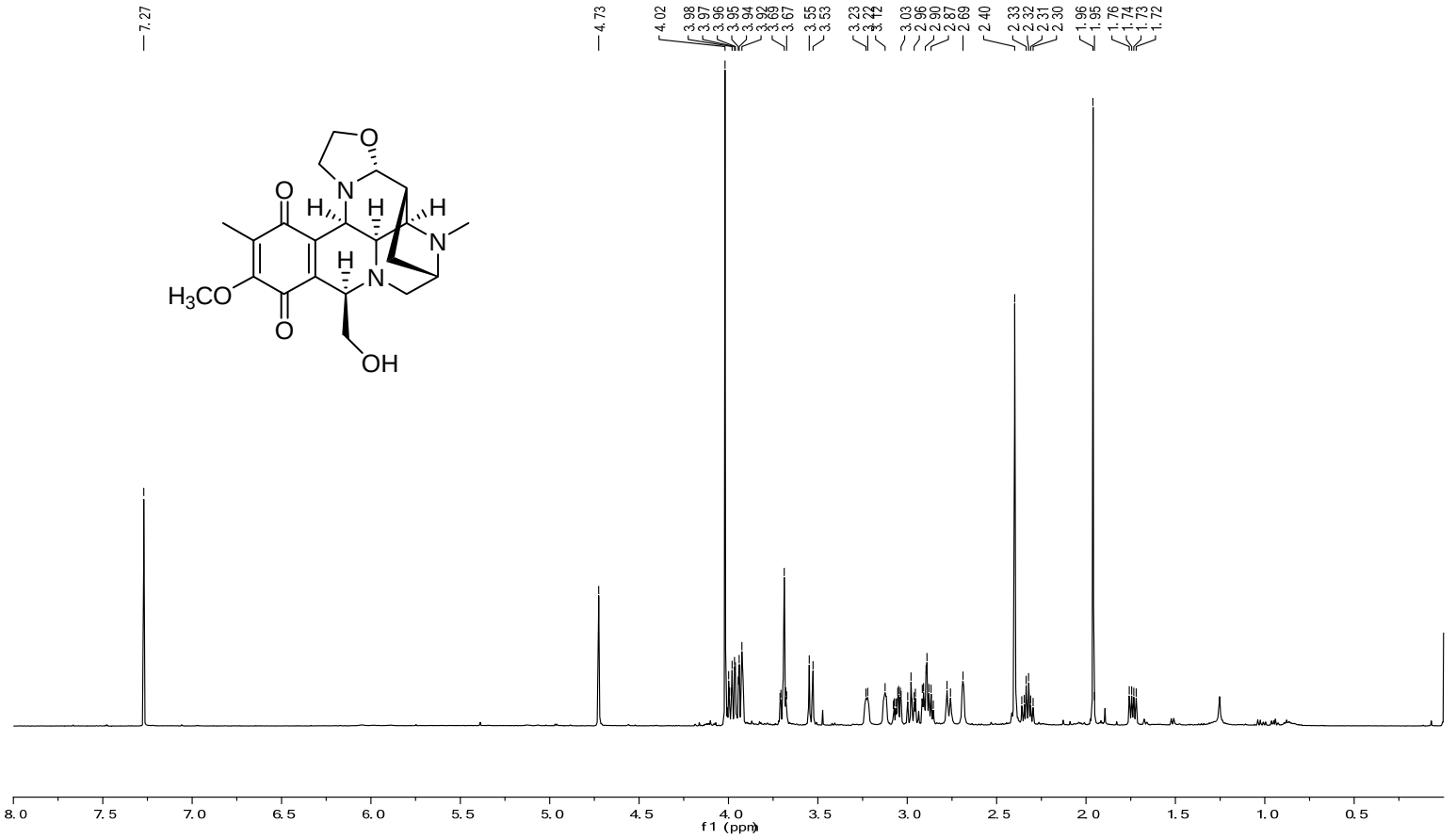
Figure S1. Identification of the genotype of *napU*-gene replacement mutant.



The genome DNA was extracted from wild type and mutant strains for PCR amplification as template using *napU*-P-for/rev as primers respectively (Table S2). (A) Replacement of *napU* gene by spectinomycin resistance gene (*aadA*) to generate double crossover mutant *S. lusitanus* TG3020 (Δ*napU*). (B) A signal of 1.62 Kb can be detected with the genome DNA of wild type strain, while gene replacement mutant strain gives1.82 Kb.

Figure S2. NMR spectra of compound **5**. (A) ^{1}H spectrum (B) ^{13}C spectrum (C) COSY spectrum (D) HMQC spectrum (E) HMBC spectrum.

A



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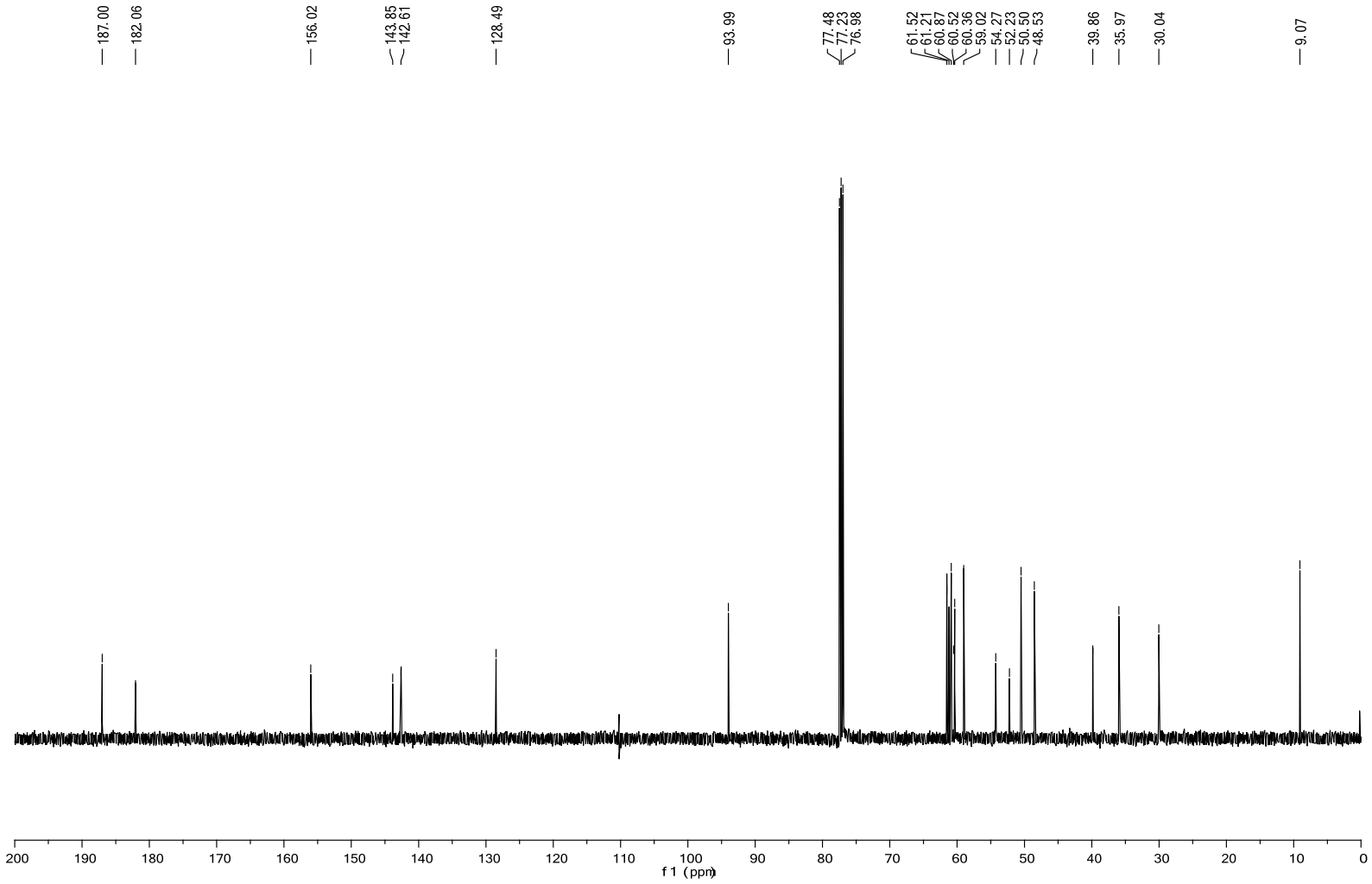
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143.85
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93.99
77.48
77.23
76.98
61.52
61.21
60.87
60.52
60.36
59.02
54.27
52.23
50.50
48.53
39.86
35.97
30.04
9.07
200
190
180
170
160
150
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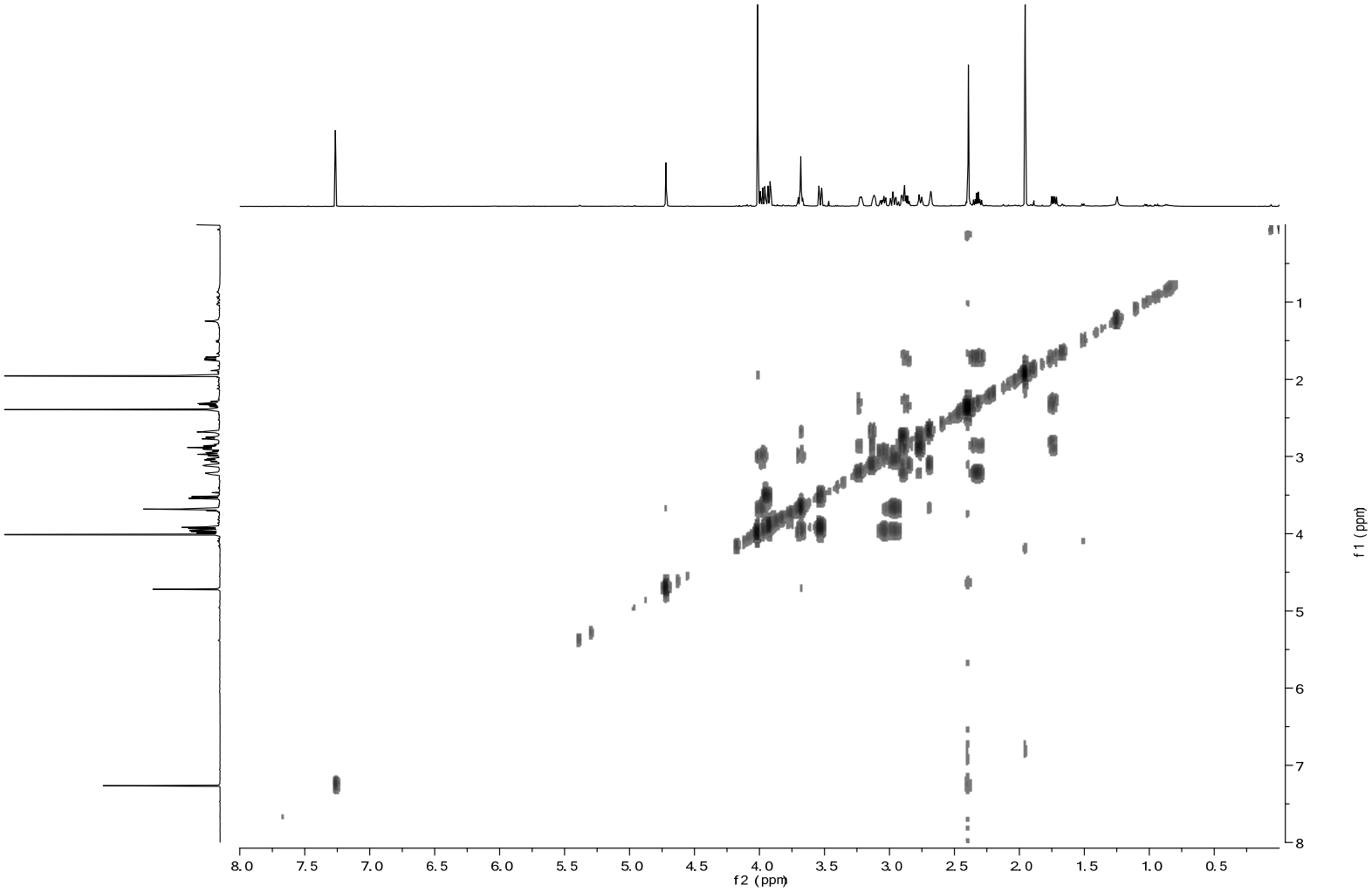
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

C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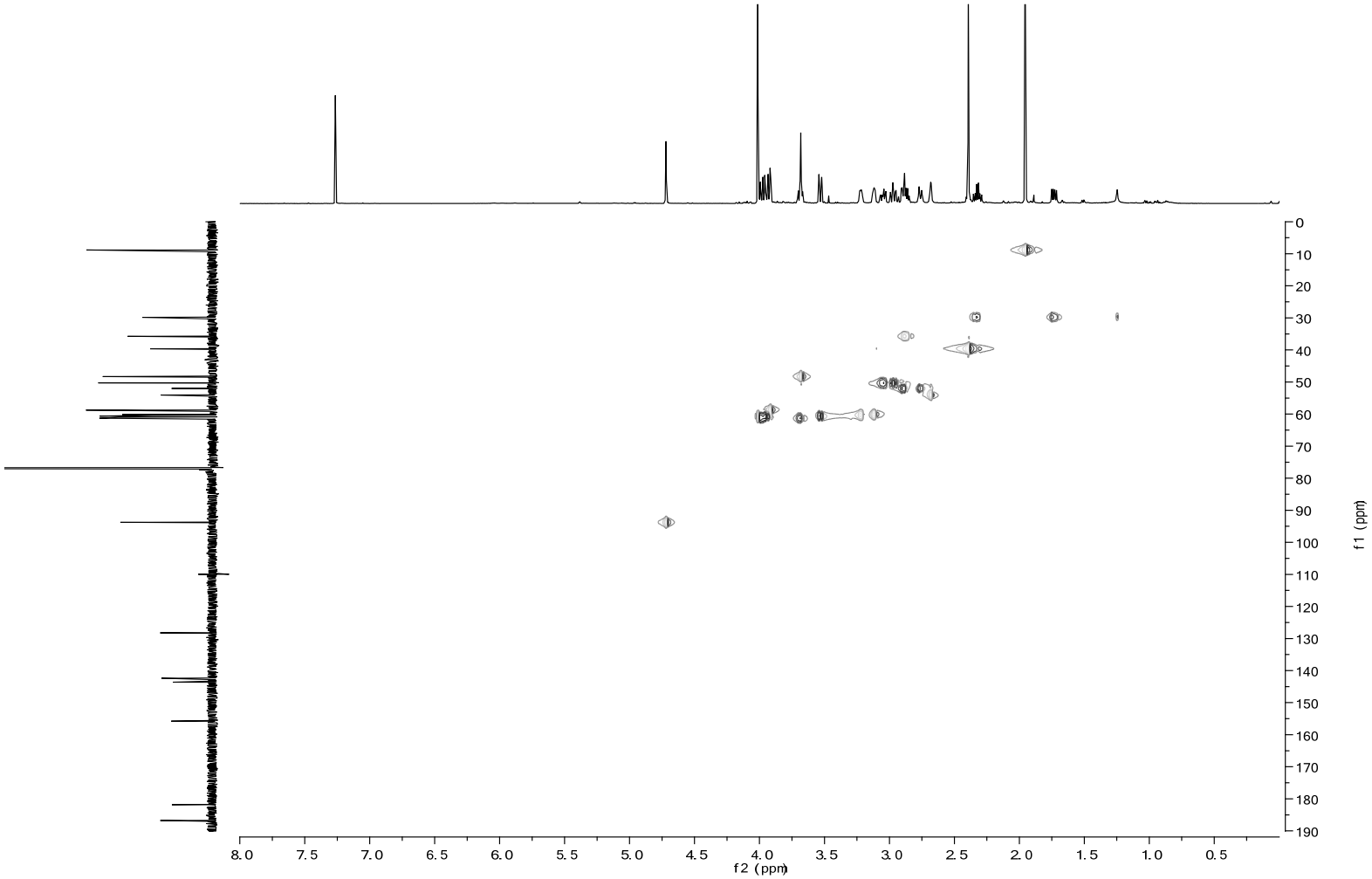
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
1
2
3
4
5
6
7
8
f1 (ppm)

D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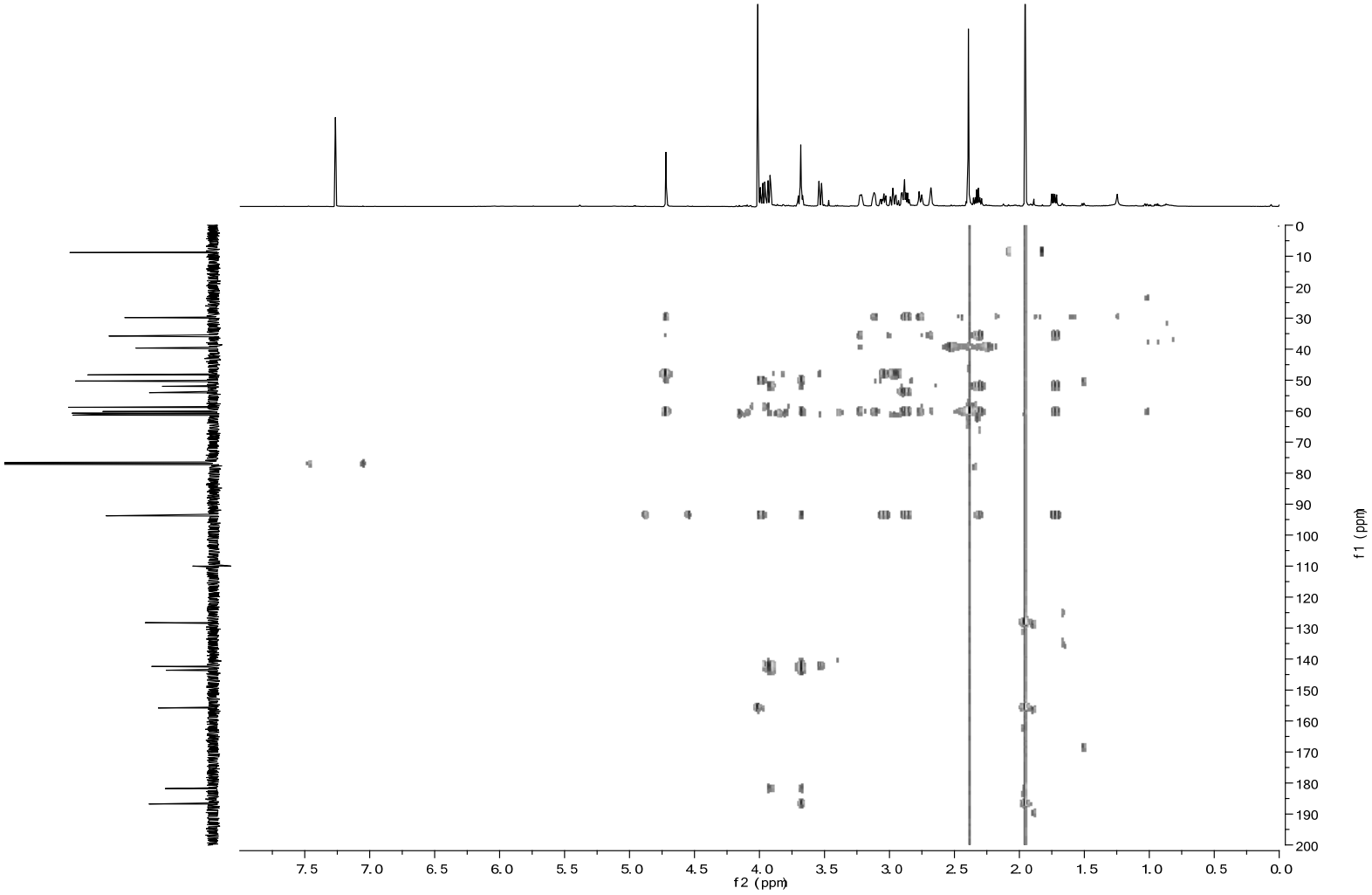
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
f1 (ppm)

E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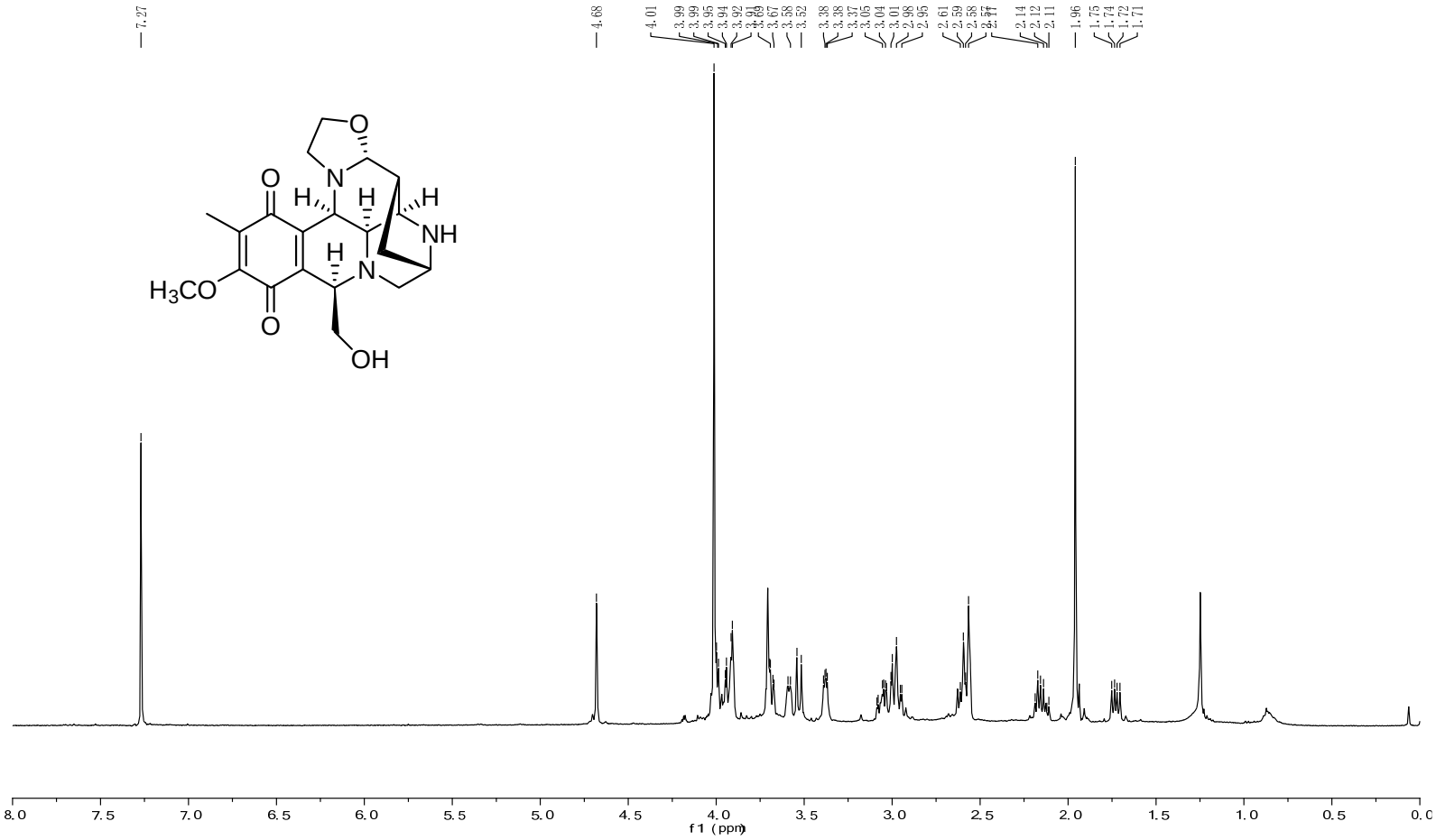
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f2 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
f1 (ppm)

Figure S3. NMR spectra of compound **6**. (A) ^{1}H spectrum (B) ^{13}C spectrum (C) COSY spectrum (D) HMQC spectrum (E) HMBC spectrum.

A



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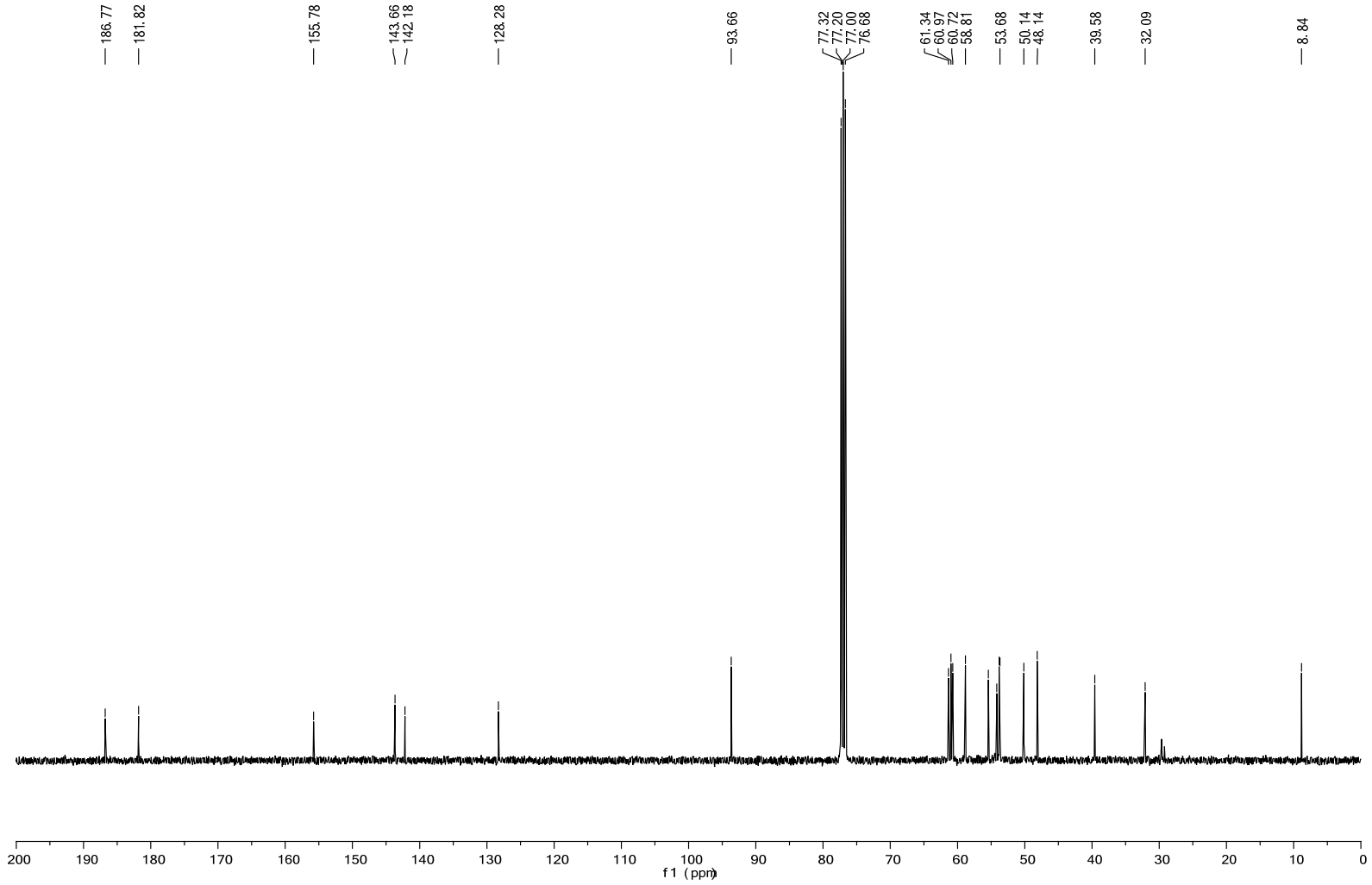
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53.68
50.14
48.14
39.58
32.09
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200
190
180
170
160
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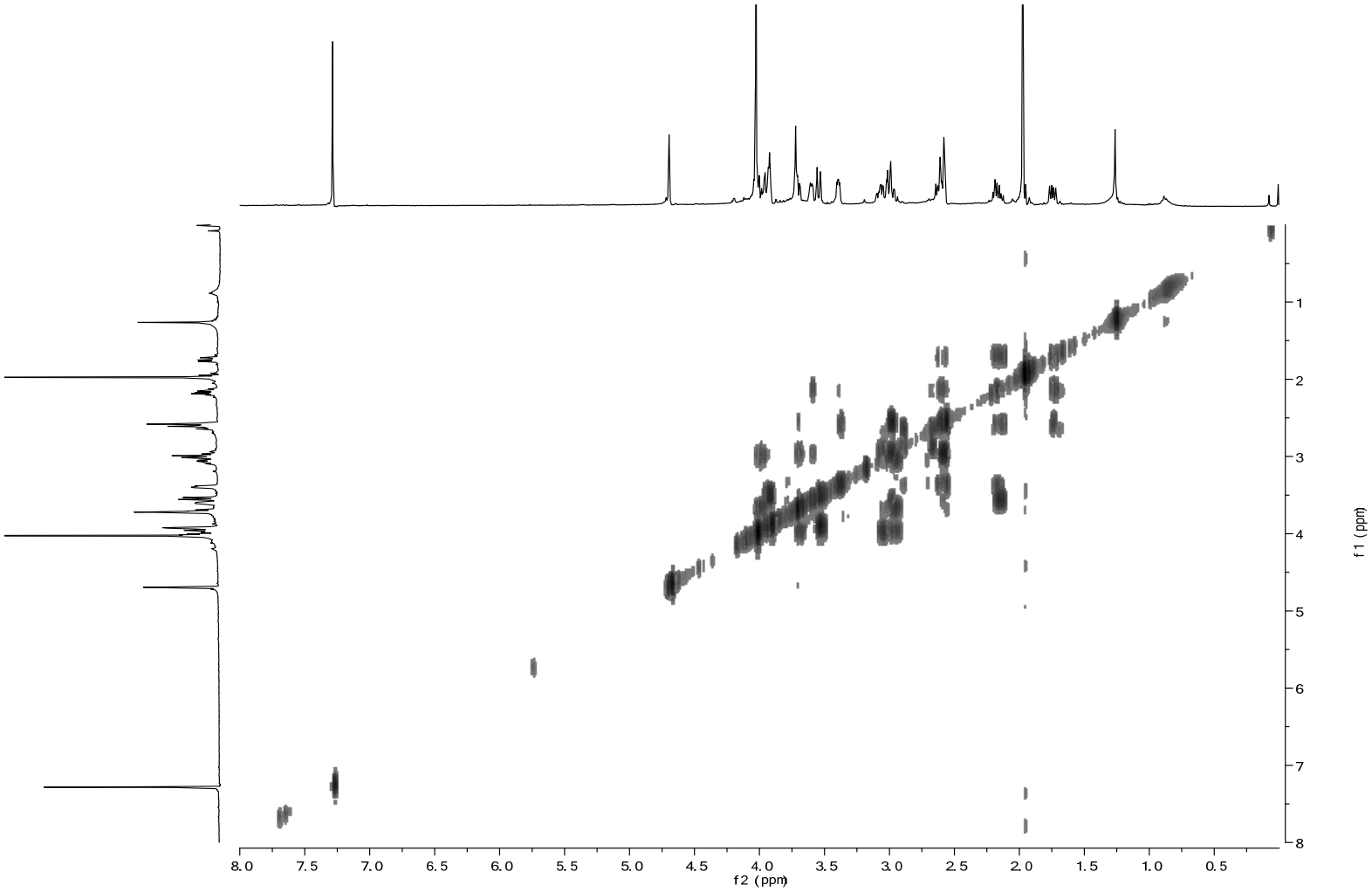
80
70
60
50
40
30
20
10
0
f1 (ppm)

C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7.0
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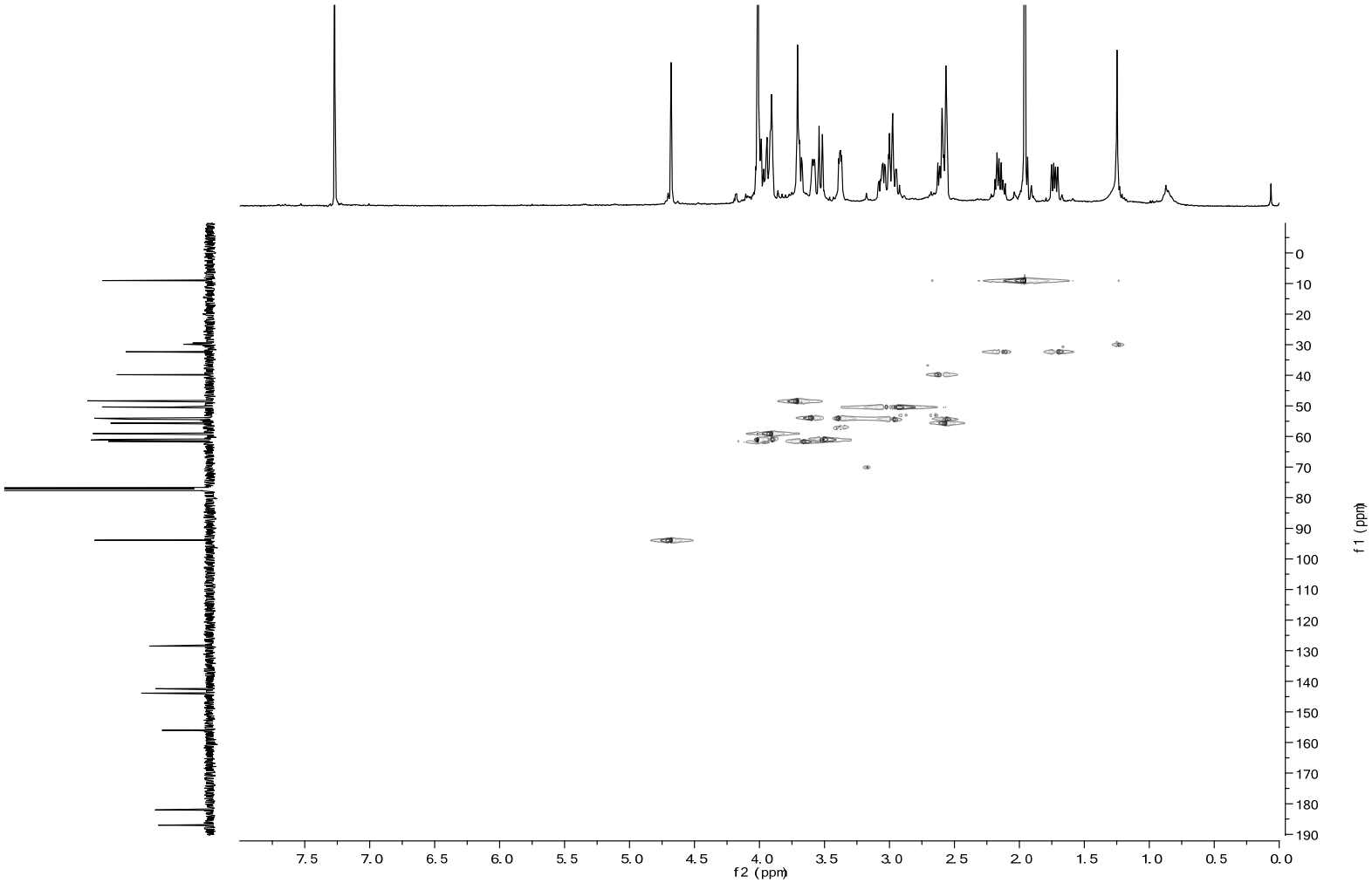
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
1
2
3
4
5
6
7
8
f1 (ppm)

D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5.0
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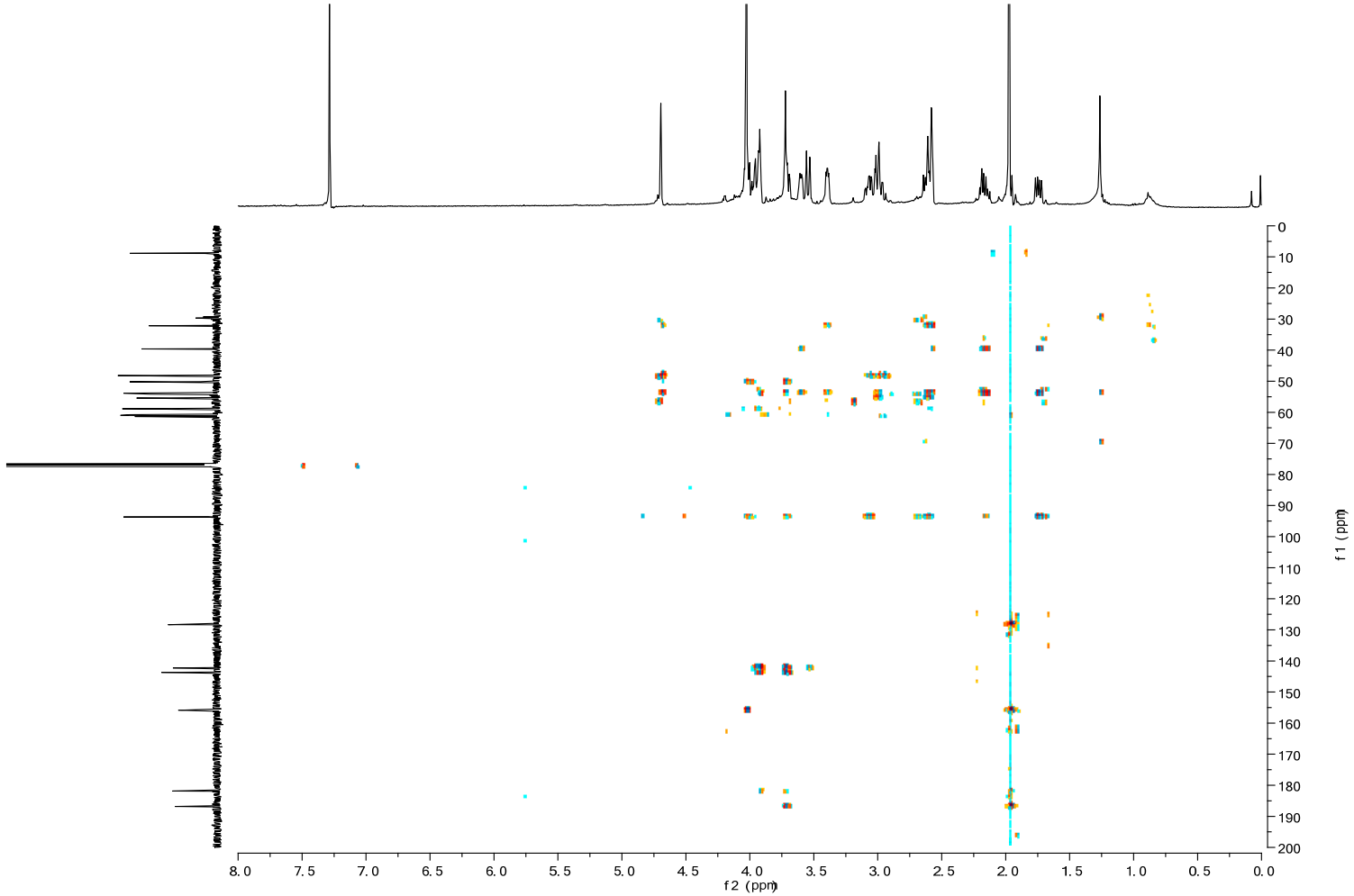
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f2 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
f1 (ppm)

E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2.0
1.5
1.0
0.5
0.0
f2 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
f1 (ppm)

Figure S4. Antibacterial activity of NDM (**1**) and new analogues (**5** and **7**) using *E. coli* BL21 (DE3) as assay strain cultured in solid medium.

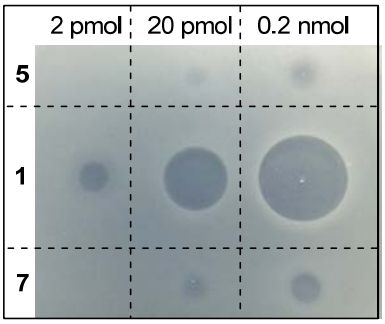
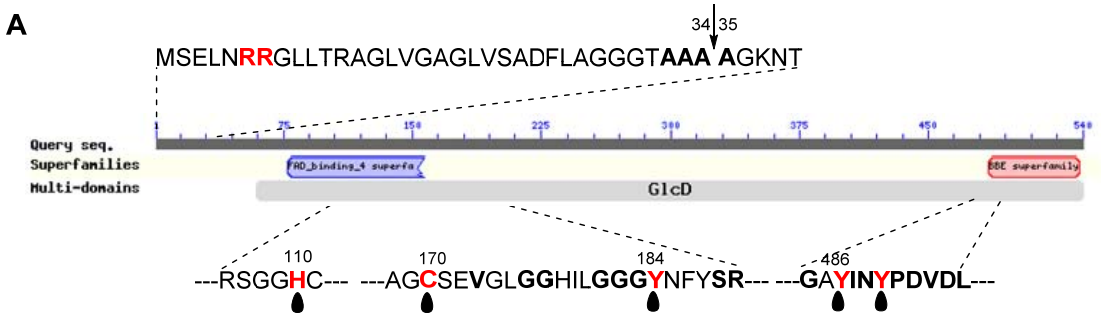


Figure S5. Bioinformatic analysis of NapU.



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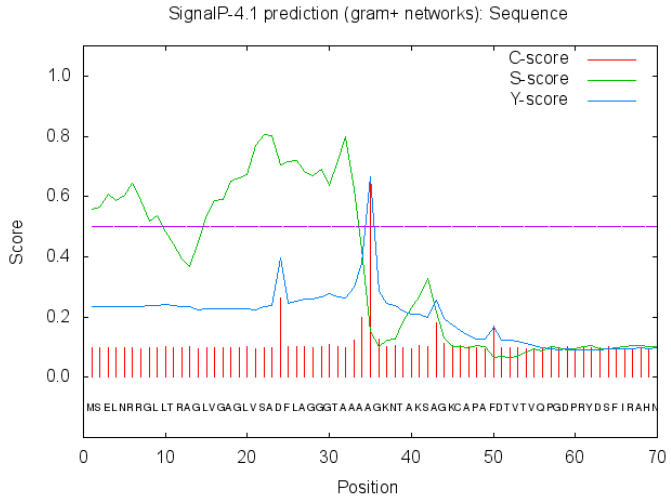


The arrow indicates the signal peptide cleavage site and the key amino acid residues for enzymatic activity are highlighted. Prediction of signal peptide cleavage site is between position 34 and 35.

Figure S6. UV-Vis absorption spectrum of recombinant NapU from *E. coli*.

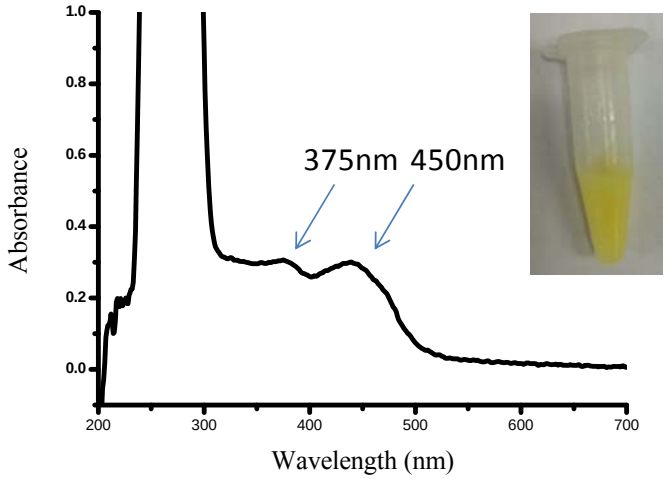
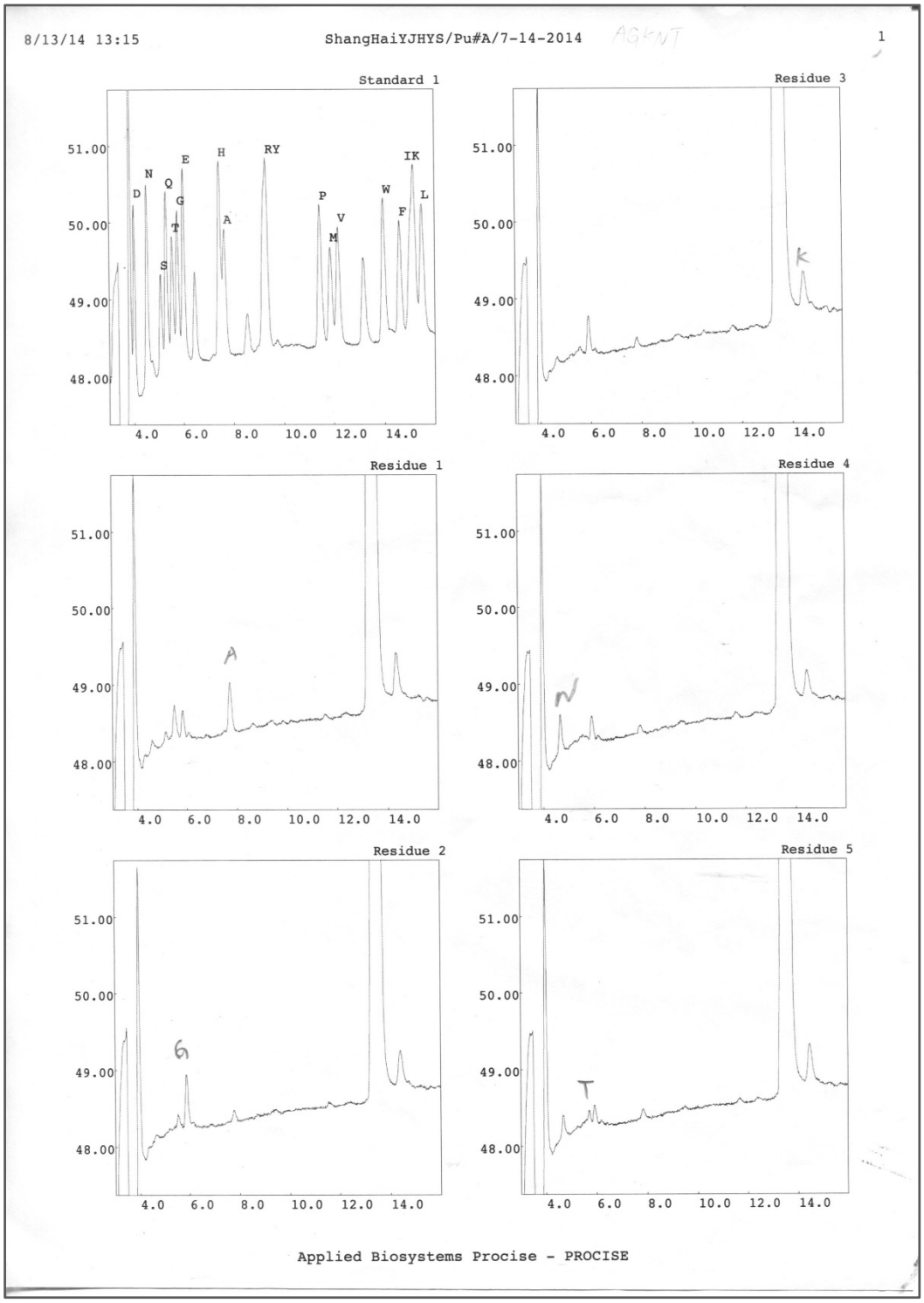


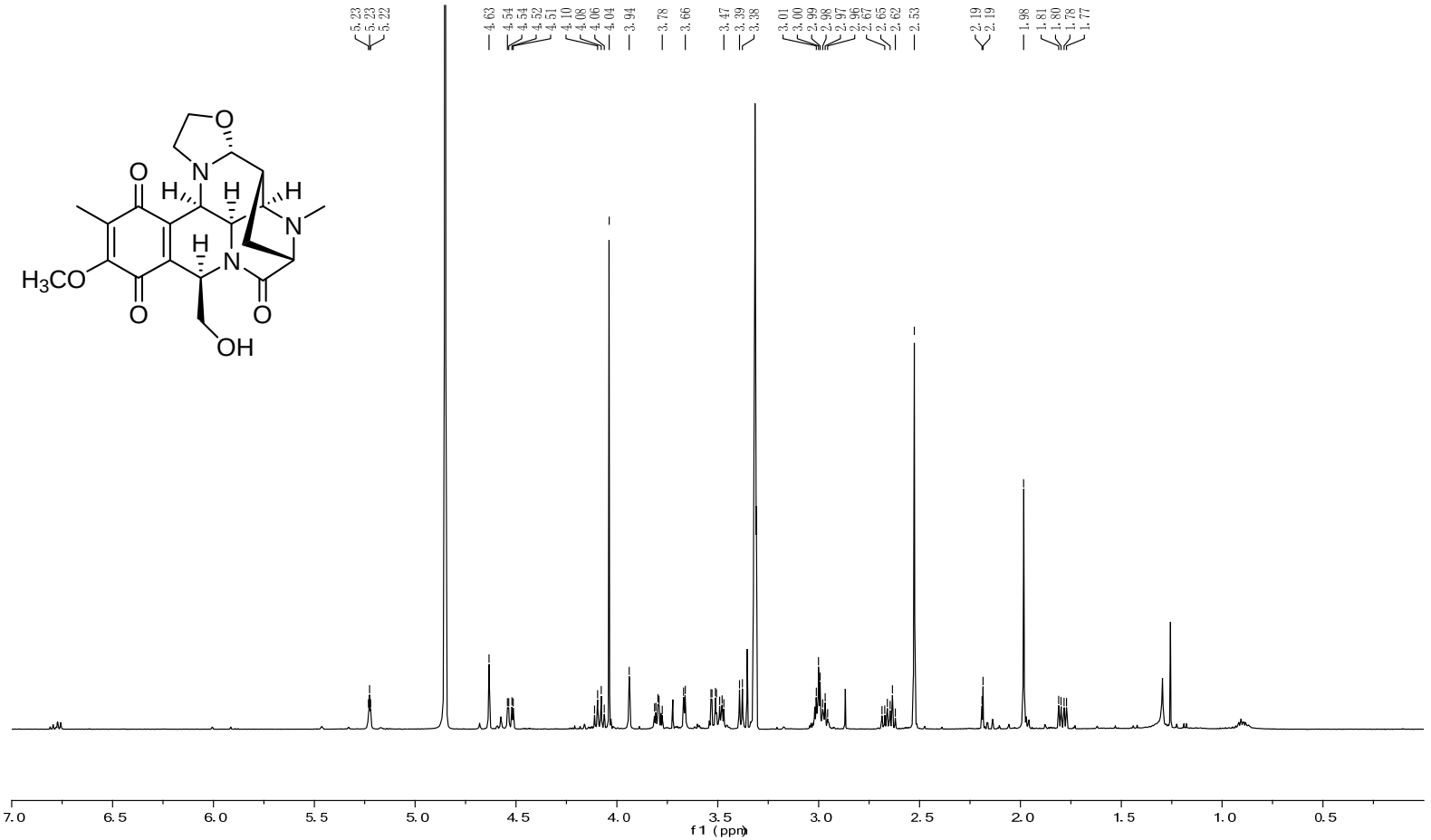
Figure S7. N-terminal sequence of NapU.



Purified 3μg of NapU was electrophoresis in 8% SDS-PAGE gels. The protein band was transferred from gels to polyvinylidene fluoride transfer membranes (PVDF, Millipore) in CAPS buffer (10 mM CAPS, 10 % ethanol, pH 11.0) subsequently. Then, the membrane with protein band was dried for sequence analysis after stained by Coomassie blue and washed several times in wash solution (40 % methanol, 1% acetic acid). The samples were sent to and N-terminal sequenced by Protein Sequencing Laboratory, School of Life Sciences, Peking University.

Figure S8. NMR spectra of compound **7**. (A) ^{1}H spectrum (B) ^{13}C spectrum (C) COSY spectrum (D) HMQC spectrum (E) HMBC spectrum.

A



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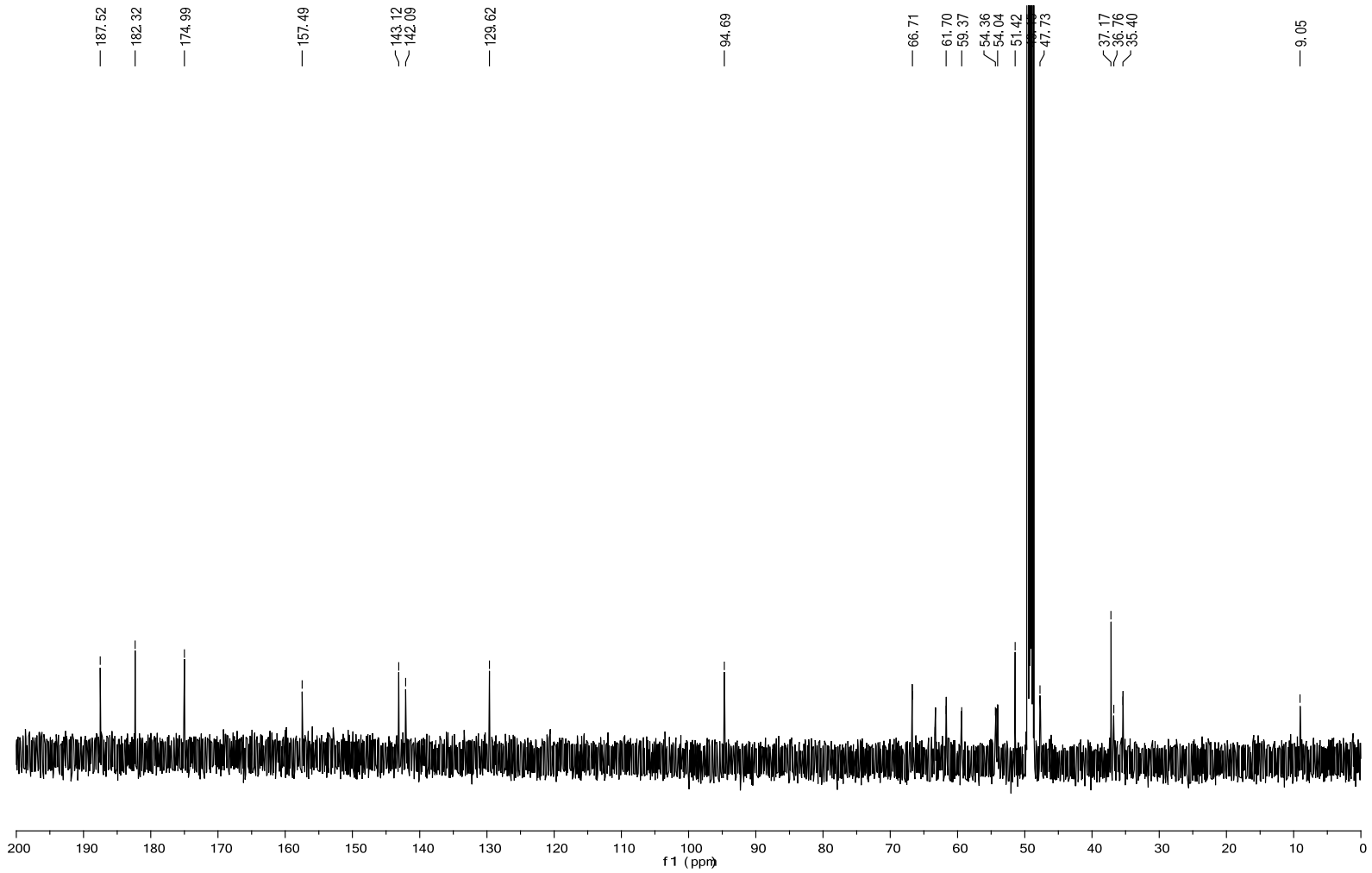
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51.42
47.73
37.17
36.76
35.40
9.05
200
190
180
170
160
150
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130
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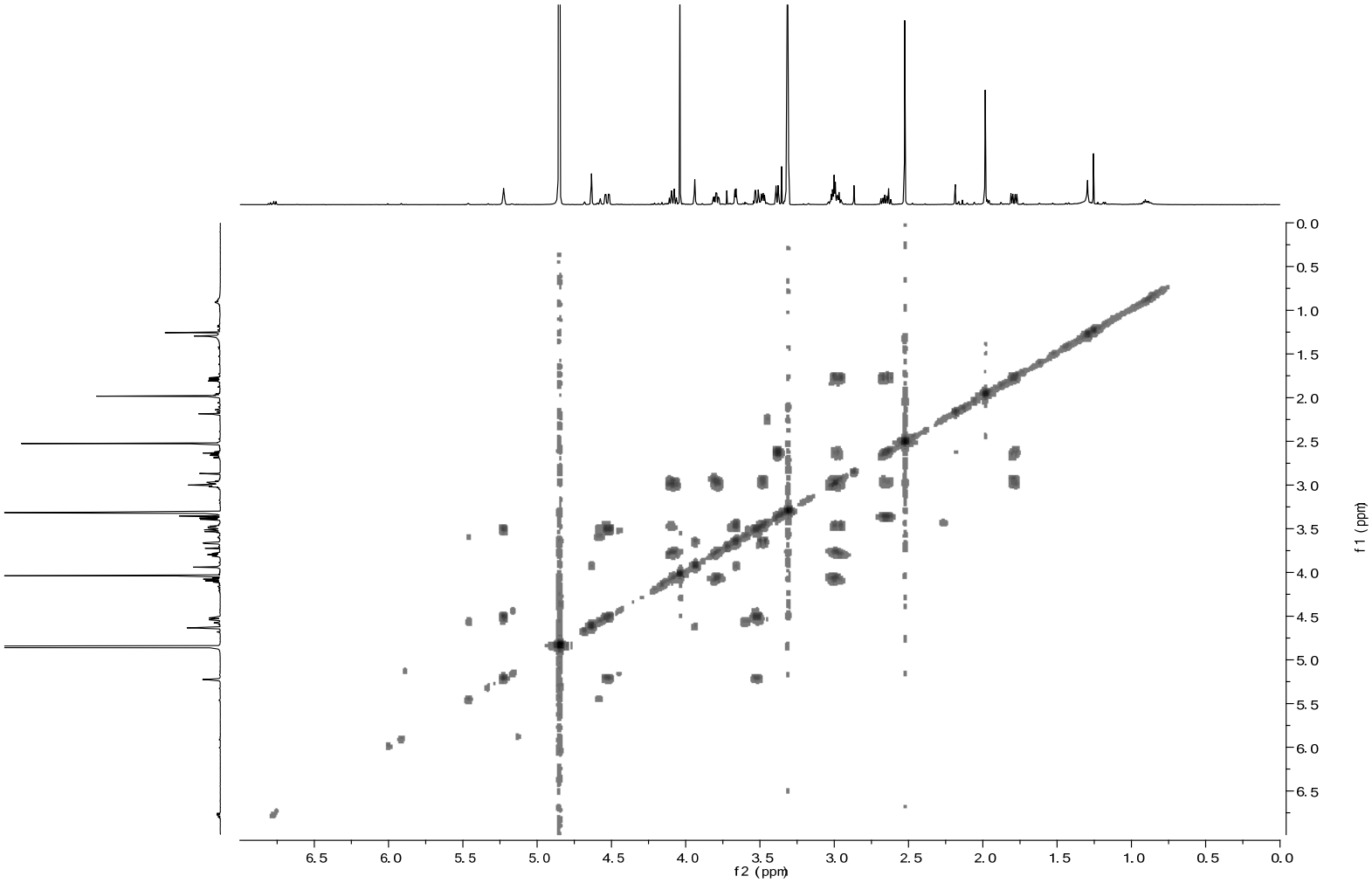
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

C



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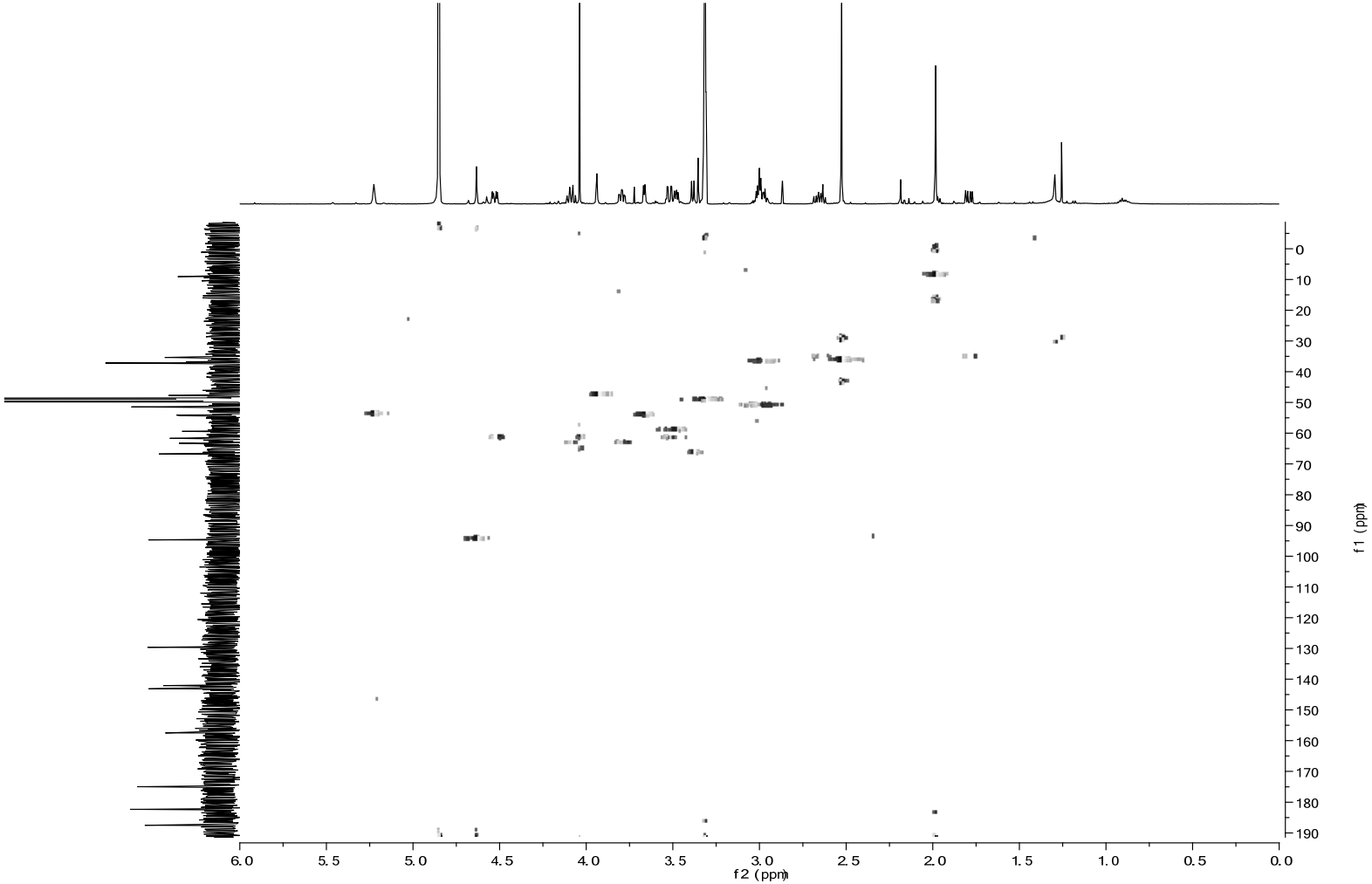
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5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f2 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
f1 (ppm)

E

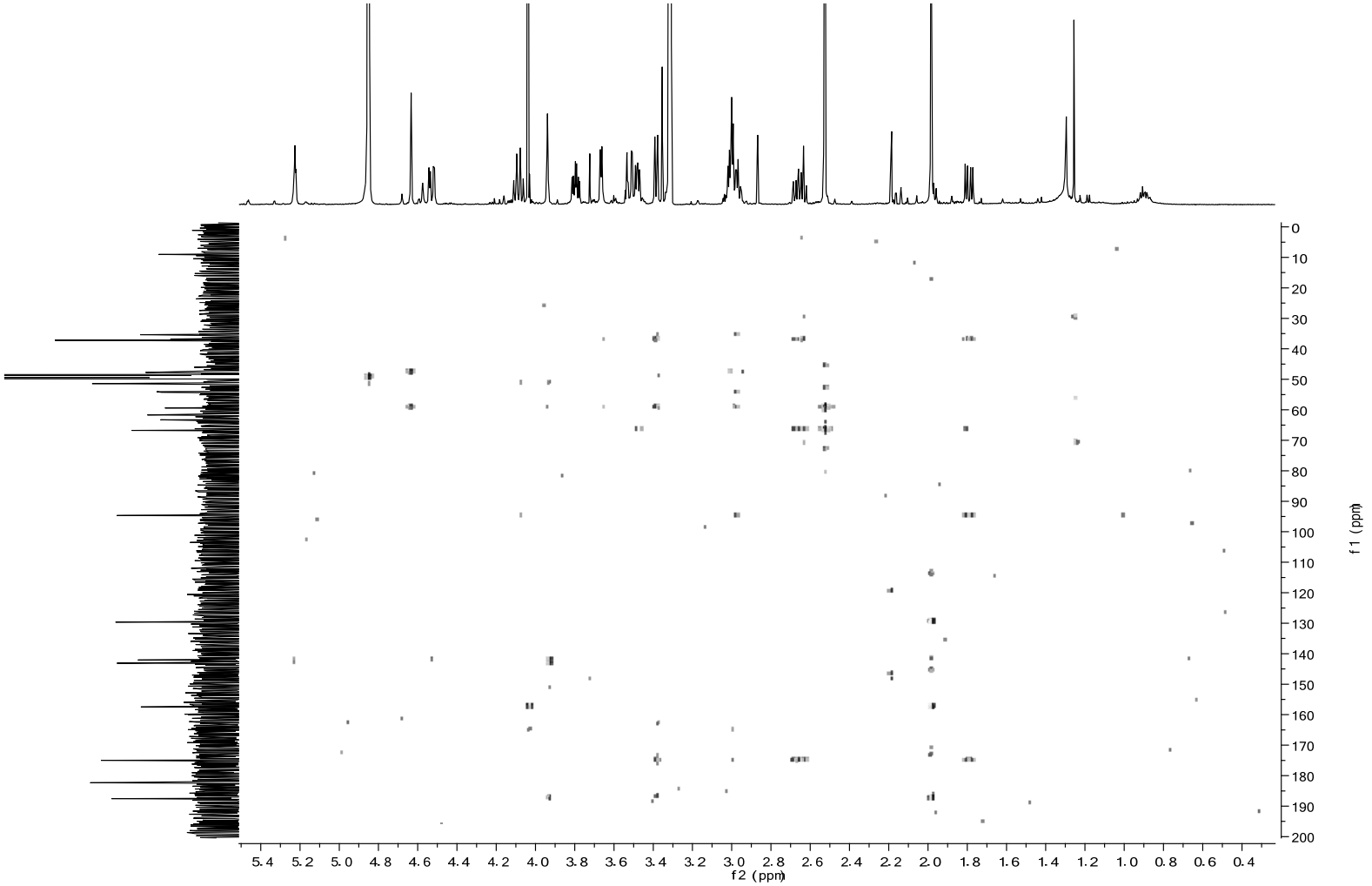

5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4
f2 (ppm)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
f1 (ppm)

Figure S9. The kinetic investigations of NapU.

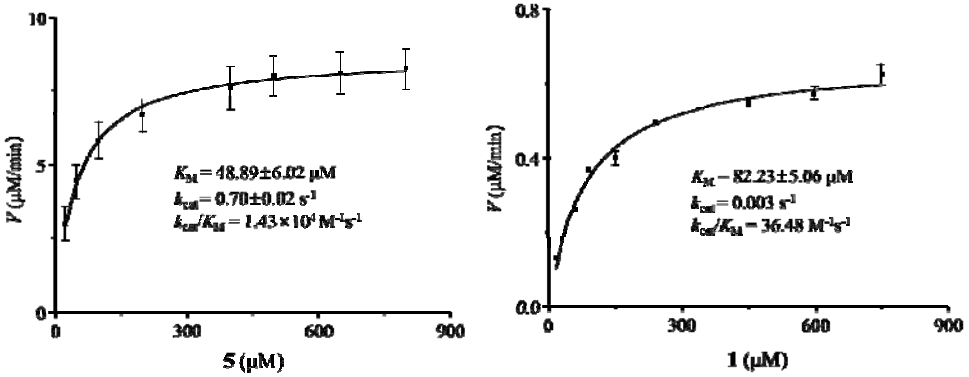
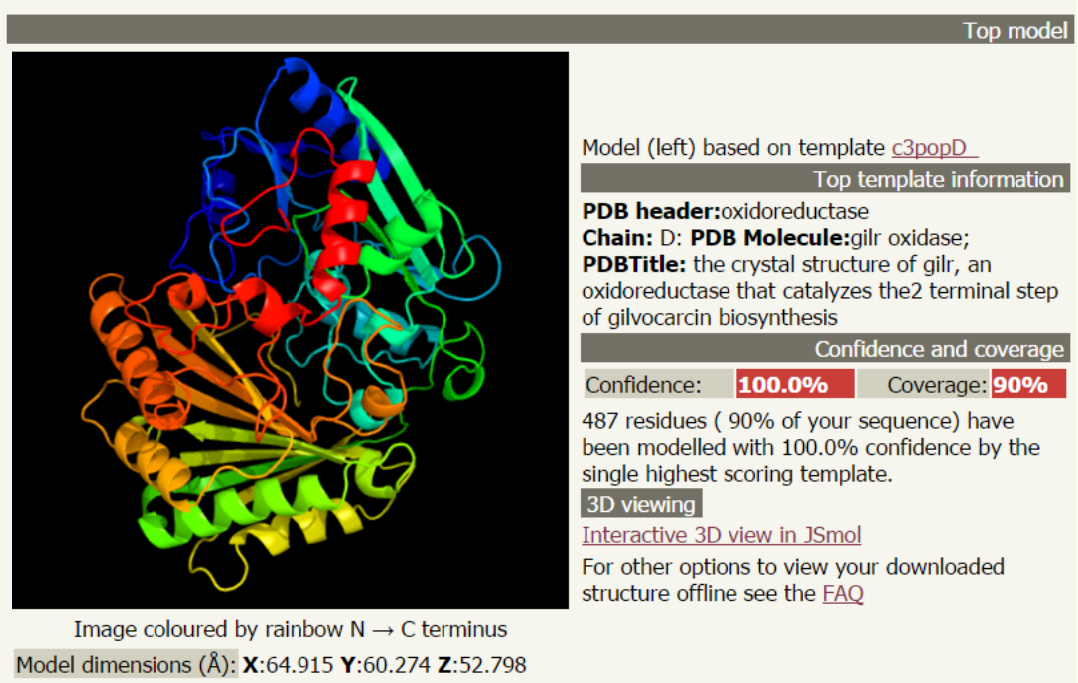
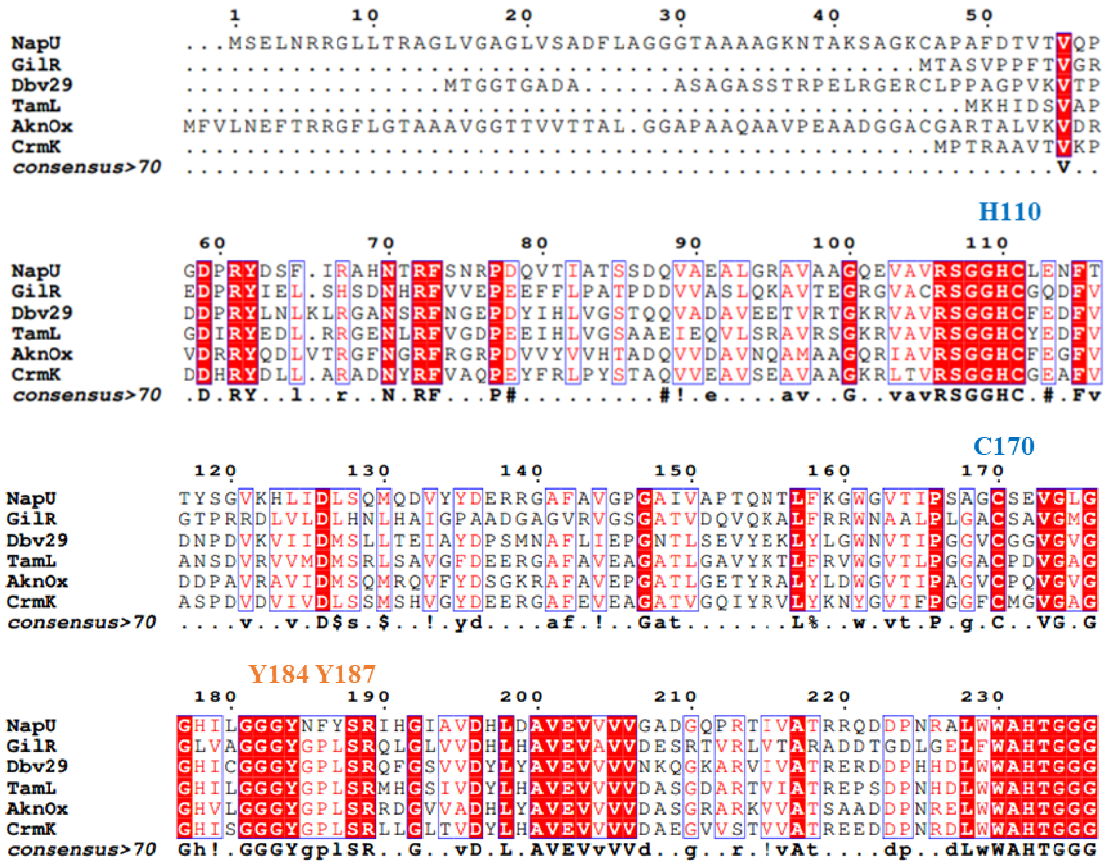


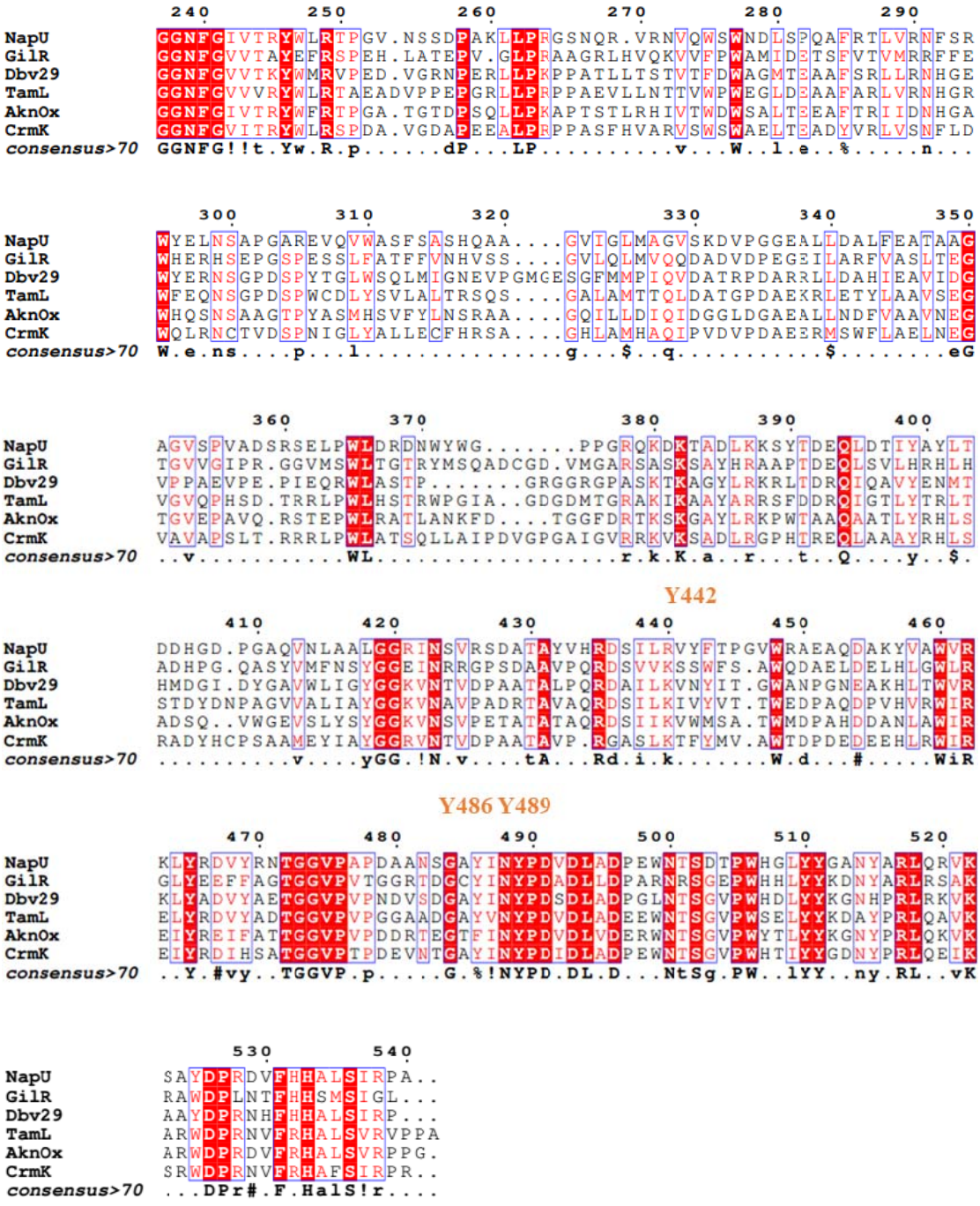
Figure S10. Proposed catalysis model of NapU.

A

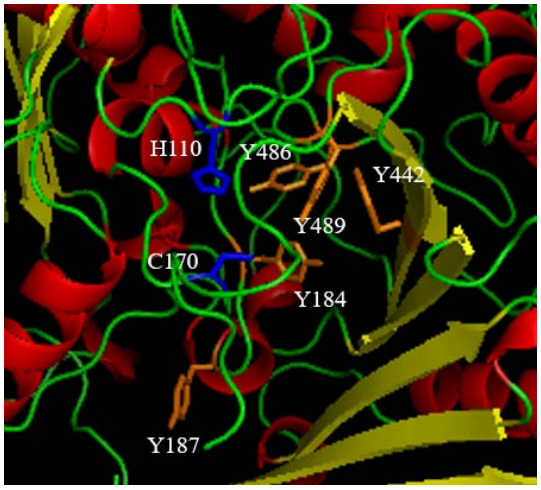


B

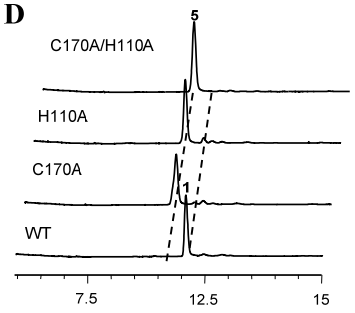



240 250 260 270 280 290
NapU GGNFGIVTRYWLRTPGV.NSSDPAKLLPRGSNQR.VRNVQWSWNDLSPQAFRTLVRNFSR
GilR GGNFGVVTAYEFRSPEH.LATEPV.GLPRAAGRLHVQKVVFPWAMIDETSFVTVMRRFFE
Dbv29 GGNFGVVTKYWMRVPED.VGRNPERLLPKPPATLLTSTVTFDWAGMTEAAFSRLLRNHGE
TamL GGNFGVVVRYWLRTAEADVPPEPGRLLPRPPAEVLLNTTVWPWEGLDEAAFARLVRNHGR
AknOx GGNFGIVTRYWFRTPGA.TGTDPSQLLPKAPTSTLRHIVTWDWSALTEEAFTRIIDNHGA
CrmK GGNFGVITRYWLRSPDA.VGDAPEEALPRPPASFHVARVSWSWAELTEADYVRLVSNFLD
consensus>70 GGNFG!!t.Yw.R.p.....dP...LP.........v...W..l.e..%.....n...
300 310 320 330 340 350
NapU WYELNSAPGARFVQVWASFSASHQAA....GVIGLMAGVSKDVPGGEALLDALFEATAAG
GilR WHERHSEPGSPESSLFATFFVNHVSS....GVLQLMVQQDADVDPEGEILARFVASLTEG
Dbv29 WYERNSGPDSPYTGLWSQLMIGNEVPGMGESGFMMPIQVDATRPDARRLLDAHIEAVIDG
TamL WFEQNSGPDSPWCDLYSVLALTRSQS....GALAMTTQLDATGPDAEKRLETYLAAVSEG
AknOx WHQSNSAAGTPYASMHSVFYLNSRAA....GQILLDIQIDGGLDGAEALLNDFVAAVNEG
CrmK WQLRNCTVDSPNIGLYALLECFHRSA....GHLAMHAQIPVDVPDAEERMSWFLAELNEG
consensus>70 W.e.ns....p...l.............g...$..q..........$........eG
360 370 380 390 400
NapU AGVSPVADSRSELPWLDRDNWYWG.......PPGRQKDKTADLKKSYTDEQLDTIYAYLT
GilR TGVVGIPR.GGVMSWLTGTRYMSQADCGD.VMGARSASKSAYHRAAPTDEQLSVLHRHLH
Dbv29 VPPAEVPE.PIEQRWLASTP.......GRGGRGPASKTKAGYLRKRLTDRQIQAVYENMT
TamL VGVQPHSD.TRRLPWLHSTRWPGIA..GDGDMTGRAKIKAAYARRSFDDRQIGTLYTRLT
AknOx TGVEPAVQ.RSTEPWLRATLANKFD....TGGFDRTKSKGAYLRKPWTAAQAATLYRHLS
CrmK VAVAPSLT.RRRLPWLATSQLLAIPDVGPGAIGVRRKVKSADLRGPHTREQLAAAYRHLS
consensus>70 ..v..........WL.................r.k.K.a..r...t..Q....y..$.
Y442
410 420 430 440 450 460
NapU DDHGD.PGAQVNLAALGGRINSVRSDATAAYVHRDSILRVYFTPGVWRAEAQDAKYVAWVR
GilR ADHPG.QASYVMFNSYGGEINRRGPSDAAVPQRDSVVKSSWFS.AWQDAELDELHLGWLR
Dbv29 HMDGI.DYGAVWLIGYGGKVNTVDPAATALPQRDAILKVNYIT.GWANPGNEAKHLTWVR
TamL STDYDNPAGVVALIAYGGKVNAVPADRTAVAQRDSILKIVYVT.TWEDPAQDPVHVRWIR
AknOx ADSQ..VWGEVSLYSYGGKVNSVPETATATAQRDSIIKVWMSA.TWMDPAHDDANLAWIR
CrmK RADYHCPSAAMEYIAYGGRVNTVDPAATAVP.RGASLKTFYMV.AWTDPDEDEEHLRWIR
consensus>70 ....v....yGG.!N.v....tA...Rd.i.k.......W.d...#.....WiR
Y486 Y489
470 480 490 500 510 520
NapU KLYRDVYRNTGGVPAPDAANSGAYINYPDVDLADPEWNTSDTPWHGLYYGANYARLQRVK
GilR GLYEEFFAGTGGVPVTGGRTDGCYINYPDADLLDPARNRSGEPWHHLYYKDNYARLRSAK
Dbv29 KLYADVYAETGGVPVPNDVSDGAYINYPDSDLADPGLNTSGVPWHDLYYKGNHPRLRKVK
TamL ELYRDVYADTGGVPVPGGAADGAYVNYPDVDLADEEWNTSGVPWSELYYKDAYPRLQAVK
AknOx EIYREIFATTGGVPVPDDRTEGTFINYPDVDLVDERWNTSGVPWYTLYYKGNYPRLQKVK
CrmK EIYRDIHSATGGVPTPDEVNTGAYINYPDIDLADPEWNTSGVPWHTIYYGDNYPRLQEIK
consensus>70 ..Y.#vy..TGGVP.p.....G.%!NYPD.DL.D...NtSg.PW..lYY..ny.RL..vK
530 540
NapU SAYDPRDVFHHALSIRPA..
GilR RAWDPLNTFHHSMSIGL...
Dbv29 AAYDPRNHFHHALSIRP...
TamL ARWDPRNVFRHALSVRVPPA
AknOx ARWDPRDVFRHALSVRPPG.
CrmK SRWDPRNVFRHAFSIRPR..
consensus>70 ...DPr#.F.HalS!r....

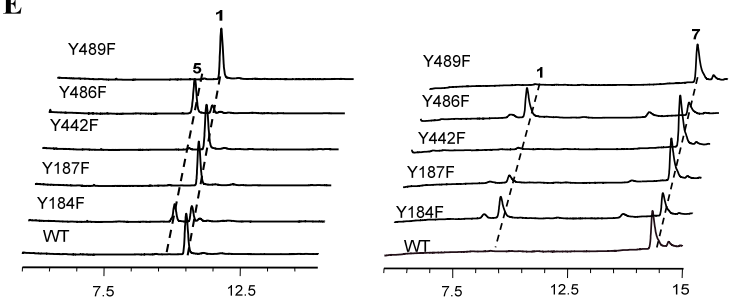
C



D



E



(A) Modeling analysis of the NapU by Phyre2 showed NapU is close to GilR. (B) Multiple sequence alignment of NapU and other homologous enzymes by Clustal Omega and colored byESPript3.0. H110 and C170 are conserved for FAD covalently binding. Y184 and Y489 are conserved for activating hydroxyl group. Other tyrosine residues are close to these conserved residues in catalyzed cavity of model and may be involved in catalysis. (C) The possible key residues are indicated in the model. H110 and C170 are shown in blue. The mentioned Tyrs are shown in orange. (D-E) Comparison of activities performed by NapU mutants and wild type.