**Supporting Information**

# Heterologous production of asperipin-2a: Proposal for sequential oxidative macrocyclizations by a fungi-specific DUF3328 oxidase.

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**General**

All reagents commercially supplied were used as received. Optical rotations were recorded on JASCO P-2200 digital polarimeter. $^{1}$H- and $^{13}$C-NMR spectra were recorded on Bruker AMX-500 spectrometer. NMR spectra were recorded in dimethyl Sulfoxide-$d_6$ (99.9%D with 0.03%TMS, kanto chemical). $^{1}$H chemical shifts were reported in δ value relative to DMSO: $\delta_H$ 2.50 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (Hz), and integration. Column chromatography was carried out on C18 open column (Wakosil, 30~50 μm). HPLC-MS analyses were conducted with QDa detector equipped with Acquity UPLC system (Waters). High resolution mass spectra were obtained with a Thermo Scientific Exactive (Thermo Fisher). Oligonucleotides for polymerase chain reactions (PCRs) were purchased from Hokkaido System Science Co., Ltd. PCRs were performed with a BioRad S1000 thermal cycler.

**Strains**

*Escherichia coli* HST08 was used for cloning and following standard recombinant DNA techniques. A fungal host strain used in this study was *Aspergillus oryzae* NSAR1, a quadruple auxotrophic mutant (*niaD*$^{-}$, *sC*$^{-}$, *ΔargB*, *adeA*$^{-}$)[1], for fungal expression. *Aspergillus flavus* CA14 was used for genomic DNA extraction.

**Preparation of expression plasmids**

The *aprA*, *aprY*, *aprR*, and *aprT* were amplified from the genomic DNA of *A. flavus* CA14 with the primer set shown in Table S1. The two mutants *aprA*/Y3F and *aprA*/Y6F were amplified from pUC57-Km-*aprA*/Y3F and pUC57-Km-*aprA*/Y6F that are synthesized by GENEWIZ, respectively (see also page S17 for each sequence). Expression plasmids were constructed as follows. Gene *aprA*, *aprA*/Y3F, and *aprA*/Y6F were inserted into the NheI site of pUSA2[1] using an In-Fusion® HD Cloning Kit (Clontech Laboratories) to construct pUSA2-*aprA*. Similary, gene *aprY* was cloned into the KpnI site of pUARA2[1] to five pUARA2-*aprY*. Genes aprR and aprT were cloned NheI and/or KpnI sites of pAdeA2[2] to construct three plasmids, pAdeA2-*aprR*, pAdeA2-*aprT*, and pAdeA2-*aprRT*. These procedures are summarized in Figure S1.

**Transformation of *A. oryzae*.**

Transformation of *A. oryzae* was performed by the protoplast-polyethylene glycol method reported previlusly[3] to construct the following transformants; AO-*aprYRT*, AO-*aprAYR*, AO-*aprART*, AO-*aprAYRT*, AO-*bscAYRT*/Y3F, and AO-*aprAYRT*/Y6F,. Plasmids used for the construction of each transformant are summarized in Table S2.

**Extraction of the metabolites from each transformant**

Mycelia of *A. oryzae* transformants were inoculated into 20 mL of potato-starch medium (potato infusion from 200 g and 20 g soluble starch for 1 L medium). Each culture was incubated at 30°C, 200 rpm for 3 days. After the fermentation, broth was extracted by 80 mL acetone. After centrifugation, the supernatants were concentrated to give the crude extract. 5-μl Aliquots of each extract were analyzed by LC-MS with Acquity UPLC BEH C18 column (ϕ2.1 x 50 mm, 1.7 μm) at the following

conditions; $CH_3CN$ and $H_2O$ (each contained 0.1% HCOOH) were used as eluents. The concentration of $CH_3CN$ was linearly increased from 5 to 50% over 2 min, 50 to 95% over 1 min. Flow rate was kept at 0.7 ml $min^{-1}$. The column temperature was kept at 40˚C. Metabolites were analyzed in ESI positive mode.

### Isolation of 1 from *A. oryzae* transformant AO-*aprA YRT*

Spore suspension of AO-*aprA YRT* was inoculated into 100 mL of potato-maltose medium (potato infusion from 200g and 30 g maltose for 1L medium) in 500 mL Erlenmeyer flasks. Each culture was incubated at 30˚C for 6 days. After filtration, broth was collected and was washed with AcOEt, and then extracted by *n*-butanol. Organic layer was subjected to C18 column chromatography (eluted by $CH_3CN/H_2O$ = 0.1–0.3). The fraction containing asperipin-2a was separated by HPLC (Shimazu Class VP system) equipped with Wakopak Navi C18-5 column (ϕ10 x 250 mm, 5 μm). MeOH and $H_2O$ (each contained 0.1% RFA) were used as eluents. The concentration of MeOH was linearly increased from 25% to 75% over 10min. Flow rate was kept at 3 mL $min^{-1}$. After several injections, the fractions containing the pure compound was collected (1.0 mg from 400-mL culture)..

### Asperipin-2a (1)

HR-ESIMS analysis (negative); calcd. for $C_{42}H_{42}O_{12}N_5$ [M-H]-: 808.2835, found: 808.2867. The NMR data are shown in page S19–S24. $[\alpha]_D^{26}$: +1162.5 (c = 0.1, DMSO). Solubility of the isolated compound in MeOH was low and we could not prepare solution at the concentration indicated in the literature ($[\alpha]_D^{23}$: +8.3 (c = 0.06, MeOH)[5]). Therefore, we chose DMSO as alternative solvent. The cause of this difference is obscure and is under investigation.

### Hydrogenolysis and hydrolysis of 1.

A mixture of 10% Pd/C (1.7 mg), ammonium formate (0.5 g, 7.9 mmol) and **1** (0.34 mg, 0.42 μmol) in MeOH (1 ml) was stirred at 70˚C until reaction was completed.[4] The mixture was filtrated through Celite. The filtrate was purified by C18 column chromatography and HPLC equipped with Wakopak Navi C18-5 (ϕ10 x 250 mm) at the conditions (a linear gradient of MeOH from 25–40% over 25min at a flow rate of 3.0 mL $min^{-1}$). To solution of the linear peptide (3% MeOH, 500 μl) was added 12 M HCl solution (500 μl) and stirred at 110˚C for 13 h.

### Chiral separation of (*S*)- and (*R*)-3-phenyllactic acid

The resulting 3-phenyllactic acid was analyzed by LC-MS equipped with chiral column (CHIRALPAK®ZWIX(+), ϕ4 x 250 mm, 3 μm) at the conditions (isocratic elution by 10 mM $HCO_2H$ and 10 mM $HCO_2NH_4$ in $MeOH/H_2O$ (98/2, v/v) at a flow rate of 0.8 ml $min^{-1}$). Commercial *S*- and *R*-3-phenyllactic acid were used as references. An ACQUITY QDa MS detector was operated in ESI-negative mode.

### Derivatization of the resultant amino acids with Marfey's reagent.

To aqueous solution of hydrolysate of **2** (50 μl) was added 20 μl of 1 M aq. $NaHCO_3$ followed by 100 μl of 1% $N^{\alpha}$-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA, advanced Marfey's reagent) in acetone.[6–8] The solution was stirred at 37˚C for 1 h. reaction was quenched by addition of 20 μl of

1M HCl. 1 μl of the resulting solution was diluted with 99 μl of MeOH and aliquot (2 μl) was analyzed by LC-MS. Authentic sample of L- and D-FDLA derivatives of amino acids were prepared under the same conditions. LC-MS was performed using ACQUITY$^{TM}$ UPLC BEH C18 column (50 mm ×2.1 mm, 1.7 μm particle size) at the following conditions: Flow rate; 0. 7 mL/min, Solvent system: A linear gradient from 5 to 50% of $CH_3CN$ with 0.1% TFA in water with 0.1% TFA over 2 min, 50% of $CH_3CN$ with 0.1% HCOOH in water with 0.1% HCOOH for 1 min. An ACQUITY QDa MS detector was operated in ESI-positive mode.

**Supplementary references**

(1) Tagami, K.; Minami, A.; Fujii, R.; Liu, C.; Tanaka, M.; Gomi, K.; Dairi, T.; Oikawa, H. *ChemBioChem* **2014**, *15*,2076–2080.

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**Table S1.** Oligonucleotides used for construction of plasmids. Homology arms for In-Fusion HD cloning are underlined.

| amplicon | sequence (from 5' to 3') | size vector |
|---|---|---|
| *aprA* | ATCGATTTGAGCTAGATGCATCTCTCGCGCTAC | bp |
| | TAGTGCGGCCGCTAGTTACTTGTCAAGATCCTCGG | pUSA2 |
| *aprY* | CGGAATTCGAGCTCGATGGAGCCCTTCTCTAAATT | bp |
| | ACTACAAGATCCCCGGCTATTCGTGCCTTTTCGTAT | pUARA2 |
| *aprR* | ATCGATTTGAGCTAGATGACTATCAAAGTGATTGTTG | bp |
| | TAGTGCGGCCGCTAGTCAACCTTTCAGAATACTAGC | pAdeA2 |
| *aprT* | CGGAATTCGAGCTCGATGGCTTCCCCTGAACTG | bp |
| | ACTACAGATCCCCGGTCAATGCGTGCTACCACTG | pAdeA2 |

**Table S2.** Summary of the transformants constructed in this study.

| **Transformants** | **Plasmids** (selection markers) | | |
|---|---|---|---|
| | (*argB*) | (*adeA*) | (*sC*) |
| AO-*aprYRT* | pUARA2-*aprY* | pAdeA2-*aprRT* | - |
| AO-*aprAYRT* | pUARA2-*aprY* | pAdeA2-*aprRT* | pUSA2-*aprA* |
| AO-*aprAYT* | pUARA2-*aprY* | pAdeA2-*aprT* | pUSA2-*aprA* |
| AO-*aprAYR* | pUARA2-*aprY* | pAdeA2-*aprR* | pUSA2-*aprA* |
| AO-*aprAYRT*//Y3F | pUARA2-*aprY* | pAdeA2-*aprRT* | pUSA2-*aprA*/Y3F |
| AO-*aprAYRT*/Y6F | pUARA2-*aprY* | pAdeA2-*aprRT* | pUSA2-*aprA*/Y6F |

**Table S3.** $^{1}$H- and $^{13}$C-chemical shifts of **1** (DMSO-$d_6$, 500 MHz).

| | measured in this study | | reported data[5] | |
|---|---|---|---|---|
| **No.** | $\delta_C$ | $\delta_H$ **(multiplicity, *J* in Hz)** | $\delta_C$ | $\delta_H$ **(multiplicity, *J* in Hz)** |
| **Phe1** | | | | |
| 1 | 74.5 | 3.74 (br. s) | 74.6 | 3.73 |
| 2 | 78.4 | 5.96 (br. s) | 78.6 | 5.93 (br. s) |
| 3 | 140.7 | | 140.9 | |
| 4, 4' | 126.8 | 7.6 (d, 7.5) | 127 | 7.59 (d, 7.5) |
| 5, 5' | 128.08 | 7.41 (t, 7.5) | 128.2 | 7.39 (t, 7.5) |
| 6 | 127.55 | 7.33 (t, 7.5) | 127.7 | 7.31 (t, 7.5) |
| 7 | 170.3 | | 170.5 | |
| **Tyr2** | | | | |
| 8 | 52.44 | 4.3 (m) | 52.6 | 4.30, (ddd, 15.0, 9.5, 7.6) |
| 8-NH | | 6.58 (d, 9.5) | | 6.55 (d, 9.5) |
| 9 | 38.76 | 2.51 (m)<br>2.31 (m) | 38.8 | 2.47 (m)<br>2.29 (dd, 13.8, 7.6) |
| 10 | 126.9 | | 127.1 | |
| 11, 11' | 130.02 | 6.83 (d, 8.5) | 130.2 | 6.80 (d, 7.2) |
| 12, 12' | 114.66 | 6.52(d, 7.5) | 114.9 | 6.50 (d, 7.2) |
| 13 | 155.68 | | 156.4 | |
| 14 | 169.79 | | 169.9 | |
| **Tyr3** | | | | |
| 15 | 58.02 | 5.01 (dd, 9.1, 9.4)) | 58.5 | 4.95 (dd, 10.2, 8.4) |
| 15-NH | | 8.22 (d, 10.5) | | 8.18 (d, 10.2) |
| 16 | 80.07 | 5.30 (d, 8.5) | 80.9 | 5.23 (d, 8.4) |
| 17 | 131.68 | | 132.2 | |
| 18 | 129.73 | 7.10 (d, 8.5) | 129.8 | 7.10, (d, 7.8) |
| 19 | 116.37 | 7.03 (m) | 116.6 | 7.01 (d, 7.8) |
| 20 | 155.69 | | 156.61 | |
| 21 | 120.64 | 7.05 (m) | 120.8 | 7.02 |
| 22 | 128.06 | 7.41 (m) | 127.7 | 7.41 (d, 7.8) |
| 23 | 169.37 | | 169.3 | |
| **Thr4** | | | | |
| 24 | 58.05 | 4.11 (d, 8.0) | 57.9 | 4.13 (d, 7.0) |
| 24-NH | | 8.08 (d, 7.5) | | 8.01 (d, 7.0) |
| 25 | 66.86 | 3.85 (m) | 67.3 | 3.83 (m) |
| 26 | 19.03 | 0.90 (d, 6.0) | 19.6 | 0.89 (d, 6.0) |
| 27 | 168.44 | | 168.4 | |
| **Gly5** | | | | |
| 28 | 42.91 | 3.67 (dd, 16.0, 6.7)<br>3.42 (dd, 17.1, 4.4) | 42.9 | 3.72<br>3.3 |

| | | | | |
|---|---|---|---|---|
| 28-NH | | 7.51 (m) | | 8.00 d (d, 7.8) |
| 29 | 168.42 | | 168.8 | |
| **Tyr6** | | | | |
| 30 | 52.42 | 4.39 (m) | 54 | 4.02 (m) |
| 30 NH | | 7.01 (m) | | 6.85 (d, 6.5) |
| 31 | 35.5 | 3.15 (dd, 13.2, 2.8)<br>2.67 (12.8, 11.7) | 36.2 | 2.97 (dd, 14.0, 6.4)<br>2.90 (dd, 14.0, 10.2) |
| 32 | 129.84 | | 130.8 | |
| 33, 33' | 130.02 | 6.82 (m) | 130.2 | 6.78, d (d, 7.2) |
| 34, 34' | 130.02 | 6.82 (m) | 129.9 | 6.75 d (d, 7.2) |
| 35 | 155.62 | | 156.4 | |
| 36 | 172.88 | | 173.3 | |

The differences observed for Gly5 and Tyr6 is likely due to the conformational flexibility of the 17-membered ring.

**Table S3.** Summary of BLAST results using AprA as a query.

| strain | Accession number |
|---|---|
| *Aspergillus oryzae* | OOO06787 |
| *Aspergillus parasiticus* SU-1 | KJK61069 |
| *Aspergillus arachidicola* | PIG85352 |

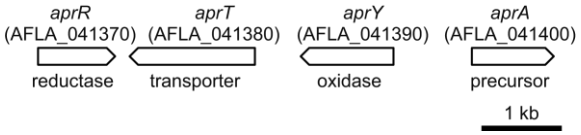


**Figure S1.** The biosynthetic gene cluster for **1**.

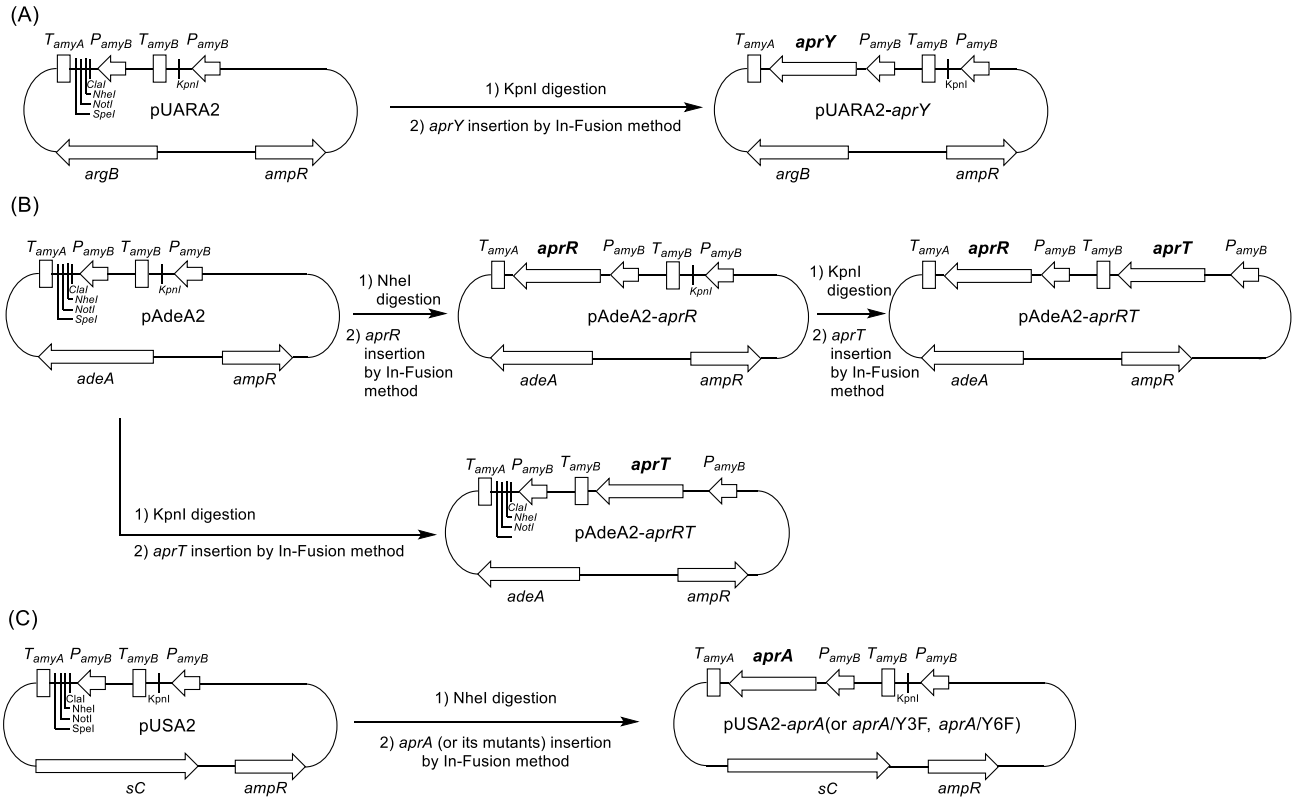


**Figure S2.** Construction of expression plasmids (A) pUARA2-*aprY*, (B) pAdeA2-*aprR*, pAdeA2-*aprRT*, and (C) pUSA2-*aprA*, pUSA2-*aprA*/Y3F, and pUSA2-*aprA*/Y6F.

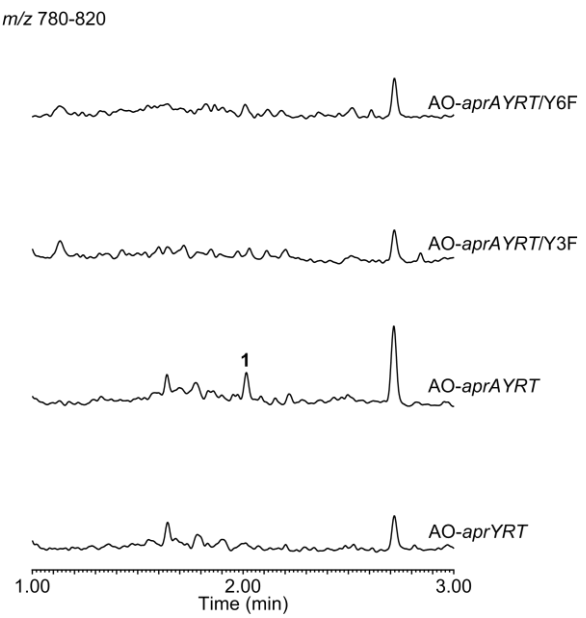


**Figure S3.** LC-MS profiles of the metabolites extracted from AO-*aprAYRT*/Y3F, AO-*aprAYRT*/Y6F. The chromatograms were extracted at *m/z* 780–820.

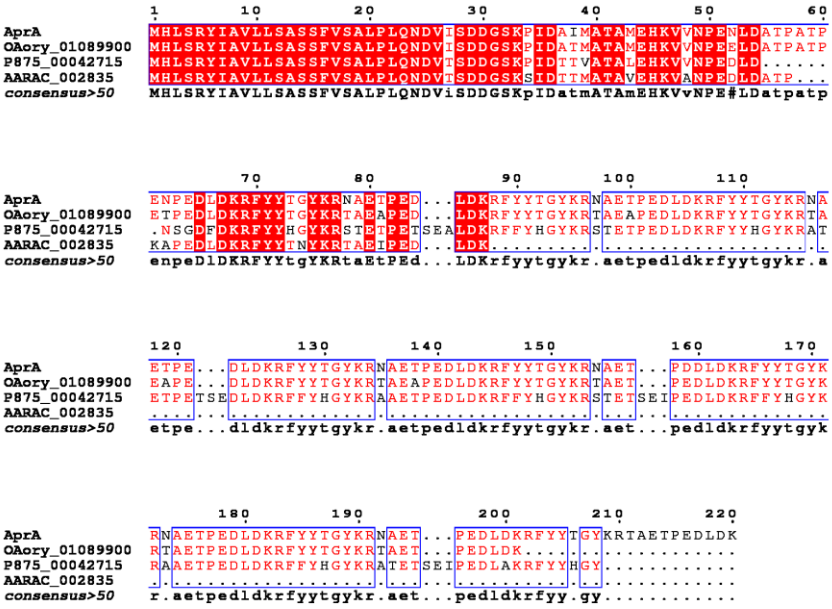


**Figure S4.** Sequence alignment of putative AprA orthologs.
The sequences were aligned by MultAlin[9] and the figure was exported by ESPript 3[10].

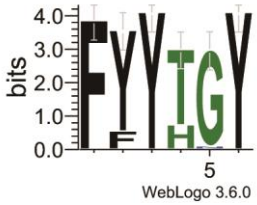


**Figure S5.** Conservation analysis of the repeated sequence of putative AprA orthologs. The logo was constructed by WebLogo 3[11,12] with sequences listed in Figure S3.

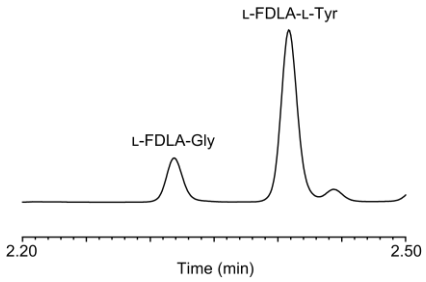


**Figure S6.** HPLC profile of L-FDLA-derivatized sample.
Chromatogram was extracted at 340 nm. The molar ratio of L-Tyr and Gly was approximately estimated to 3:1.

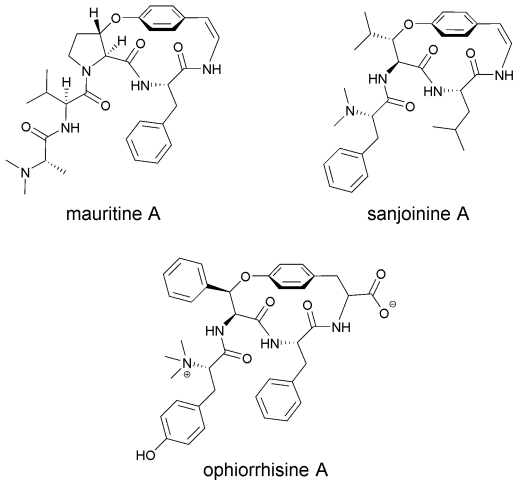


**Figure S7.** Cyclopeptide alkaloids that are structurally-related to **1**.

The nucleotide and corresponding amino acid sequences of revised *aprA*, *aprA*/Y3F and *aprA*/Y6F. Core sequences were shown by bold letters and mutated residues were colored red.

>Revised *aprA*

ATGCATCTCTCGCGCTACATCGCTGTACTGTTGTCGGCATCGAGCTTTGTCTCTGCTCTTC
CACTTCAAAATGATGTGATCTCCGACGACGGATCTAAGCCCATAGATGCGATAATGGCCA
CCGCAATGGAGCACAAGGTAGTTAACCCGGAGAACCTTGATGCGACCCCTGCAACCCCT
GAGAACCCCGAGGATCTTGACAAACGATTCTACTATACCGGCTATAAACGTAACGCTGAG
ACCCCTGAGGATCTTGACAAACGATTCTACTATACCGGCTATAAACGCAACGCTGAGACC
CCCGAGGATCTTGATAAACGATTCTACTATACCGGCTATAAACGTAACGCTGAGACCCCC
GAGGATCTTGACAAACGATTCTACTATACCGGCTATAAACGCAACGCTGAGACCCCTGAG
GATCTTGACAAACGATTCTACTATACCGGCTATAAACGCAATGCTGAGACCCCTGATGATC
TTGACAAACGATTTTACTATACCGGCTATAAACGCAATGCTGAGACCCCTGAGGATCTTG
ACAAACGATTCTACTATACCGGCTATAAACGCAATGCTGAGACCCCTGAGGATCTTGACA
AACGATTCTACTATACCGGCTATAAACGCACCGCTGAGACCCCCGAGGATCTTGACAAGT
AA

>Revised AprA

MHLSRYIAVLLSASSFVSALPLQNDVISDDGSKPIDAIMATAMEHKVVNPENLDATPATPENP
EDLDKR**FYYTGY**KRNAETPEDLDKR**FYYTGY**KRNAETPEDLDKR**FYYTGY**KRNAETPED
LDKR**FYYTGY**KRNAETPEDLDKR**FYYTGY**KRNAETPDDLDKR**FYYTGY**KRNAETPEDLD
KR**FYYTGY**KRNAETPEDLDKR**FYYTGY**KRTAETPEDLDK

>*aprA*/Y3F

ATGCATCTCTCGCGCTACATCGCTGTACTGTTGTCGGCATCGAGCTTTGTCTCTGCTCTTC
CACTTCAAAATGATGTGATCTCCGACGACGGATCTAAGCCCATAGATGCGATAATGGCCA
CCGCAATGGAGCACAAGGTAGTTAACCCGGAGAACCTTGATGCGACCCCTGCAACCCCT
GAGAACCCCGAGGATCTTGACAAACGATTCTACTTCACCGGCTATAAACGTAACGCTGAG
ACCCCTGAGGATCTTGACAAACGATTCTACTTCACCGGCTATAAACGCAACGCTGAGACC
CCCGAGGATCTTGATAAACGATTCTACTTCACCGGCTATAAACGTAACGCTGAGACCCCC
GAGGATCTTGACAAACGATTCTACTTCACCGGCTATAAACGCAACGCTGAGACCCCTGA
GGATCTTGACAAACGATTCTACTTCACCGGCTATAAACGCAATGCTGAGACCCCTGATGA
TCTTGACAAACGATTTTACTTCACCGGCTATAAACGCAATGCTGAGACCCCTGAGGATCT
TGACAAACGATTCTACTTCACCGGCTATAAACGCAATGCTGAGACCCCTGAGGATCTTGA
CAAACGATTCTACTTTACCGGCTATAAACGCACCGCTGAGACCCCCGAGGATCTTGACAA
GTAA

>AprA/Y3F

MHLSRYIAVLLSASSFVSALPLQNDVISDDGSKPIDAIMATAMEHKVVNPENLDATPATPENP
EDLDKR**FY<u>F</u>TGY**KRNAETPEDLDKR**FY<u>F</u>TGY**KRNAETPEDLDKR**FY<u>F</u>TGY**KRNAETPEDL
DKR**FY<u>F</u>TGY**KRNAETPEDLDKR**FY<u>F</u>TGY**KRNAETPDDLDKR**FY<u>F</u>TGY**KRNAETPEDLDK
R**FY<u>F</u>TGY**KRNAETPEDLDKR**FY<u>F</u>TGY**KRTAETPEDLDK

>*aprA*/Y6F

ATGCATCTCTCGCGCTACATCGCTGTACTGTTGTCGGCATCGAGCTTTGTCTCTGCTCTTC
CACTTCAAAATGATGTGATCTCCGACGACGGATCTAAGCCCATAGATGCGATAATGGCCA
CCGCAATGGAGCACAAGGTAGTTAACCCGGAGAACCTTGATGCGACCCCTGCAACCCCT
GAGAACCCCGAGGATCTTGACAAACGATTCTACTATACCGGCTTCAAACGTAACGCTGAG
ACCCCTGAGGATCTTGACAAACGATTCTACTATACCGGCTTCAAACGCAACGCTGAGACC
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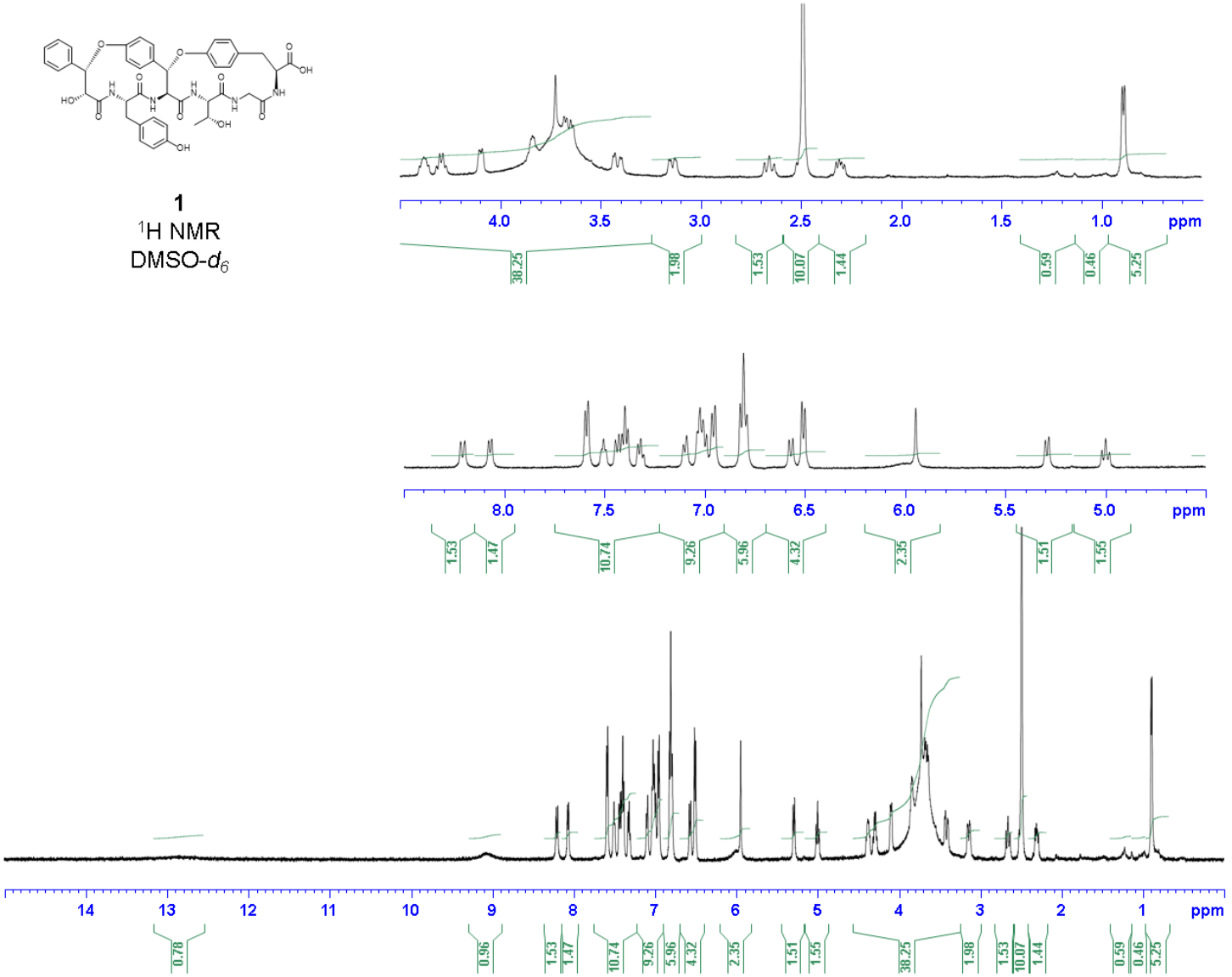
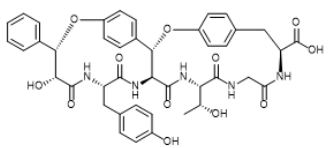
TGACAAACGATTCTACTATACCGGCTTCAAACGCAATGCTGAGACCCCTGAGGATCTTGA
CAAACGATTCTACTATACCGGCTTCAAACGCACCGCTGAGACCCCCGAGGATCTTGACAA
GTAA

>AprA/Y6F

MHLSRYIAVLLSASSFVSALPLQNDVISDDGSKPIDAIMATAMEHKVVNPENLDATPATPENP
EDLDKR**FYYTGF**KRNAETPEDLDKR**FYYTGF**KRNAETPEDLDKR**FYYTGF**KRNAETPEDL
DKR**FYYTGF**KRNAETPEDLDKR**FYYTGF**KRNAETPDDLDKR**FYYTGF**KRNAETPEDLDK
R**FYYTGF**KRNAETPEDLDKR**FYYTGF**KRTAETPEDLDK



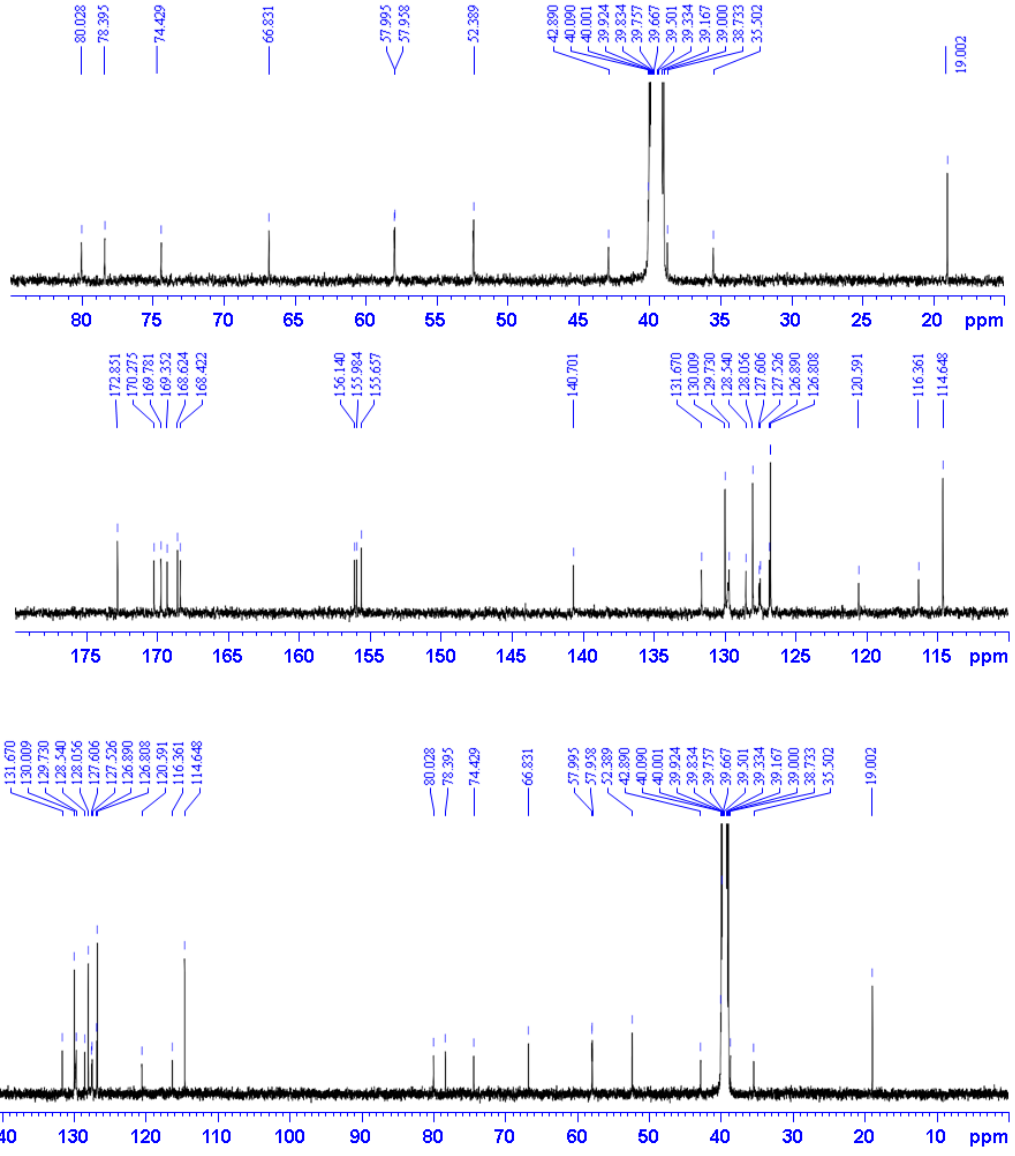
**1**
$^{1}$H NMR
DMSO-$d_6$

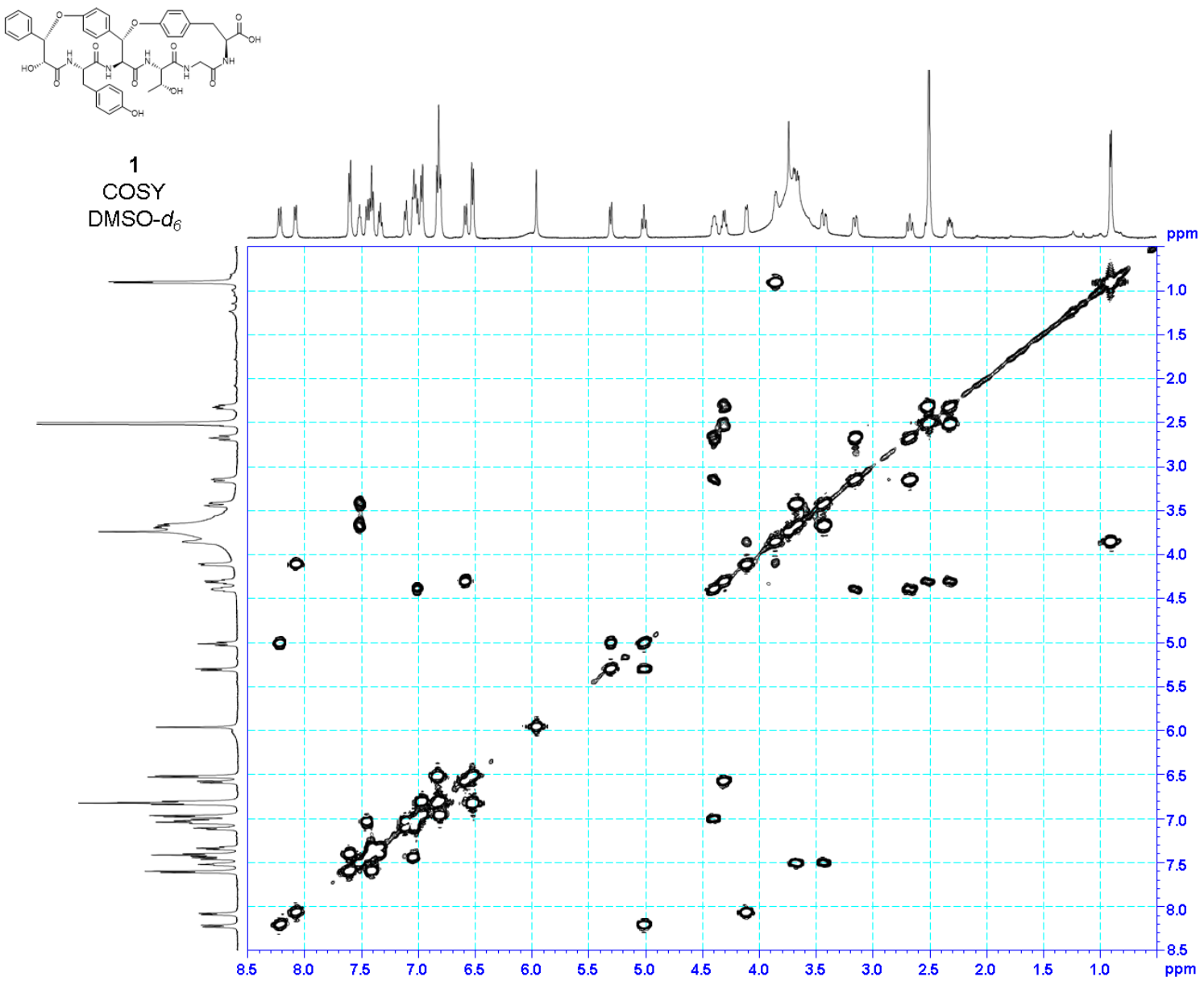


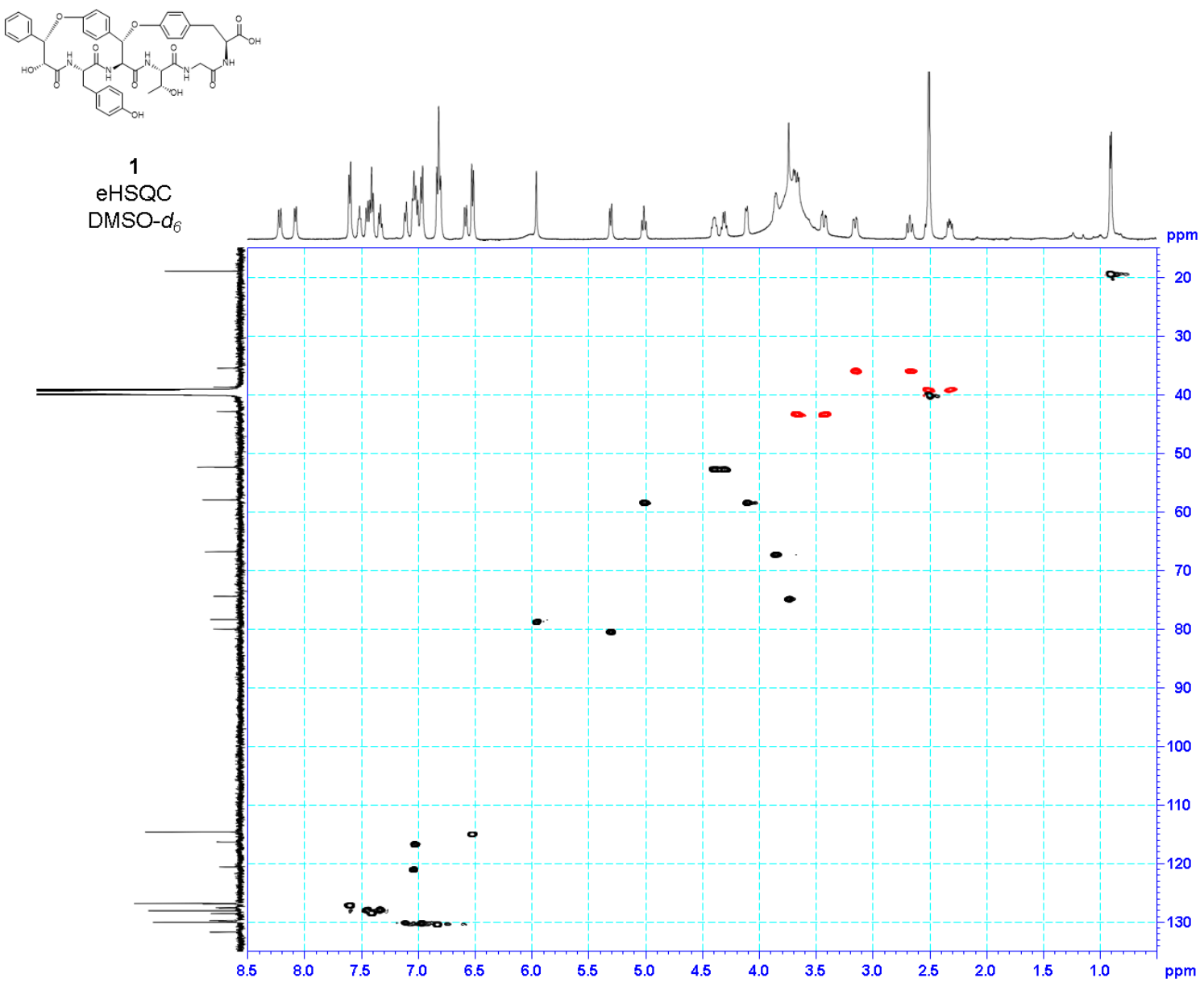
**1**

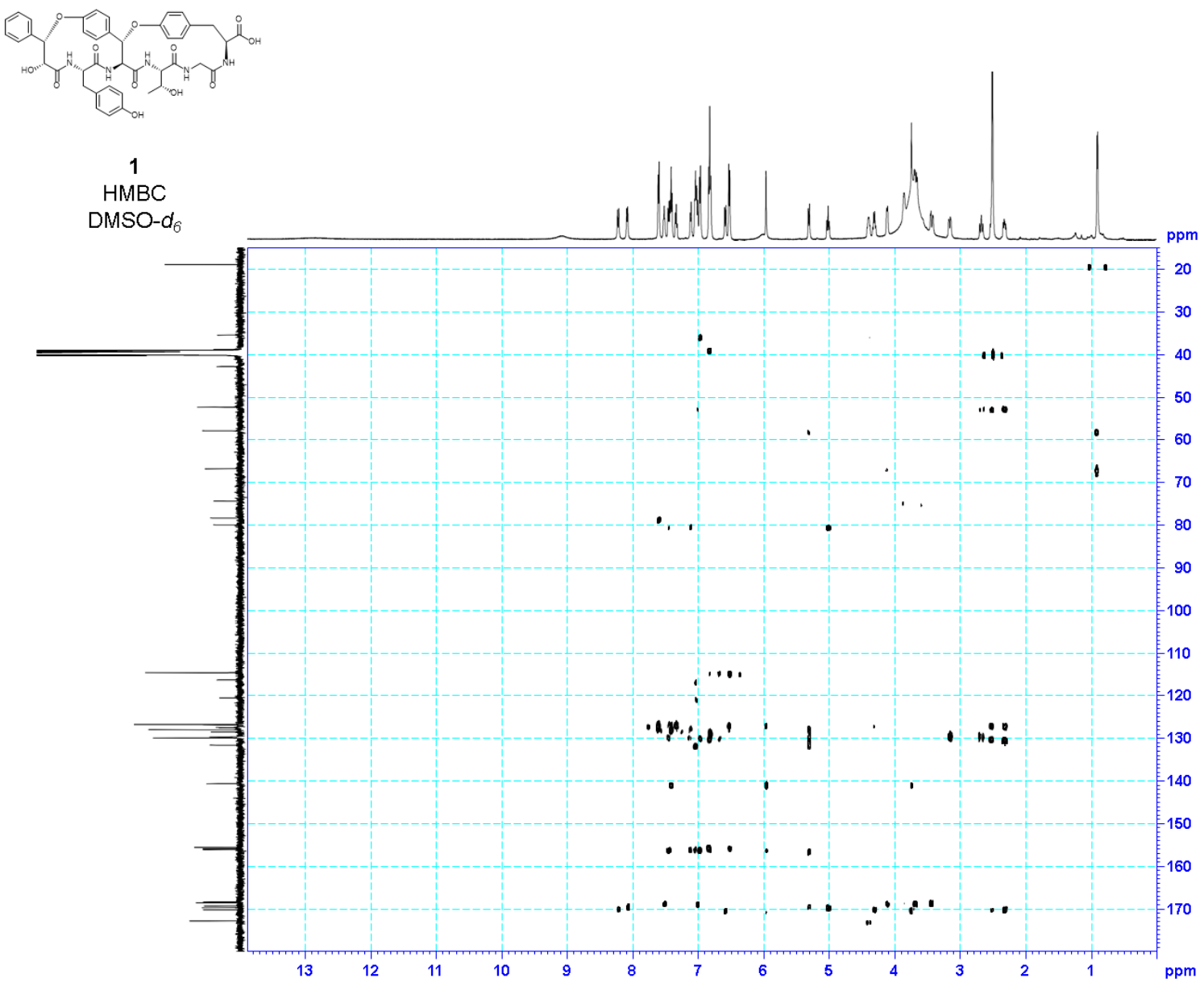
$^{13}$C NMR

DMSO-$d_6$




1
COSY
DMSO-$d_6$
ppm
8.5
8.0
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
ppm


1
eHSQC
DMSO-$d_6$
ppm
20
30
40
50
60
70
80
90
100
110
120
130
8.5
8.0
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
ppm


1
HMBC
DMSO-$d_6$
ppm
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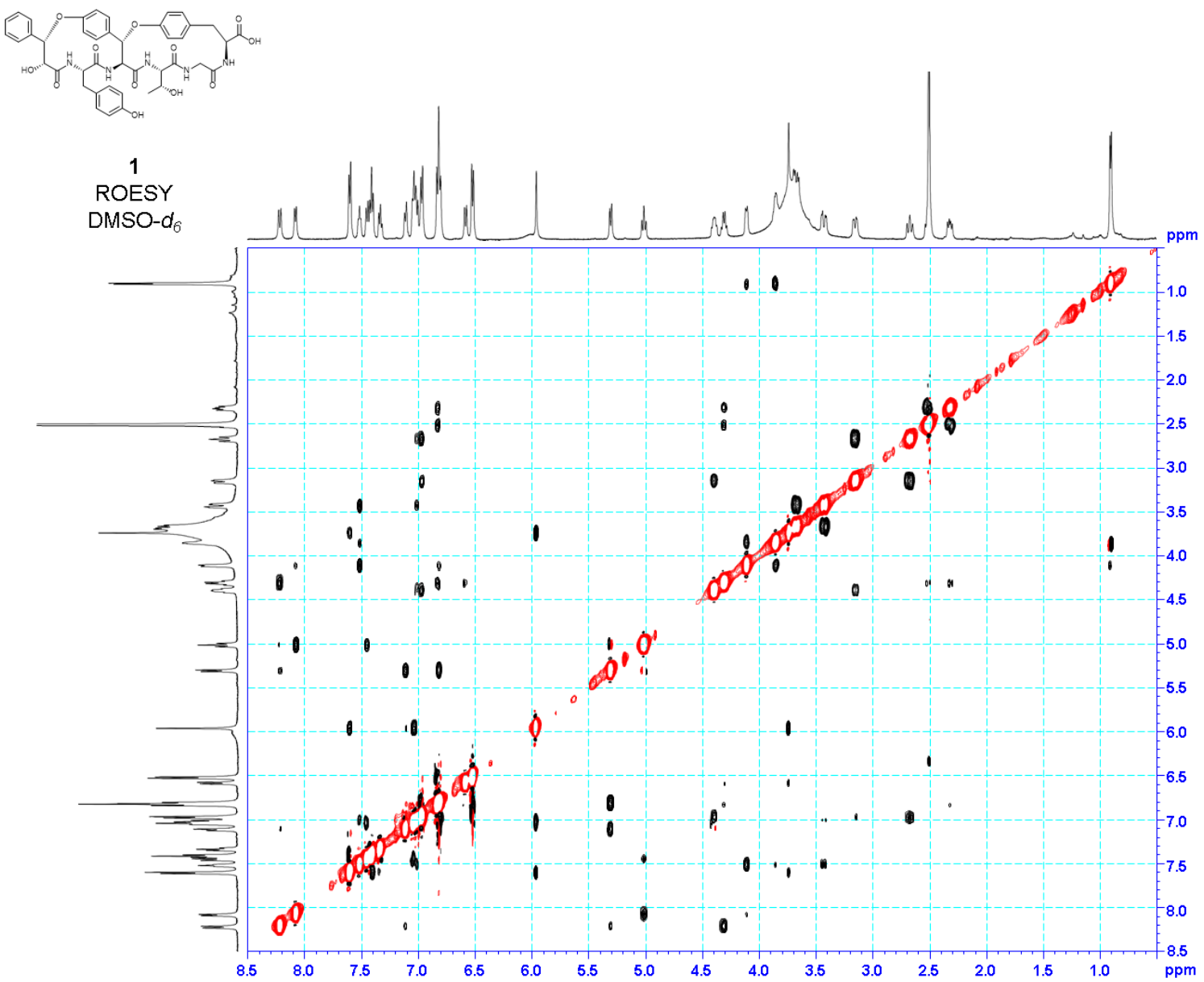
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
13
12
11
10
9
8
7
6
5
4
3
2
1
ppm


1
ROESY
DMSO-$d_6$
ppm
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8.0
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
ppm