Supplementary Information

Construction of a hybrid gene cluster to reveal a coupled ring formation-hydroxylation in the biosynthesis of the antifungal HSAF and analogous from *Lysobacter enzymogenes*†‡

Xue Li,[a] Haoxin Wang,[a] Yaoyao Li*[b] and Liangcheng Du*[c]

[a] State Key Laboratory of Microbial Technology, Shandong University, Qingdao, Shandong 266237, P. R. China

[b] Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong 250012, P. R. China

[c] Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

*Yaoyao Li (Corresponding author)

E-mail: liyaoyao@sdu.edu.cn

ORCID: 0000-0002-8762-9615

*Liangcheng Du (Corresponding author)

E-mail: ldu3@unl.edu

ORCID: 0000-0003-4048-1008

1. Microbial strains, plasmids and primers

Escherichia coli DH5α was used as the host for general plasmid DNA propagation. *E. coli* ET121567/pUZ8002 was used as the donor strain for conjugation between *E. coli* and *Streptomyces*. *Streptomyces* sp. SR111[1] and *Streptomyces* sp. S001 were used as the hosts for heterologous production of HSAF analogues. Strain S001 was isolated from the intertidal soil collected at Laoshan, Qingdao, China, which produces few secondary metabolites and has clean metabolic background. Vector pSET5035 was derived from pSET152ER, used for integration of various HSAF gene clusters into strains SR111 and S001. Vectors pUC-EE and pUC-KT were derived from pUC18 with convergent promoters *ermEp**/*erm21p* and *kasOp**/*tcp830*, respectively, which were used to clone the biosynthetic genes.

Table S1. Primers used in this study.

Gene	Sequence of oligos (5'-3')
For *cbmB* gene	
cbmB-F	cggacatatgcggcgtagtaacagggtg
cbmB-R	attactagttcacctcatgctcttgtcgagc
For verification of the recombinant strain	
ermE4GB-F：	gtgcacgcggtcgatcttgac
kas4GB-R	aactcccccagtcctgcac
For OX1 site mutagenesis	
OX1-F	caaaggaggcggacatatgtcgcctgattcggccg
R218P-R	tgttcgggaaaccgcggcgcagcag
R218P-F	gcggtttcccgaacatgttcttccaggacccgg
OX1-R	gcctgcagttatgcattactagttcaggcggccggaagcg
K116T-R	tcgacgaccgtgttgaacagttcgaagttgcgc
K116T-F	tcaacacggtcgtcgacgagcgcggc
P225H-R	ggtcctggaagaacatgttgcg
P225H-F	aacatgttcttccaggaccacgg
G337K-R	gcgctccttcggccgcggcagcacttc
G337K-F	ggccgaaggagcgctatcccggcgtg
S518A-R	gtccgctggccgcggccttgatcaggct
S518A-F	gcggccagcggacgcttctgcgc

2. Construction of HSAF$_{hybrid}$ expression vector and heterologous production in *Streptomyces*

The 1681 bp *cbmB* gene was amplified by PCR from fosmid XIV-12H using the primer pairs of cbmB-F/cbmB-R.[2] The PCR product was digested with *Nde*I and *Spe*I, and inserted into the same sites of pUC-EE to yield pUC-*ermEp*-cbmB*. A 14534 bp fragment harboring the HSAF PKS-NRPS gene and *OX3-4* genes was cut off from pIB-HSAF1ΔOX12 by digestion with *Nde*I and *Nhe*I, and ligated to the *Nde*I/*Spe*I-digested pUC-KT to yield pUC-*kasOp**-HSAF1ΔOX12.[1] The *ermEp*-cbmB* fragment from pUC-*ermEp*-cbmB* by digestion with *Eco*RI and *Spe*I, along with the *kasOp**-HSAF1ΔOX12 fragment from pUC-*kasOp**-HSAF1ΔOX12 by digestion with *Xba*I and *Nsi*I, were ligated to the *Eco*RI/*Pst*I digested pSET5035 to yield pSET5035-HSAF$_{hybrid}$ (Figure S1). The construct pSET5035-HSAF$_{hybrid}$ was transformed into *E. coli* ET121567/pUZ8002, which was then conjugated with strain SR111 and S001 to produce the recombinant strain SR111-HSAF$_{hybrid}$ and S001-HSAF$_{hybrid}$, respectively. Each of the recombinant strains was verified by PCR with primers listed in Table S1.

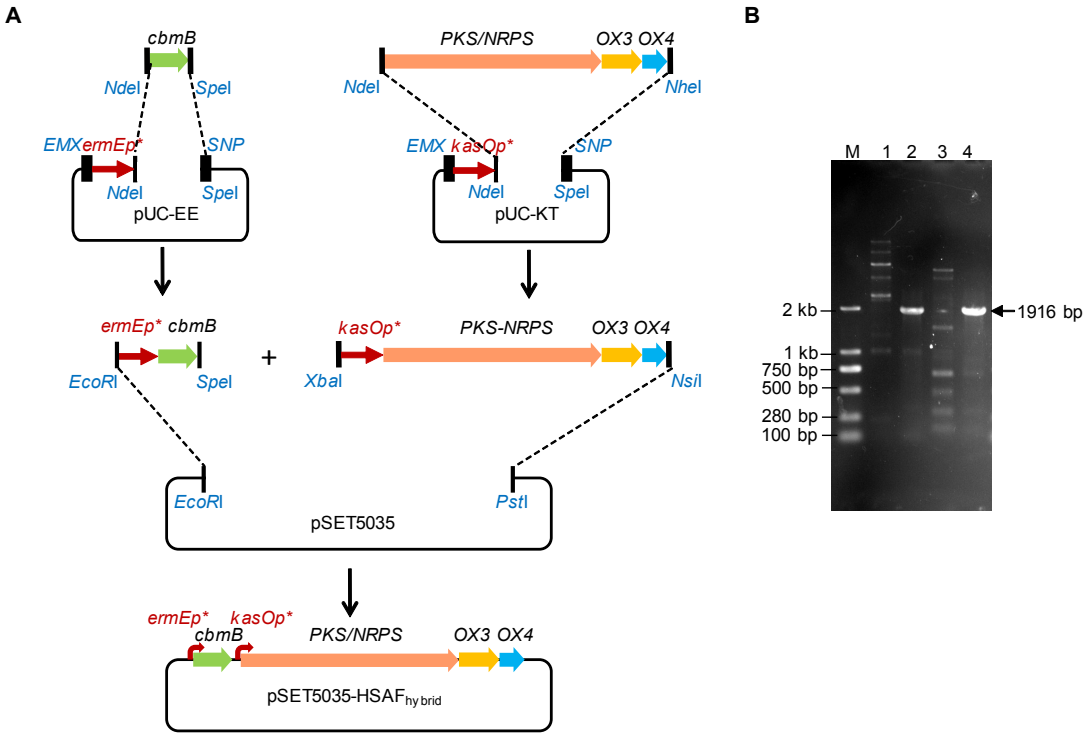

Figure S1. (A) Schematic illustration for the construction of the $HSAF_{hybrid}$ gene cluster; *EMX*, *Eco*RI/*Mfe*I/*Xba*I; *SNP*, *Spe*I/*Nsi*I/*Pst*I; (B) Verification of the recombinant strains by PCR (primer pairs used for PCR are listed in Table S1). M: DNA marker, DL2000; 1, SR111; 2, SR111-$HSAF_{hybrid}$ (1916 bp expected); 3, S001 wild type; 4, S001-$HSAF_{hybrid}$ (1916 bp expected).

3. Production and analysis of metabolites in recombination strains

For production of secondary metabolites, strains SR111, SR111-HSAF1 and SR111-$HSAF_{hybrid}$ were cultivated on M4 agar medium (25 g soluble starch, 15 g bean cake powder, 2 g yeast extract, 15 g agar, pH 7.2, 1 L) for 7 days at 30 °C; strains S001, S001-$HSAF_{hybrid}$, S001-MT1 and S001-MT2 were cultivated on YMG agar medium (10 g malt extract, 4 g glucose, 4 g yeast extract, 15 g agar, pH 7.2, 1 L) for 7 days at 30 °C. The whole agar cultures were diced, and then extracted with AcOEt/MeOH/AcOH (80:15:5, v/v/v) at room temperature. The crude extract was dried under reduced pressure, and the resulting residues were dissolved in 2 ml DMSO. An aliquot (5 μl) of each of the extracts was analyzed by HPLC (DIONEX Ultimate 3000 instrument; YMC-Pack Pro C18, 4.6×250 mm, 5 μm, flow rate 1 ml/min, UV detection at 305 nm). Chromatographic condition was as follows: solvents: A) water with 0.1% formic acid (FA), and B) acetonitrile with 0.1% FA; solvent gradient from 20% B to 35% B in the first 5 min, increased to 75% at 12 min, to 90% B at 20 min, to 100% B at 23 min, followed by 4 min with 100% B. To analyze the molecule weight of compounds **3**-**5**. LC-HRMS analysis was carried on an ESI QTOF high resolution mass spectrometer (Bruker) coupled with HPLC (DIONEX Ultimate 3000 instrument). Chromatographic condition was as described above.

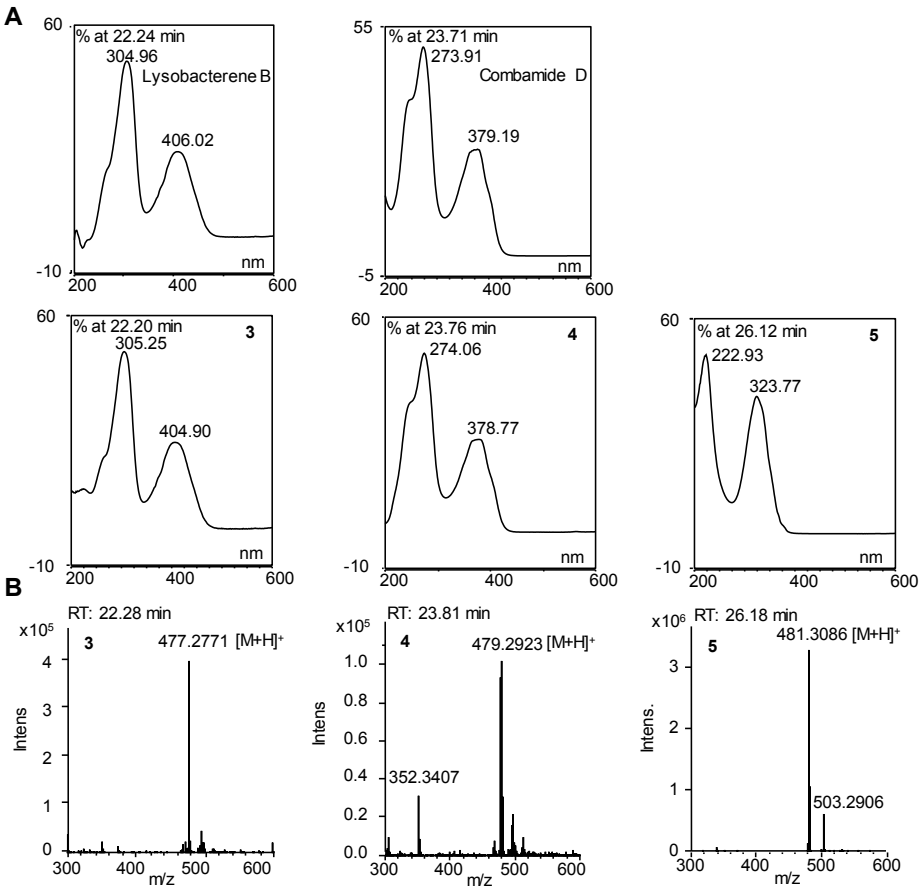

Figure S2. (A) UV-vis spectra of **3**-**5**, and reference compounds Lysobacterene B and Combamide D; (B) Mass spectra of **3**-**5**.

4. Fermentation and isolation of compound 5

The fermentation of strain S001-HSAF$_{hybrid}$ was performed on YMG agar medium (10 liters) for 7 days at 30 °C. The agar culture was diced and extracted three times with AcOEt/MeOH/AcOH (80:15:5, v/v/v) at room temperature, and the crude extract was concentrated under reduced pressure. The crude extract was sequentially solvent partitioned into AcOEt-soluble and H_2O-soluble extracts. The AcOEt extract was concentrated and was loaded to a Sephadex LH-20 column for separation. The column was eluted with MeOH/$CHCl_3$ (1:1, v/v) to obtain two fractions, Fr.1-2. After concentrated under reduced pressure, Fr.2 was dissolved in DMSO, and

yellow precipitate was observed. The yellow precipitate was collected and washed with DMSO several times to give **5** (~15 mg).

5. Structural elucidation of compound 5

To analyze the structure of compound **5**, 1D- and 2D-NMR were performed on a Bruker DRX-600 spectrometer. The molecular formula of **5** was established as $C_{29}H_{40}N_2O_4$ by high resolution ESIMS (*m/z* 481.3086, calculated 481.3066 for $[M + H]^+$, Figure S2B). The 1D- and 2D- NMR data (Table S2, Figures S3A, S7-S12) established the structure of compound **5**, which is identical to pactamide A.[3] In addition, the ECD spectrum of compound **5** is most close to that of pactamide A, indicating that the absolute configuration of **5** is the same as pactamide A.

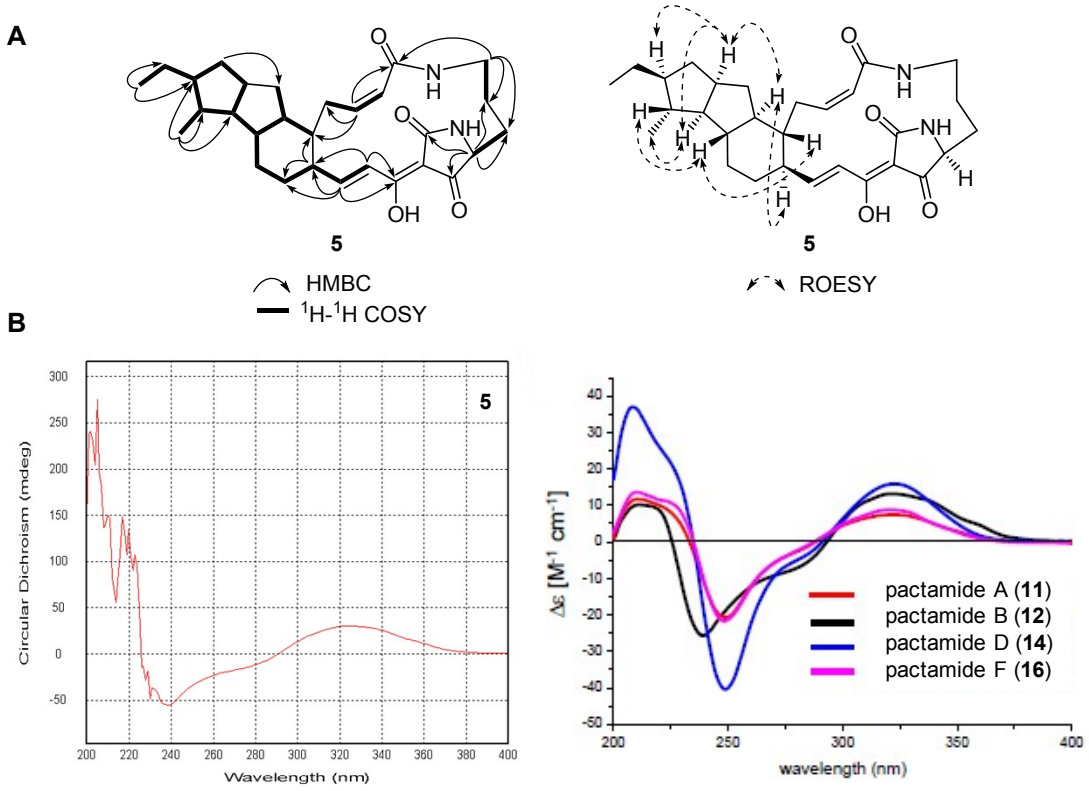

Figure S3. (A) The key HMBC,^{1}H-^{1}H COSY, and ROESY correlations of **5**; (B) The ECD spectra of **5** and pactamides.

Table S2. ^{1}H- and ^{13}C-NMR date of compound **5** at 600 MHz and 150 MHz, respectively, in pyridine-d_5; *J* in ppm.

No.	^{1}H	^{13}C	No.	^{1}H	^{13}C
1		196.8s	17	1.14 - 1.00 (m)	46.8d
2	4.13 - 4.09(m)	62.2d	18	1.57 - 1.52 (m)	59.1d
3	2.18 - 2.16 (m)	27.4t	19	0.95 - 0.90 (m)	53.4d
	2.06 - 2.01 (m)				
4	1.87 - 1.83 (m)	21.5t	20	1.88 - 1.82 (m)	30.5t
	1.51 - 1.47 (m)			1.07 - 1.01 (m)	
5	3.88 - 3.85 (m)	39.2t	21	1.67 (br d, *J* = 13.0)	32.7t
	2.73 (d, *J* = 12.7)			1.27 - 1.20 (m)	
6	8.83 br s		22	2.02 - 1.95 (m)	46.7d
7		166.9s	23	6.95 (dd, *J* = 15.5, 10.5)	151.1d
8	6.23 (d, *J* = 12.0)	125.0d	24	7.65 (d, *J* = 15.5)	123.1d
9	5.99 (t, *J* = 11.2)	140.2d	25		173.7s
10	4.09 - 4.07 (m)	29.6t	26		101.8s
	2.36 (br d, *J* = 18.6)				
11	1.24 - 1.20 (m)	44.8d	27		177.1s
12	1.45 - 1.41 (m)	49.8d	28		
13	1.97 - 1.93 (m)	37.5t	29	0.98 (d, *J* = 6.5)	19.4q
	0.76 - 0.70 (m)				
14	2.30 - 2.26 (m)	41.7d	30	1.57 - 1.49 (m)	26.3t
				1.07 - 1.01 (m)	
15	2.01 - 1.95 (m)	40.8t	31	0.88 (t, *J* = 7.4)	12.9q
	0.78 - 0.72 (m)				
16	1.32 - 1.24 (m)	54.1d			

6. Phylogenetic analysis of NAD(P)H-dependent flavin oxidoreductases in PoTeM biosynthesis

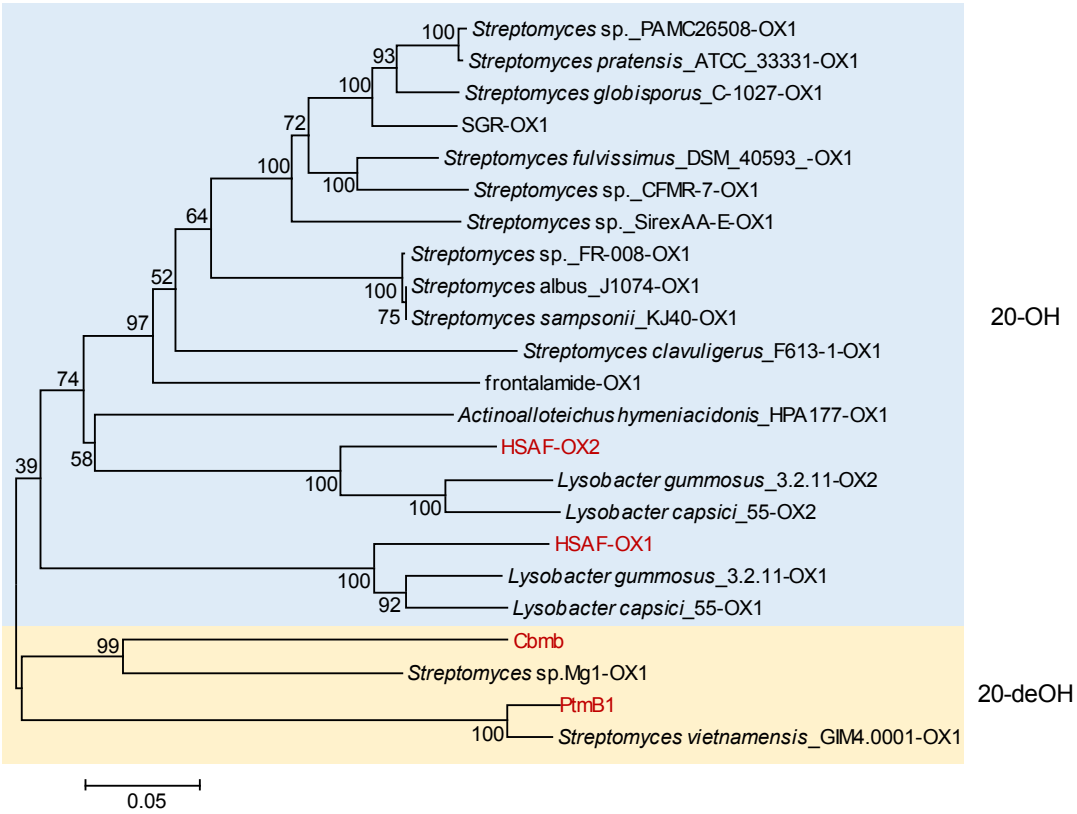

Figure S4. Phylogenetic analysis of the NAD(P)H-dependent flavin oxidoreductases, HSAF OX1-2, CbmB, PtmB1, and their homologs in databases. The oxidoreductases are generally grouped into the "20-OH clade", which are known or predicted to catalyze a C-20 hydroxylation during the biosynthesis of PoTeM compounds, and the "20-deOH clade", which are known or predicted not to catalyze the hydroxylation.

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                                                  116
HSAF-OX1                          LFNKVVDERGREVNFYNDPDRLERELIAVSPQDAPLIKAFCADFRRFTKLALHPFLKPPP  172
HSAF-OX2                          MFNKVVDEDGRSVTFYNDPDRLEAHLLEISPADAEPIRAFCADLRRFVKIELYPFLTPDP  163
frontalamide-OX1                  LFNKVVGRDGREVVFWNDPDRLEAHLLELSPADAPHIRAYCRDLRRFQKIELYPFLTAPA  174
SGR-OX1                           LFNKVEDASGRSVTFYNDPDRLEAHLLEVSPADAPLIRAFTRDLRRFIDIELYPFLTAPA  163
L. gummosus 3.2.11-OX1            LFNKVVDEHGREVRFYNDPDRLEQHLREVSPADAPLIKAFCDDLRRFAKLTLHPFLTPPP  142
L. gummosus 3.2.11-OX2            MFNKVTDENGRSVIFYNDPDRLERHLLEISPADHALIRSFCADLRRFIKIEMYPFLKPDP  163
L. capsici 55-OX1                 LFNKVVDEHGREVRFYNDPDRLEQHLIEVSPVDAPLIKAFCDDLRRFTKLALHPFLKPAP  171
L. capsici 55-OX2                 MFNKVVDEHGRSVTFYNDPDRLERHLLEISPADEALIRSFCADLRRFIKIELYPFLKPDP  163
A. hymeniacidonis HPA177-OX1      MFNKVVDSDGRSVVFYNDPDRLERHLLEISPADAKLIRAFCRDLRRFIPMNLYPFLTPPP  163
Streptomyces sp. SirexAA-E-OX1    LFNKVEDASGRSVTFYNDPDRLEAHLLEVSPADSAPVRAFTRDLRRFTGIDLYPFLTAPP  163
Streptomyces sp. PAMC26508-OX1    LFNKVEDEHGRSVTFYNDPGRLETHLLELSPADAPHIRAFTRDLRRFIDIELYPFLTAPA  163
S. pratensis ATCC 33331-OX1       LFNKVEDEHGRSVTFYNDPGRLETHLLELSPADAPHIRAFTRDLRRFIDIELYPFLTAPA  163
S. fulvissimus DSM 40593-OX1      LFNKVEDESGRSVTFYNDPDRLEAHLLAVSPADAPLIRAFTRDLKRFIAIDLYPFLTAPA  169
S. globisporus C-1027-OX1         LFNKVEDEHGRSVTFYNDPDRLEAHLLDLSPADAPLIRAFTRDLRRFIDIELYPFLTAPA  172
S. albus J1074-OX1                LFNKVTDESGRSVVFYNDPGRLEAHLLEVSPADARLIRSFCRDLRRFTEIELYPFLTAPA  163
Streptomyces sp. FR-008-OX1       LFNKVTDESGRSVVFYNDPGRLEAHLLEVSPADARLIRSFCRDLRRFTEIELYPFLTAPA  163
S. sampsonii KJ40-OX1             LFNKVTDESGRSVVFYNDPGRLEAHLLEVSPADARLIRSFCRDLRRFTEIELYPFLTAPA  163
Streptomyces sp. CFMR-7-OX1       LFNKVEDENGRSVTFYNDPDRLEAHLLALSPADAPLIRAFTRDLRRFIAIELYPFLTAPA  169
CbmB                              VFNTVVDDDGRSVVFYNDPDRLERHLLRLSPADAPLIRSFCRDLRRFTDIDIFPFLKPAP  163
PtmB1                             QFNRVVAEDGRSVTFYNDPDRLQNHLLELSPGDGRLIRAFCRDLRRFAGLAPHWELKPPP  162
S. vietnamensis GIM4.0001-OX1     QFNRVVAEDGRSVTFYNDPDRLQKHLLELSPGDARLIRAFCRDLRRFAELDPHWELKPPP  162
Streptomyces sp. Mg1-OX1          MFNTVVDEHGRSVTFYNDPGRLERHLLEISPADARPIRAFCRDLRRFTDTNLYPFLTPPP  163
                                   ** *    **.* *:***.**: .*  :** *   ::::  *::**     .  *.
                                                                    218     225
HSAF-OX1                          LENWKEKLATLRQVLPAFALFWRTGAAQMGPYADRFADPLLRRGFRNMFFQDPGNFAVLP  232
HSAF-OX2                          LKSVGEKLRTLLKVLPAFRLFWRNAATPMHRFADRFSDPLLRRAFRNIFYQDPETFPVLP  223
frontalamide-OX1                  LKTLGEKLRTLRTVLPAFRLFWRNAATPMHAFADKFQDPLLRTAFRNIFFQDPEGFPVLP  234
SGR-OX1                           LRTVGERARTLRTVLPAFRLFWRTAATPMHVFADRFQDPLLRRAFRNIFFQDPEGFPMLP  223
L. gummosus 3.2.11-OX1            LETWREKLATLRQVLPAFRLFWRTGAAQMGAYADRFADPLLRRGFRNMFFQDPQNFAVLP  202
L. gummosus 3.2.11-OX2            LKSVGEKLKTLWEILPAFRLFWRNAATPMHAFADKFQDPLLRRAFRNIFYQDPECFPLLP  223
L. capsici 55-OX1                 LENWREKLAMMRQVLPAFRLFWRTGAAQMGPYADRFADPLLRRGFRNMFFQDPQNFAVLP  231
L. capsici 55-OX2                 LKSAGEKIKTMLEILPAFRLFWRNAATPMHDFADKFQDPLLKRAFRNIFYQDPECFPLLP  223
A. hymeniacidonis HPA177-OX1      LESVGEKIAKLRTILPAFVLYWRTAATQMKTFADKFADPLLRKAFRNIFFQDPEDFPLLP  223
Streptomyces sp. SirexAA-E-OX1    LRTVRERAALLRTVLPAFRLFWRTAATPMHTFADTFQDPLLRQAFRNIFFQDPEGFPLLP  223
Streptomyces sp. PAMC26508-OX1    LRTVRERAGTLRTVLPAFRLFWRTAATPMHVFADRFEDPLLRKAFRNIFFQDPGGFPLLP  223
S. pratensis ATCC 33331-OX1       LRTVRERAGTLRTVLPAFRLFWRTAATPMHVFADRFEDPLLRKAFRNIFFQDPGGFPLLP  223
S. fulvissimus DSM 40593-OX1      LRTVRERAATLRTVLPAFRLFWRTAATPMHVFADKFQDPLLRRAFRNIFFQDPEGFPLLP  229
S. globisporus C-1027-OX1         LRTVRERAETLRTVLPAFRLFWRTAATPMHAFADRFQDPLLRKAFRNIFFQDPGGFPLLP  232
S. albus J1074-OX1                LRTVREKAATLRTVLPAFRLFWRTAATPMHRFADRFEDPLLRKAFRNIFFQDPEGFPLLP  223
Streptomyces sp. FR-008-OX1       LRTVREKAATLRTVLPAFRLFWRTAATPMHRFADRFEDPLLRKAFRNIFFQDPEGFPLLP  223
S. sampsonii KJ40-OX1             LRTVREKAATLRTVLPAFRLFWRTAATPMHRFADRFEDPLLRKAFRNIFFQDPEGFPLLP  223
Streptomyces sp. CFMR-7-OX1       LRTVRERAATLRTVLPAFRLFWRTAATPMHVFADRFQDPLLRRAFRNIFFQDPEGFPLLP  229
CbmB                              LLTLREKARALRKILPLLNLFRRTAGTSMESFAARFEDPLLRRALPFVFFQDHEVFPLLP  223
PtmB1                             LKSLTEKARTLLAILPAFRLYWRTAATPMRRFADRFEDPLLRQAFPNMFLQELTGFPLLP  222
S. vietnamensis GIM4.0001-OX1     LKSPAEKVRTLLAILPAFRLYWRTAATPMRRFADRFEDPLLRRAFPNVFLQELTGFPLLP  222
Streptomyces sp. Mg1-OX1          LQTPREKLATLRAVLPDFLLFWRTAATRMERFAGRFQDPLLRRAFPFIFFQDHEVFPLLP  223
                                  * .  *:   :  :** : *: *...: *  :*  * ****: .:  :* *:   * :**
                                                                   337
HSAF-OX1                          NGECHYADHVVSACDGLFTLNTLLDGQYSNPRLDKLFKEVLPRPGERYPGVVSAFVGFDG  352
HSAF-OX2                          GGARHYADIVVSAADGYTTLYGMLEGKYTSPAIETLYGEMLDKPGILFPAVVSAFVGLTG  343
frontalamide-OX1                  GGKTLYAEHVISAADGDTTIKGLLGGRYTGPRIDKLYEELLDQEGTLFPAVVSAFVGIEG  354
SGR-OX1                           NGKQYFAEHVVSACDGHTTVYGLLGGRYTGPRIDKLYTDLLHRPGTLFPAVVSAFVGLRG  343
L. gummosus 3.2.11-OX1            NGERHYSDHVVAACDGVFTLHNLLDGKYSNPRLDKLFNDVLHRPGERYPGVVSAFVGFEG  322
L. gummosus 3.2.11-OX2            GGARHYADVVVSAADGHTTIYGMLEGKYTNPTIDTLYEEMLNKPGILFPAVVSAFVGLHG  343
L. capsici 55-OX1                 NGEKHYADHVVAACDGMFTLHGLLDGKYTNPRLDKLFNDVLHRPGERYPGVVSAFVGFEG  351
L. capsici 55-OX2                 GGARHYADFVVSAADGYTTIYGMLEGKYKNKTIDTLYDEMLNKPGILFPAVVSAFVGLEG  343
A. hymeniacidonis HPA177-OX1      GGERLYADHVVAACDGRTTIYDFLDGRYTGPTIDRLYDELLTKPGTLFPAVVSAFVGIEG  343
Streptomyces sp. SirexAA-E-OX1    NGRRYFAEHIVSACDGHTTVYKLLGGRYTGPRIEKLYGDLIERPGTLFPAVVSAFVGIRG  343
Streptomyces sp. PAMC26508-OX1    NGNRYFAEHVVSACDGHTTVYGLLGGRYTGPRIDKLYTDLLHRPGTLFPAVVSAFVGLRG  343
S. pratensis ATCC 33331-OX1       NGKRYFAEHVVSACDGHTTVYGLLGGRYTGPRIDKLYTDLLHRPGTLFPAVVSAFVGLRG  343
S. fulvissimus DSM 40593-OX1      NGKRYFAEHVVSAADGHTTVYGLLGGRYTGPRIDKLYTDLLQRPGTLFPAVVSAFVGLRG  349
S. globisporus C-1027-OX1         GGRRYFAEHVVSACDGHTTVYGLLGGRYTGPRIDRLYTDLLHRPGTLFPAVVSAFVGLRG  352
S. albus J1074-OX1                GGRRYYAEHVVSACDGHTTIRGLLDGKYTGPRVDKLYDELLESPGTLFPAVVSAFVGVEG  343
Streptomyces sp. FR-008-OX1       GGRRYYAEHVVSACDGHTTIRGLLDGKYTGPRVDKLYGELLESPGTLFPAVVSAFVGVEG  343
S. sampsonii KJ40-OX1             GGRRYYAEHVVSACDGHTTIRGLLDGKYTGPRVDKLYDELLESPGTLFPAVVSAFVGVEG  343
Streptomyces sp. CFMR-7-OX1       NGQRYFAEHIVSACDGRTTVHGLLDGRYTSPRIDKLYNDLIERPGTLFPAVVSAFVGLRG  349
CbmB                              GGERHYADHVVAACDGATTLDRLLKGRYSSPRTDRLFQSVLGTPKLVYPGVVSVFVGFAG  343
PtmB1                             DGQEHFADHVIAACDGPTVLDRLLEGRWSSPHTERLYTELLDRPDNLYPAVVSAFVGIDG  342
S. vietnamensis GIM4.0001-OX1     DGQEHFADHVIAACDGPTVLDRLLEGRWSSPRTERLYKELLDRPDNLYPAVVSAFVGIDG  342
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Streptomyces sp.Mg1-OX1          GGERHYADHVVSACDGHTTIHGLLEGRYGSPRVDALFQEMMNRPELVYPGVVSAFVGLDG   343
                                 .*   ::: :::*.**  .:  :* *:: .   : *: .::      :*.***.*.*. *
                                                                          518
HSAF-OX1                         LAWKS-PDADDLIAALIAKDRLRLQGLRGFSMAGHWVAGGSLIKAASSGRFCAQYLCEEL   531
HSAF-OX2                         LAWKAFSDAEDLANELVNKGRMQLPGLKGFYMAGQWVGLGGLIRAASTGRFAMQYICKEL   523
frontalamide-OX1                 LAWKAFSEADDISAKLVGKDRMRLPGLAGFSMAGQWVGMGGLIRAASTGRFAVQYLCDEL   534
SGR-OX1                          LAWKAFSDADDVAARLVGKDRMRLPGLSNFSMAGQWVGMGGLIRAASTGRFAVQYLCRDL   523
L.gummosus 3.2.11-OX1            LAWKS-PDADDLLADLIKKDRMRLQGLSGFSMAGHWINGGSLIKAASSGRFAARFLCEEL   501
L.gummosus 3.2.11-OX2            LAWKAFSDAEDLANELVNKGRMQLPGLSGFYMAGQWVGLGGLIRAASSGRFVMQFICKEI   523
L.capsici 55-OX1                 LAWKS-PDADDLLAALVNKDRMRLKGLSGFSMAGHWINGGSLIKAASSGRFAAQYLCEEL   530
L.capsici 55-OX2                 LAWKAFSDAEDLANKLVNKGRMQLPGLGGFYMAGQWVGLGGLIRAASSGRFVMQFICKEL   523
A.hymeniacidonis HPA177-OX1      LAWKAFSDADDLAAKLINKEHMRLPGLRGFSMAGQWVGLGGLIRAASTGRFVTQFLCAEL   523
Streptomyces sp. SirexAA-E-OX1   LAWKAFSDADDVAARLVGKDRMRLPGLSGFSMAGQWVGMGGLIRAASTGRFATQYLCREL   523
Streptomyces sp. PAMC26508-OX1   LAWKAFSEADDVAARLVGKDRMRLPGLDGFSMAGQWIGMGGLIRAASTGRFAVQYLCREL   523
S.pratensis ATCC 33331-OX1       LAWKAFSDADDVAARLVGKDRMRLPGLDGFSMAGQWIGMGGLIRAASTGRFAVQYLCREL   523
S.fulvissimus DSM 40593-OX1      LAWKAFSDADDVAARLVGRDRMRLPGLSGFSMAGQWVGMGGLIRAASTGRFATQYLCREL   529
S.globisporus C-1027-OX1         LAWKAFSDADDVAARLVGKDRMRLPGLSGFSMAGQWVGMGGLIRAASTGRFAVQYLCREL   532
S.albus J1074-OX1                LAWKAFSDADDLAAGLVGKDRMRLPGLAGFSMAGQWVGMGGLIRAASTGRFAVQYLCAEL   523
Streptomyces sp.FR-008-OX1       LAWKAFSDADDLAAGLVGKDRMRLPGLAGFSMAGQWVGMGGLIRAASTGRFAVQYLCAEL   523
S.sampsonii KJ40-OX1             LAWKAFSDADDLAAGLVGKDRMRLPGLAGFSMAGQWVGMGGLIRAASTGRFAVQYLCAEL   523
Streptomyces sp.CFMR-7-OX1       LAWKAFSDADDVAARLVGRDRMRLPGLSGFSMAGQWVGMGGLIRAASTGRFAVQYLCREL   529
CbmB                             LAWKSYTEADGLIQHLIEKDRLRLPGLDGLSLAGQWFTGGGLIRVAAGGRFVAQYLCEEL   523
PtmB1                            LAWKAFSEADDVSTALVDRGRMRLPGLSGFSMAGQWTGMGGLIRAATSGRYAVQFLCDEL   522
S.vietnamensis GIM4.0001-OX1     LAWKAFSEADDVSTALVDRDRMRLPGLSGFSMAGQWTGMGGLIRAATSGRYAVQFLCDEL   522
Streptomyces sp.Mg1-OX1          LGWKSFTEADDLITGLINKDRMRLPGLRGLSLAGQWFGGGSLIRAAASGRFVTQYVCREL   523
                                 *.**:  :*:.:   *: : :::* ** .: :**:*   *.**:.*: **:  :::* :
```

Figure S5. Multiple alignment of the amino acid sequences of HSAF OX1-2, CbmB, PtmB, and their homologs shown in Figure S4 using Clustal Omega. The residues that are highly conserved in the "20-OH clade" are highlighted in green color and the corresponding residues in the "20-deOH clade" are highlighted in purple, if conserved.

7. Site-directed mutagenesis of *OX1* gene and heterologous production in *Streptomyces*

Based on the phylogenetic analysis of OX1 and homologs, the PoTeM oxidoreductases are grouped into the "20-OH clade" and the "20-deOH clade" (Figure S4). Further sequence analysis of the clades revealed residues that are highly conserved in the "20-OH clade" but not in the "20-deOH clade" (Figure S5). These conserved residues in the "20-OH clade" were changed to the corresponding ones in CbmB of the "20-deOH clade", using PCR coupled Gibson assembly strategy (Figure S6A, B). The primers used in the experiments are summarized in Table S1. For the one-site point-mutagenesis, OX1-R218P, primer pairs OX1-F/R218P-R and R218P-F/OX1-R were used. The resulting two fragments (673 bp and 1134 bp) were ligated to the *Nde*I/SpeI

digested vector pUC-EE to generate the construct pUC-*ermEp**-MT1. For the four-site point-mutagenesis, OX1-K116T/P225H/G337K/S518A, primer pairs OX1-F/K116T-R, K116T-F/P225H-R, P225H-F/G337K-R, G337K-F/S518A-R, S518A-F/OX1-R were used respectively. The resulting five fragments (312 bp, 332 bp, 363 bp, 558 bp and 230 bp) were ligated to the *Nde*I/SpeI digested vector pUC-EE to generate the construct pUC-*ermEp**-MT2. All mutated sites were confirmed by DNA sequencing. After digested with *Mfe*I and *Spe*I, the point-mutated *OX1* genes were further recombined with HSAF PKS-NRPS and *OX3-4* as described for the construction of S001-HSAF$_{hybrid}$. The final constructs were transformed to strain S001 to produce the recombinant strain S001-MT1 and S001-MT2, respectively. Each of the recombinant strains was verified by PCR with primers listed in Table S1 (Figure S6C).

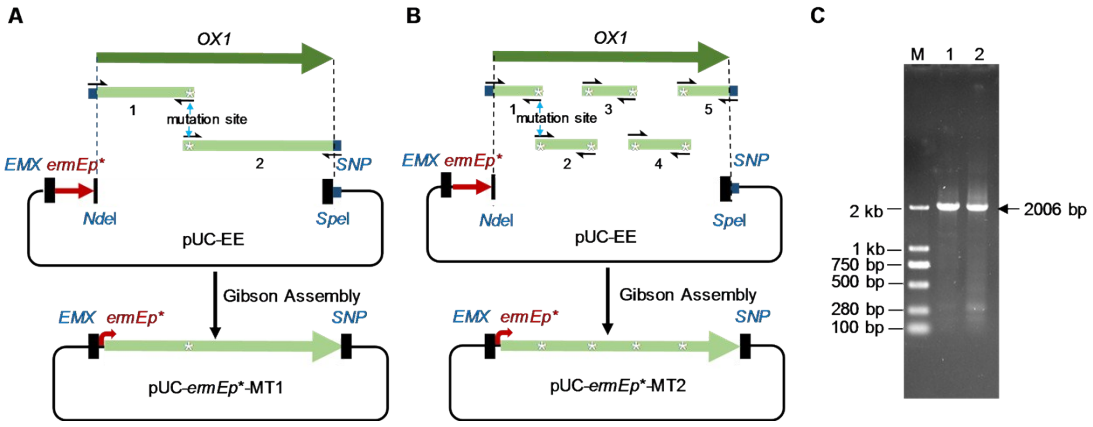

Figure S6. Schematic illustration for the introduction of the desired point-mutagenesis in *OX1* gene. (A) OX1 with one site of mutagenesis (R218P); (B) OX1 with four sites of mutagenesis (K116T, P225H, G337K, S518A); (C) Verification of the recombinant strains with mutated *OX1* gene by PCR (primer pairs used for PCR are listed in Table S1). M: DNA marker, DL2000; 1, S001-MT1 (2006 bp expected); 2, S001-MT1 (2006 bp expected).

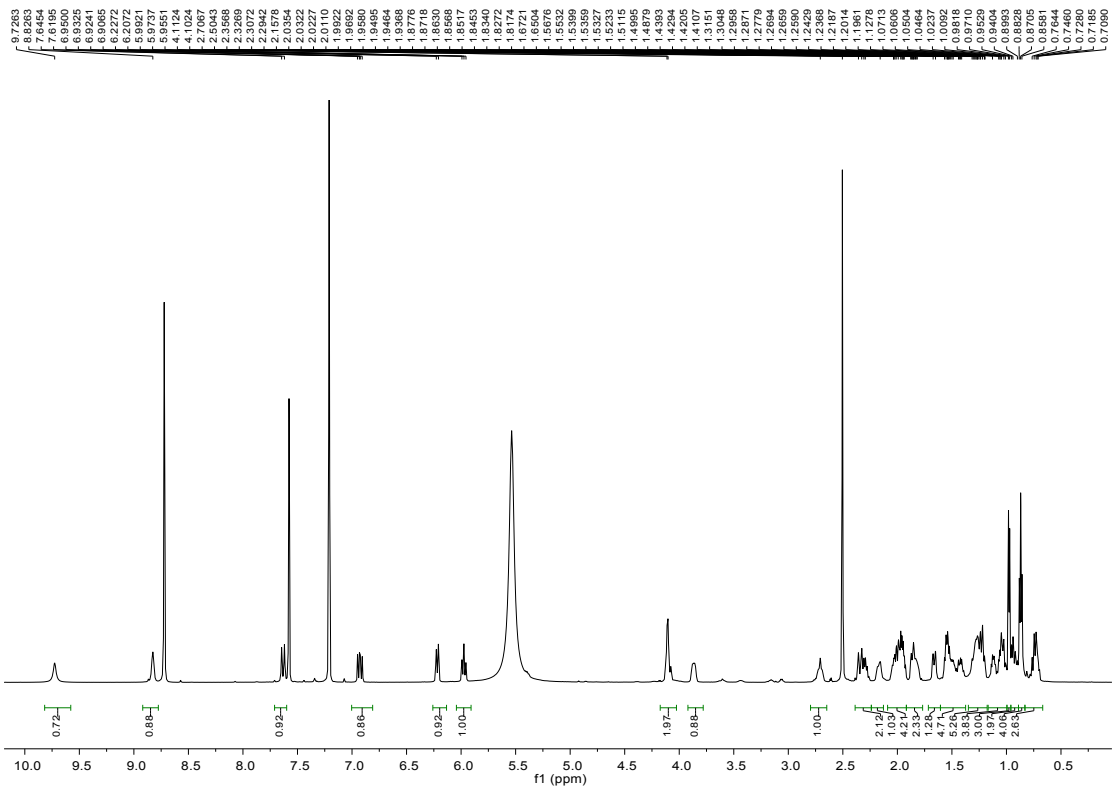

Figure S7. The ^{1}H NMR spectrum of **5** in pyridine-d_5.

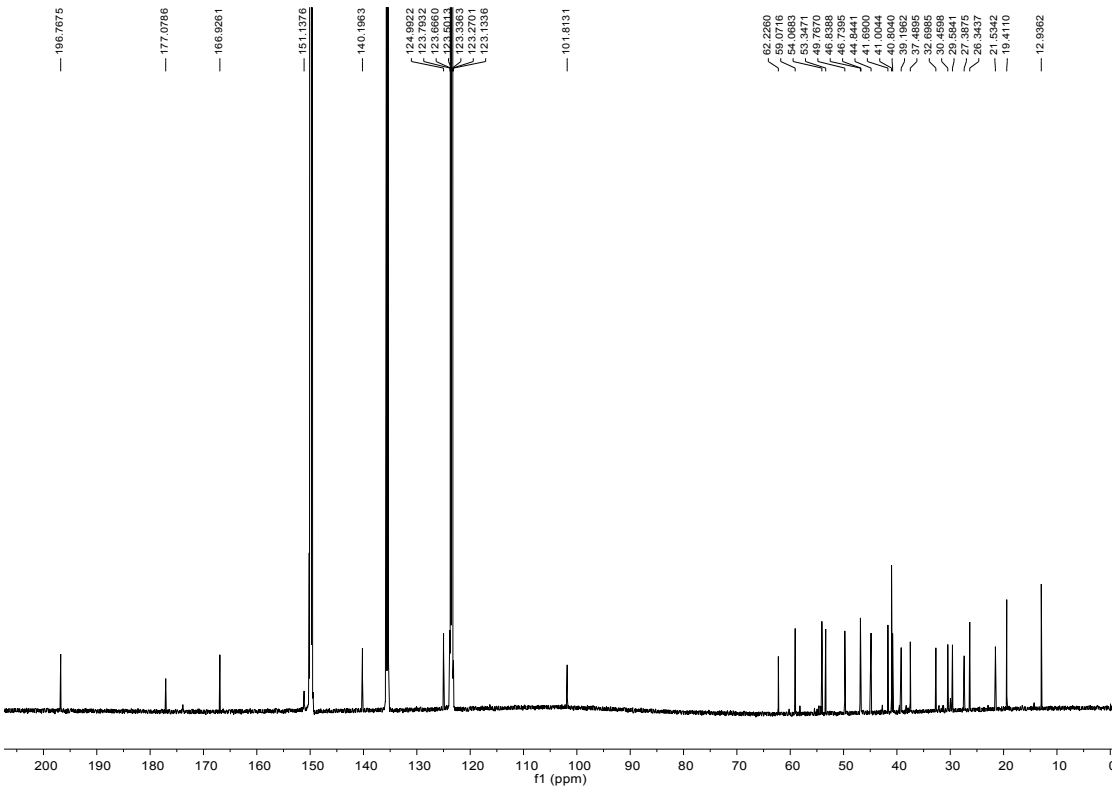

Figure S8. The ^{13}C NMR spectrum of **5** in pyridine-d_5.

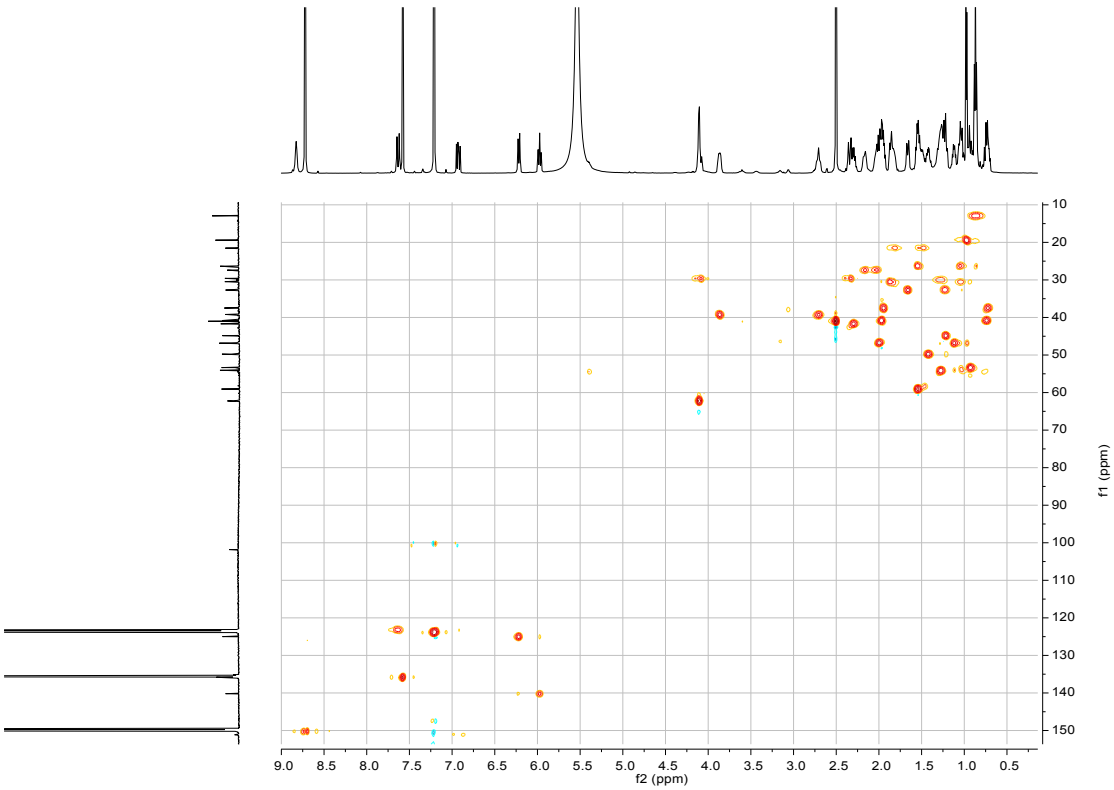

Figure S9. The HSQC spectrum of **5** in pyridine-d_5.

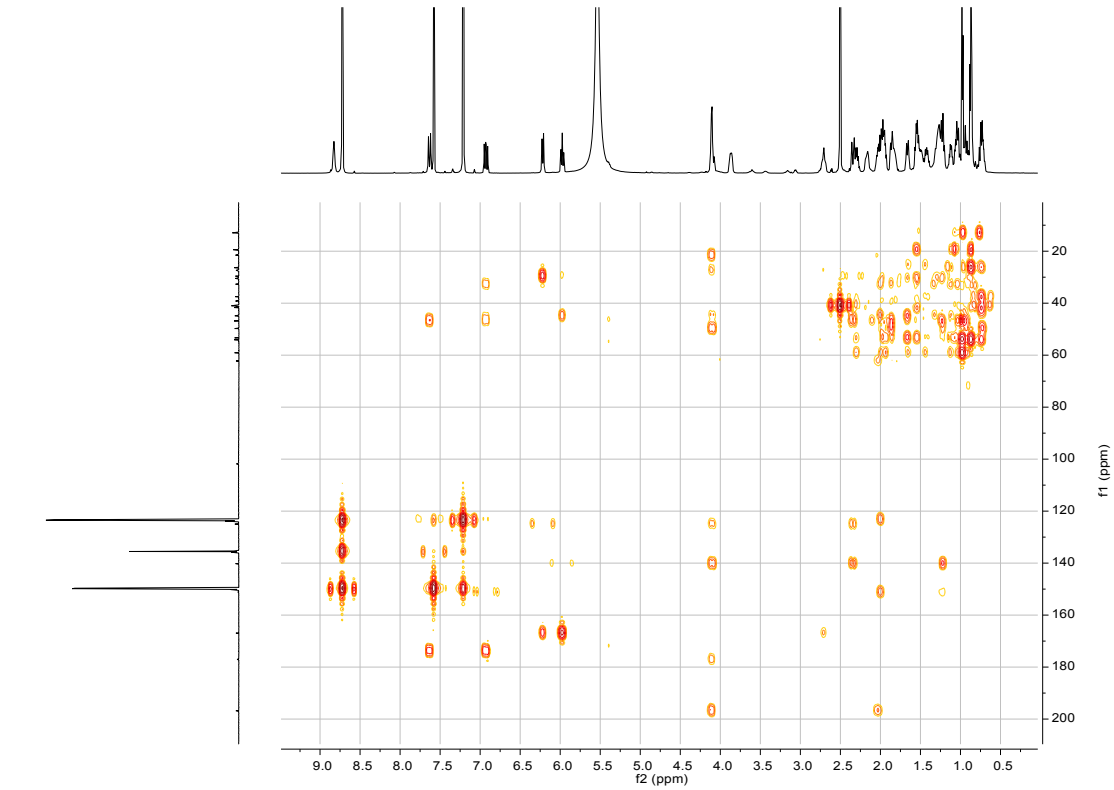

Figure S10. The HMBC spectrum of **5** in pyridine-d_5.

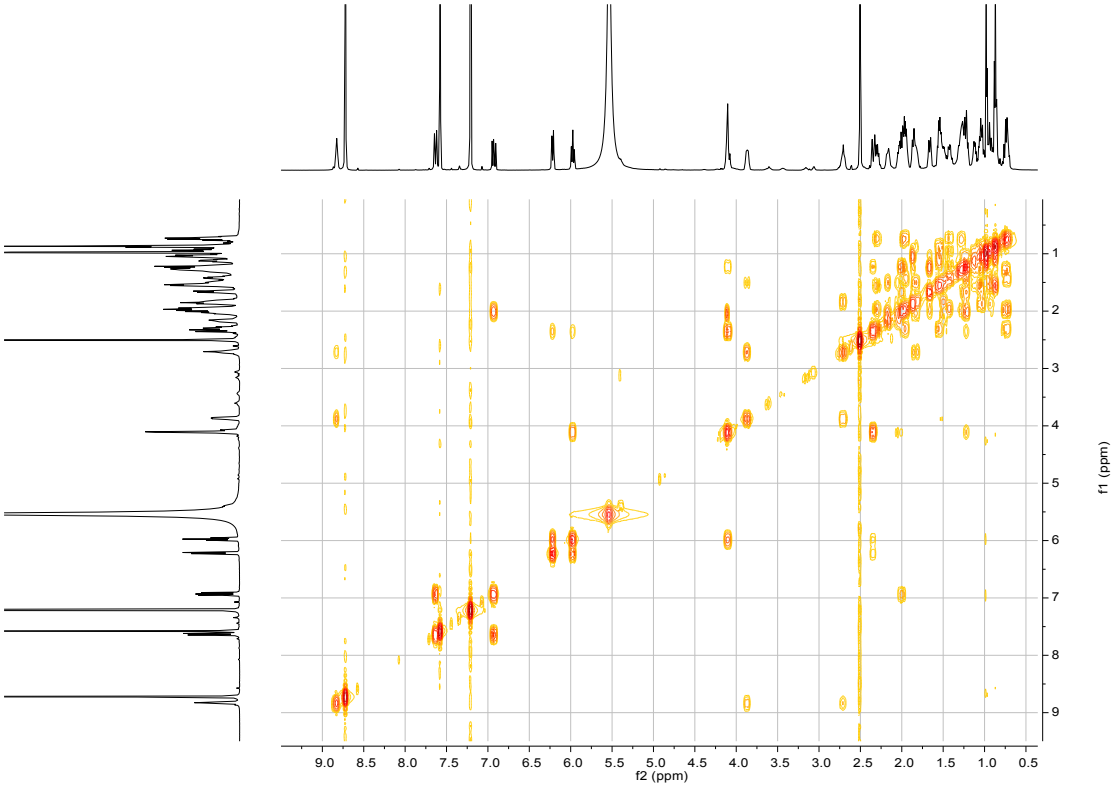

Figure S11. The ^{1}H-^{1}H COSY spectrum of **5** in pyridine-d_5.

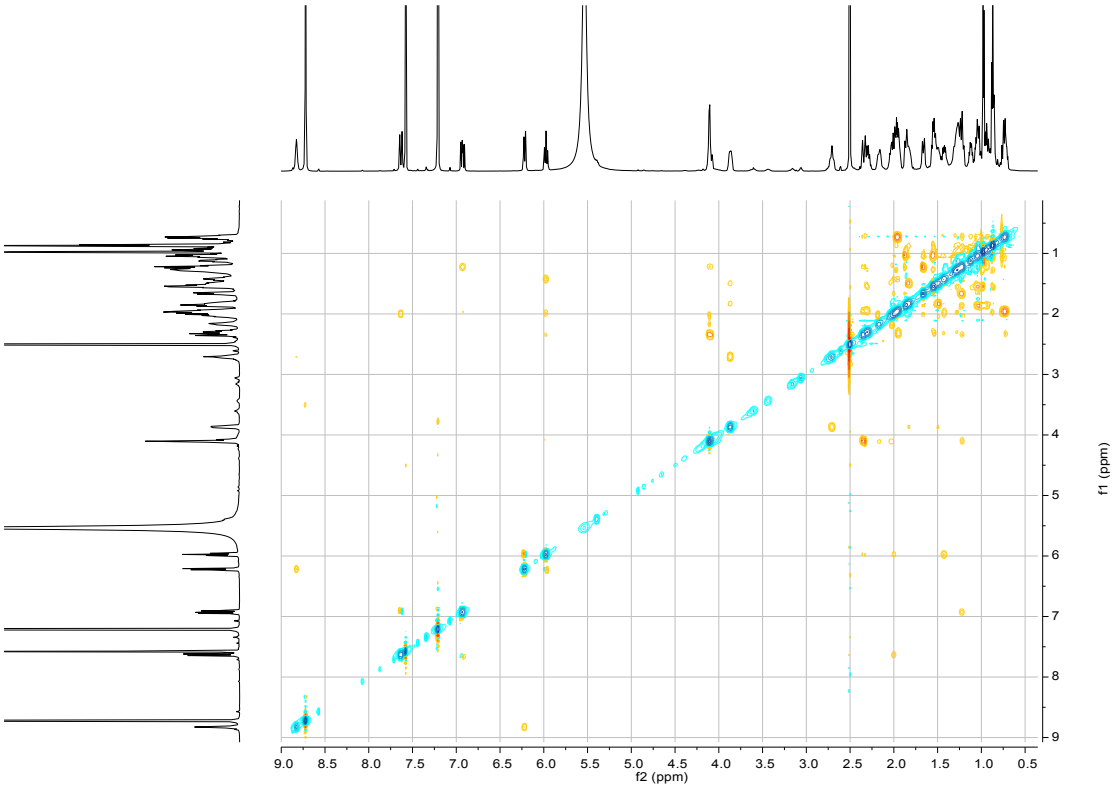

Figure S12. The ROESY spectrum of **5** in pyridine-d_5.

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