Supporting Information

Heterologous Expression of Highly Reducing Polyketide Synthase Involved in Betaenone Biosynthesis

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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, Bruker DRX-500. NMR spectra were recorded in $CDCl_3$ (99.8 atom % enriched, Kanto) or C_6D_6 (99.5 atom % enriched, Kanto). ^{1}H chemical shifts were reported in δ value based on internal TMS (0 ppm), $CDCl_3$ (7.26 ppm), or benzene (7.15 ppm) as references. ^{13}C chemical shifts were reported in δ value based on chloroform (77.0 ppm) or benzene (128.0 ppm) as references. 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. Mass spectra were obtained with a JEOL JMS-T100LP (ESI and APCI mode). Column chromatography was carried out on 60N silica gel (Kanto Chemicals).

Strain and Culture Conditions.

Phoma betae Fr. PS-13 was obtained from the Hokkaido National Agricultural Experimental Station, Sapporo, and cultured at 30 °C in potato glucose medium. The host strain used in this study was *A. oryzae* NSAR1, a quadruple auxotrophic mutant (*niaD*$^{-}$, *sC*$^{-}$, Δ*argB*, *adeA*$^{-}$) for fungal expression.

General technique for DNA manipulation.

Genomic DNA of *Phoma betae* Fr. PS-13 was extracted according to the literature procedure. PCR reactions were performed with the KOD-Plus-Neo (TOYOBO). The primers used to amplify the genes were purchased from Hokkaido System Science Co., Ltd.

Accession number.

The betaenone biosynthetic gene cluster sequence has been deposited in the DNA Data Bank of Japan (DDBJ) with the accession number LC011911.

Construction of expression vector.

The pAdeA2, an expression vector carrying *Aspergillus nidulans adeA* gene as selectable marker, was prepared as follows: The *adeA* gene was amplified from genomic DNA of *A. nidulans* with primer set as shown in Supplemental Table S3 and inserted into the *SphI*/*XbaI*-digested pUSA2 using Gibson assembly cloning kit to give pAdeA2 expression vector.

Preparation of expression plasmids.

The *bet1* fragments (Fr1-Fr3) were amplified from genomic DNA of *P. betae* with primer set as shown in Supplemental Table S3. Fr1 was inserted into the *ClaI*-digested pUARA2 using In-Fusion method to obtain pUARA2-*bet1*-Fr1. Fr2 and Fr3 was inserted into the resultant plasmid in a stepwise manner to afford pUARA2-*bet1*. The strategy is summarized in Figure S9. The *bet2*, *bet3* and *bet4* were also amplified from genomic DNA of *P. betae* with primer set as shown in Supplemental Table S3. Each gene was inserted into the *ClaI*-digested pUSA2 or *NheI*-digested pAdeA2 using In-Fusion method to obtain pAdeA2-*bet2*, pUSA2-*bet3* and pAdeA2-*bet4*.

The *bet1* mutant genes were prepared by fusion PCR; two flanking sequences were initially

amplified from pUARA2-*bet1* plasmid with primer set as shown in Supplemental Table S3. Then, amplification using nested primers fuses the two fragments into a single molecule (Fr3-S2705A, Fr3-Y2737F, and Fr3-K2741A). Resultant fragment was inserted into the pUARA2-*bet1*-Fr1, 2 to afford pUARA2-*bet1-mutant*.

Transformation of *Aspergillus oryzae*.

A spore suspension of *A. oryzae* NSAR1 or dehydroprobetaenone I-producing transformant (1.0 x 10^8 cells) were inoculated into DPY (dextrin-polypeptone-yeast extract: 2% dextrin, 1% polypeptone, 0.5% yeast extract, 100 mL) medium supplemented with appropriate nutrients. After 3 days incubation at 30 °C, mycelia was collected by filtration and washed with water. Protoplasting was performed using Yatalase (Takara; 5 mg mL^{-1}) in Solution 1 (0.8 mM of NaCl, 10 mM of NaH_2PO_4, pH 6.0) at 30 °C for 2 h. Protoplasts were centrifuged at 2,000 rpm (Beckman JLA10.500) for 5 min and washed with 0.8 M of NaCl solution. Then, protoplasts were adjusted to 2.0 x 10^8 cells/mL by adding Solution 2 (0.8 M of NaCl, 10 mM of $CaCl_2$, 10 mM of Tris-HCl, pH 8.0) and Solution 3 (40% (w/v) of PEG4000, 50 mM of $CaCl_2$, 50 mM of Tris-HCl, pH 8.0) in 4/1 volume ratio. Plasmids (pUARA2-*bet1* and pUSA2-*bet3* for the first transformation of wild type strain. pAdeA2-*bet2* or pAdeA2-*bet4* for the second transformation of dehydroprobetaenone I-producing strain. 12 μg each) added to the protoplast solution (200 μL). The aliquot was incubated on ice for 20 min and then Solution 3 (1 mL) added to the aliquot. After 20 min incubation at room temperature, Solution 2 (10 mL) added to the mixtures and the mixture was centrifuged at 2,000 rpm (Beckman JLA10.500) for 5 min. The transformation mixture was poured onto the Czapek-Dox (3.5%) agar plate supplemented with 0.8 M of NaCl and appropriate nutrients and then overlaid with the soft-top agar (1.2 M of sorbitol, 3.5% of Czapek-Dox, 0.6% of agar). The plates were incubated at 30 °C for 3-7 days.

Production and HPLC analysis of the metabolites.

Mycelia of *A. oryzae* transformants were inoculated into a solid medium containing polished rice (100 g) and adenine (10 mg) in 500 mL Erlenmeyer flasks. Each culture was incubated at 30 °C for 2 weeks. After extraction with ethyl acetate, the extract was then concentrated in vacuo and the residues were extracted with ethyl acetate (100 mL x 2). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The crude extracts were directly analyzed by LC-MS equipped with ZORBAX XDB-C18 column (50 mm x 2.1 mm) at the following conditions:

LC conditions to analyze dehydroprobetaenone I and probetaenone I;

A linear gradient from 50% to 100% acetonitrile for 15 min and 100% acetonitrile for 15 min at a flow rate of 0.2 mL/min.

LC conditions to analyze betaenone B;

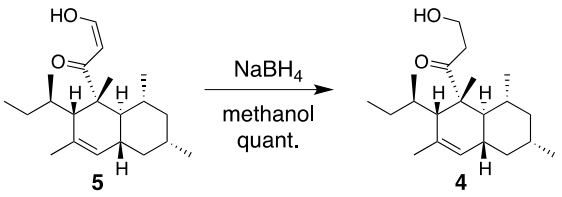
A linear gradient from 30% to 85% acetonitrile for 15 min and 100% acetonitrile for 15 min at a flow rate of 0.2 mL/min.

Dehydroprobetaenone I (5).

HR-ESIMS analysis of **5**; calcd. for $C_{21}H_{35}O_2$ $[M+H]^+$: 319.2632, found: 319.2682. $[\alpha]_D^{23}$ -19.3 (c 0.3 $CHCl_3$). ^{1}H-NMR (500 MHz, C_6D_6) δ 15.5 (s, 1H), 7.39 (s, 1H), 5.41 (d, J = 4.9 Hz, 1H), 5.18 (s, 1H),

1.96 (t, *J* = 9.5), 1.85 (m, 1H), 1.71 (m, 1H), 1.67 (s, 3H), 1.66-1.60 (m, 2H), 1.59 (s, 1H), 1.53 (m, 1H), 1.45 (m, 1H), 1.20 (m, 1H), 1.00 (d, *J* = 7.1 Hz, 3H), 0.99 (s, 3H), 1.05-0.97 (m, 2H), 0.96 (s, 1H), 0.92 (t, *J* = 7.2, 3H), 0.86 (d, *J* = 6.5, 3H), 0.85 (d, *J* = 7.0, 3H). ^{13}C-NMR (125 MHz, C_6D_6) δ 207.7, 173.5, 131.2, 127.4, 100.1, 58.8, 50.7, 46.8, 44.1, 42.7, 42.5, 38.1, 35.3, 34.0, 27.1, 25.9, 22.4, 22.3, 22.0, 18.7, 13.4.

Betaenone B (2).
HR-APCIMS analysis of **2**; calcd. for $C_{21}H_{35}O_5$ $[M+H]^+$: 369.2636, found: 369.2599. $[\alpha]_D^{24}$ 24.1 (c 0.3 $CHCl_3$).



Reduction of dehydroprobetaenone I (5)
To a solution of dehydroprobetaenone I (28.6 mg, 89.9 μmol) in methanol (2.0 mL) was added $NaBH_4$ (1.3 mg, 30 μmol) in methanol (0.5 mL) and the mixture was stirred for 50 min at room temperature. After concentration, the crude residue was dissolved in ethyl acetate. The solution was washed with water, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude residue was purified by silica gel chromatography (hexane/EtOAc 10:1) to give probetaenone I (28.6 mg, quant.).

Conformational search of dehydroprobetaenone I (5)
The lowest energy conformer of **5** was determined by a conformational search. Calculations were performed using Maestro v9.8 (Force field: OPLS_2005; Solvent: None; Conformational search: MonteCarlo Multiple Minimum (MCMM) method; Minimization method: Powell-Reeves conjugate gradient (PRCG); Steps: 10000). 6 conformers were found within a range up to 8 kJ/mol above the global minimum. The conformers are summarized in Supplementary Figure S6.

Figure S1. Gene integration analysis of (A) *bet1*/*3* gene in double transformants, (B) *bet4* in *bet1*/*3*/*4* triple transformants, and (C) *bet2* in *bet1*/*2*/*3* triple transformants.

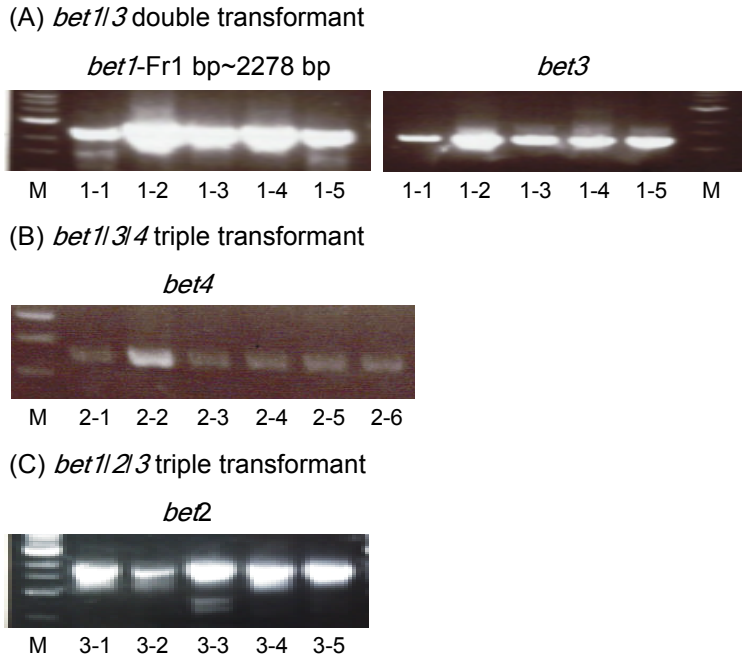


Figure S2. MS spectra of metabolites. (A) **5** from *bet1*/*3* transformant, (B) **4** from *bet1*/*3*/*4* transformant, (C) **2** from *bet1*/*2*/*3* transformant, and (D) authentic **2** from *P. betae*.

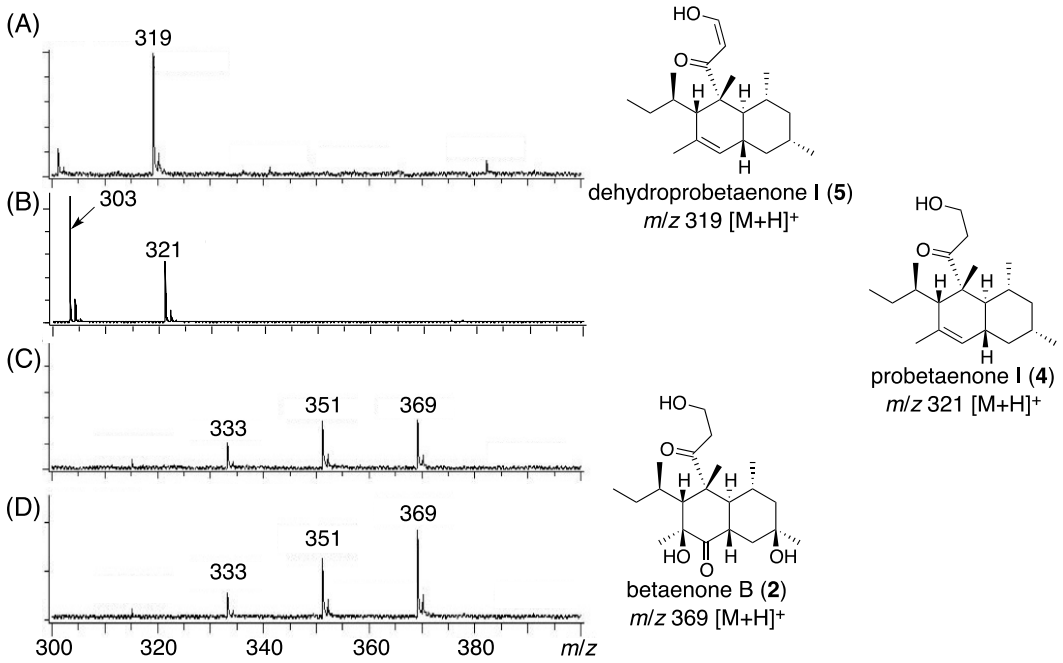


Figure S3. Schematic representation of the fungal expression vector, pAdeA2. P_{amyB}, amyB promoter; T_{amyA}, amyA terminator; T_{amyB}, amyB terminator; *adeA*, phosphoribosylaminoimidazolesuccinocarboxamide synthase.

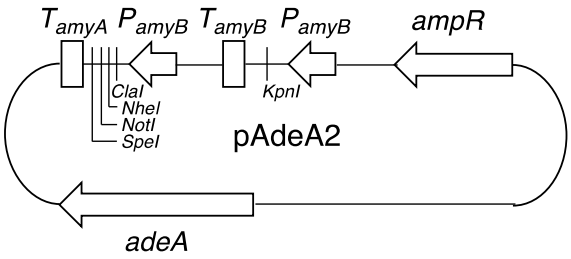


Figure S4. Multiple sequence alignment of R domains. The sequences referred to are as follows; AfoE (XM_653546) from asperfuranone biosynthetic gene cluster and AzaA (AGN71604) from azaphilone biosynthetic gene ccluster.

Figure S5. LC-MS profiles monitored at *m*/*z* 319 of the metabolites from R domain mutant strains. (A) *A. oryzae* NSAR1. (B) *bet1* transformant. (C) *bet1*/*3* transformant. (D) *bet1*-S2705A/*bet3*. (E) *bet1*-Y2737F/*bet3*. (F) *bet1*-K2741A/ *bet3*.

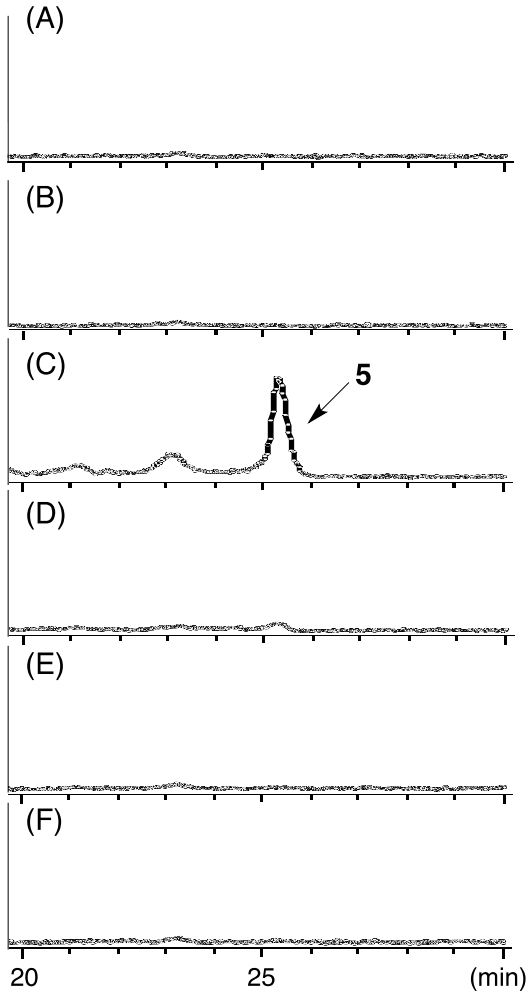
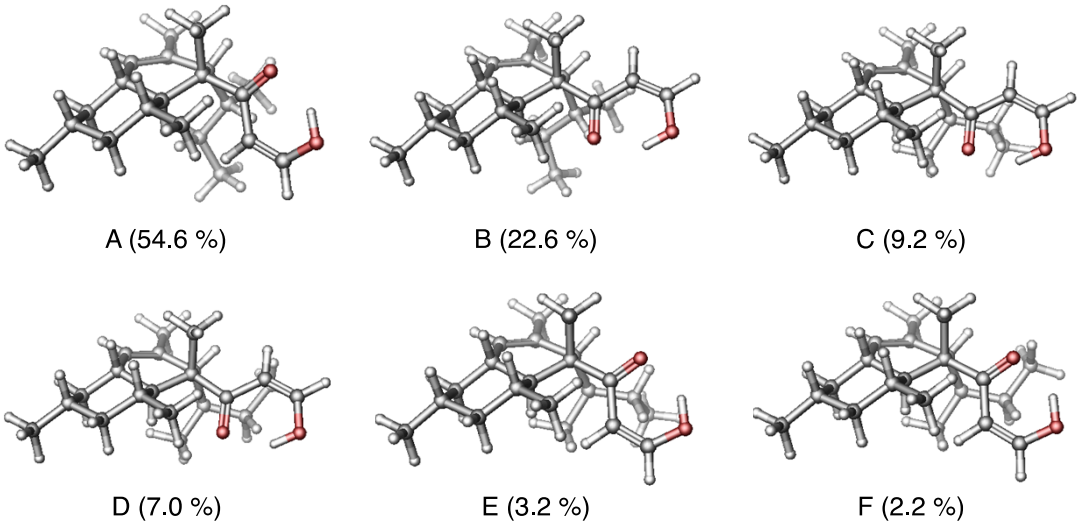


Figure S6. (A) calculated conformations of **5**. (B) Detailed analysis of conformer B.

(A)



Structure	Potential energy (kJ/mol)	Relative potential energy (kJ/mol)	Boltzmann populations (%)
A	202.835	0	54.6
B	205.025	2.19	22.6
C	207.247	4.412	9.2
D	207.928	5.093	7
E	209.848	7.013	3.2
F	210.763	7.928	2.2

(B)

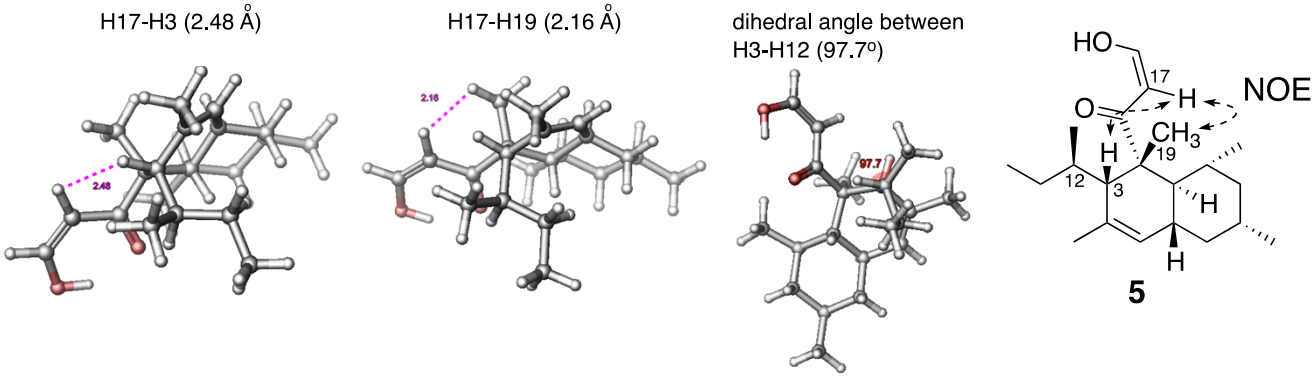


Figure S7. A phylogenetic tree of trans-ER and cis-ER domain of HR-PKS and PKS-NRPS in biosynthetic gene cluster of various polyketide metabolites. * shows inactive cis-ERs because of the point mutation on NADPH binding motif.

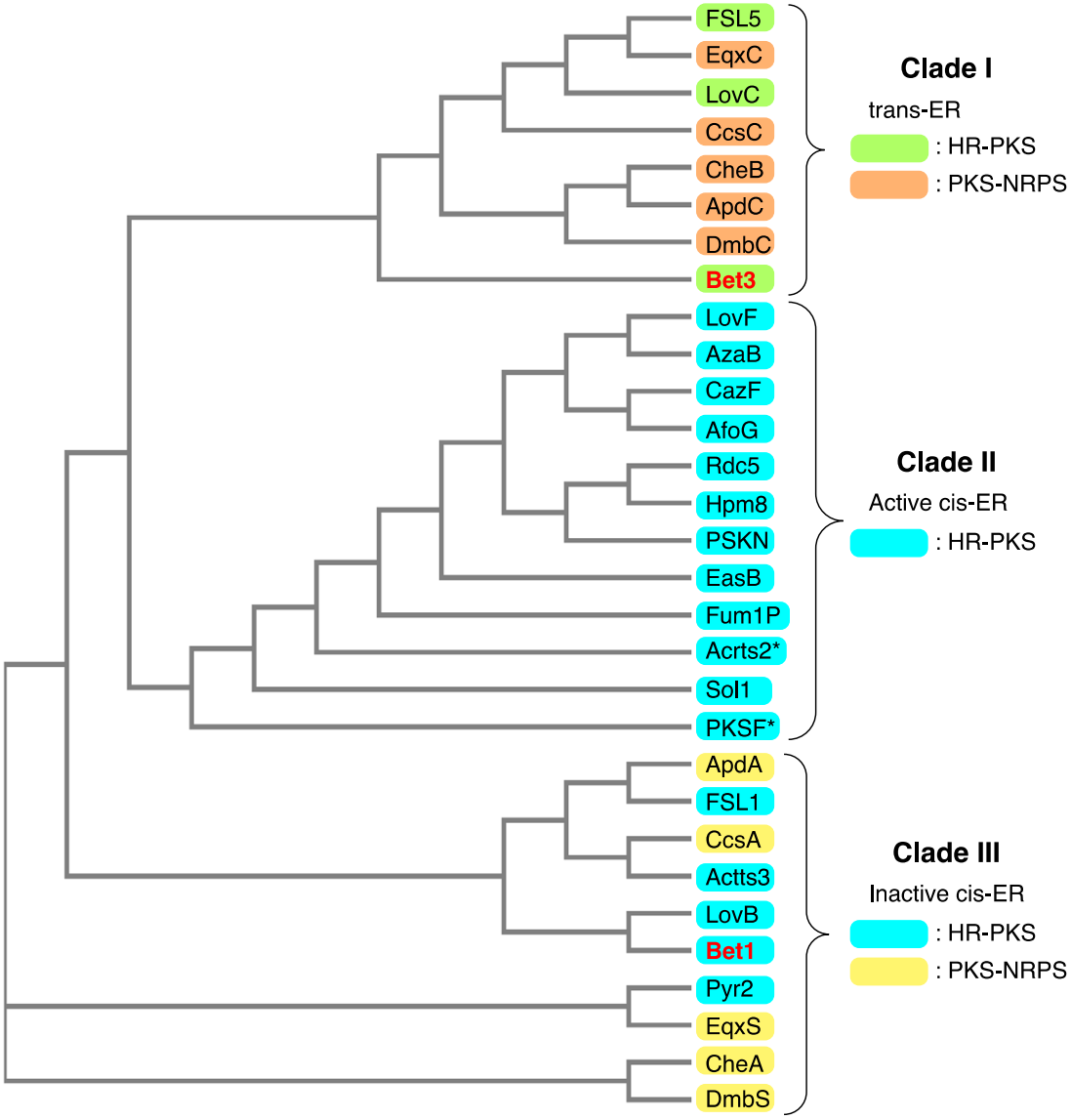


Figure S8. Multiple sequence alignment of ER domains. The sequences referred to are summarized in Table S2.

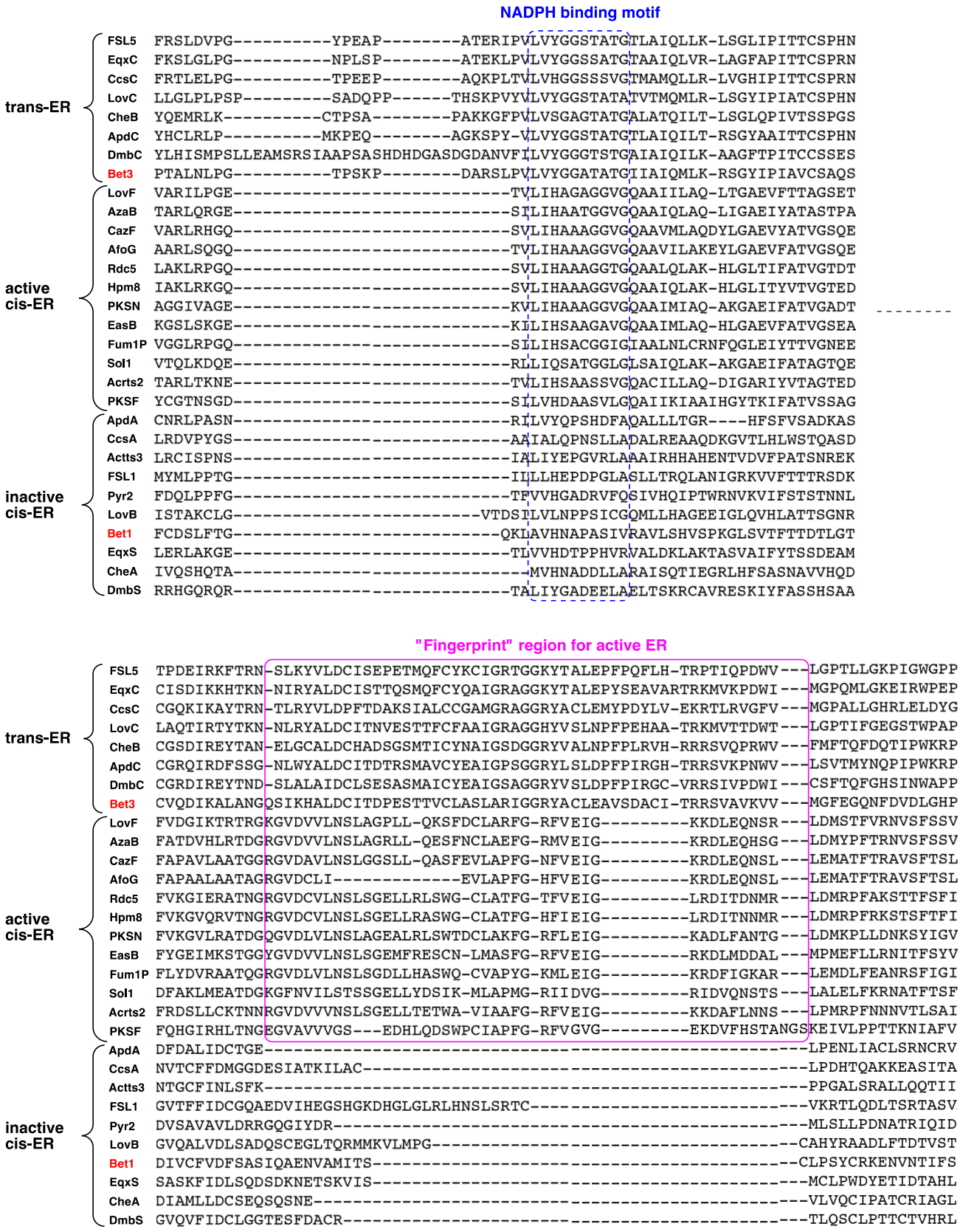


Figure S9. Construction of expression plasmid, pUARA2-*bet1*.

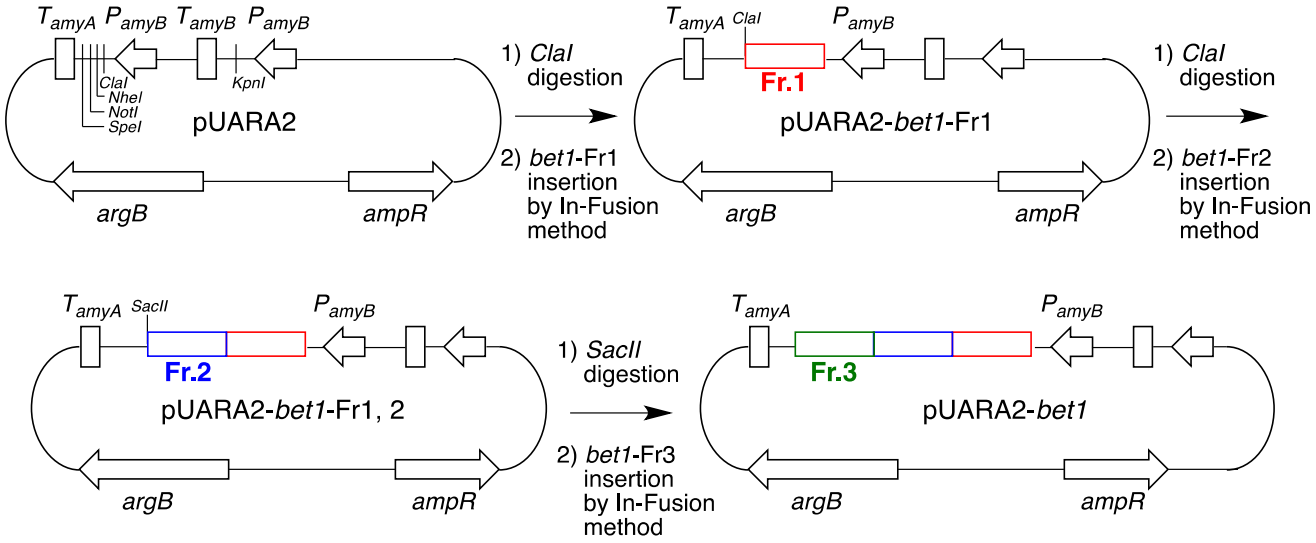


Table S1. Proposed function for betaenone biosynthetic genes from *Phoma betae*.

Gene	Size (bp)	Homolog		Identity (%)/ Similality (%)
orf2	1839	dehydrogenase	XP_007587319	67%/79%
orf1	1761	FAD binding domain protein	XP_008031658	71%/82%
bet4	1041	short-chain dehydrogenase	XP_008031659	83%/90%
bet3	1095	enoyl reductase (trans-ER)	XP_003306797	82%/88%
bet2	1566	cytochrome P450	EMD93172	87%/91%
bet1	8715	polyketide synthase [KS-AT-DH-MT-ER^0-KR-ACP-R]	AFN68297	84%/90%
orf3	405	hypothetical protein	XP_007688029	49%/52%
orf4	3369	hypothetical protein	XP_003303789	39%/54%

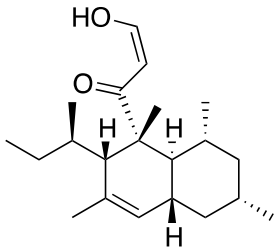
Table S2. Summary of functionally characterized HR-PKSs.

Compound	HR-PKS	Accession No.	trans-ER	Accession No.	Chain release mechanism	Reference
solanapyrone	Sol1 [KS-MAT-DH-MT-ER-KR-ACP]	BAJ09789	—	—	spontaneous cleavage	ChemBioChem 2010, 11, 1245.
alternapyrone	PKSN [KS-MAT-DH-MT-ER-KR-ACP]	BAD83684	—	—	spontaneous cleavage	Chem. Biol. 2005, 12, 1301.
aslanipyrone	PKSF [KS-MAT-DH-ER-KR-ACP]	BAE80697	—	—	spontaneous cleavage	ChemBioChem 2006, 7, 920.
pyripyropene	Pyr2 [KS-MAT-DH^{o}-MT^{o}-ER^{o}-KR^{o}-ACP]	XP_751268	—	—	spontaneous cleavage	Nat. Chem. 2010, 2, 858.
hypothemycin	Hpm8 [KS-MAT-DH-MT^{o}-ER-KR-ACP]	ACD39767	—	—	transfer to NR-PKS	J. Am. Chem. Soc. 2013, 135, 1735.
asperfuranone	AfoG [KS-MAT-DH-MT-ER-KR-ACP]	XP_658640	—	—	transfer to NR-PKS	J. Am. Chem. Soc. 2009, 131, 2965.
radicicol	Rdc5 [KS-MAT-DH-MT^{o}-ER-KR-ACP]	ACD39774	—	—	transfer to NR-PKS	J. Biol. Chem. 2010, 285, 41412.
azanigerone A	AzaB [KS-MAT-DH-MT-ER-KR-ACP]	EHA28244	—	—	transfer to NR-PKS	Chem. Biol. 2012, 19, 1049.
chaetomugilin A	CazF [KS-MAT-DH-MT-ER-KR-ACP]	XP_001216280	—	—	transfer to NR-PKS	J. Am. Chem. Soc. 2012, 134, 17900.
emericellamide	EasB [KS-MAT-MT-DH-ER-KR-ACP]	XP_660151	—	—	unknown	Chem. Biol. 2008, 15, 527.
ACR-toxin	Acrts2 [KS-MAT-DH-MT-ER^{o}-KR-ACP]	BAN19720	—	—	unknown	Mol Plant Microbe Interact. 2012, 25, 1419.
ACT-toxin	Actts3 [KS-MAT-DH-MT-ER^{o}-KR-ACP]	BAJ14522	—	—	unknown	Mol Plant Microbe Interact. 2010, 23, 406.
fumonisin B1	Fum1p [KS-MAT-DH-MT-ER-KR-ACP]	AAD43562	—	—	unknown	FEMS Microbiol. Lett. 2005, 248, 257.
lovastatin	LovF [KS-MAT-DH-MT-ER-KR-ACP]	AAD34559	—	—	LovD catalyzing acyl transfer	Science 2009, 326, 589.
lovastatin	LovB [KS-MAT-DH-MT-ER^{o}-KR-ACP-CON]	AAD39830	LovC	AAD34554	LovG catalyzing hydrolysis	Science 2009, 326, 589.
fusarielin F	FSL1 [KS-MAT-DH-MT-ER^{o}-KR-ACP]	ESU17181	FSL5	EYB31404	unknown	Environ. Microbiol. 2012, 14, 1159.

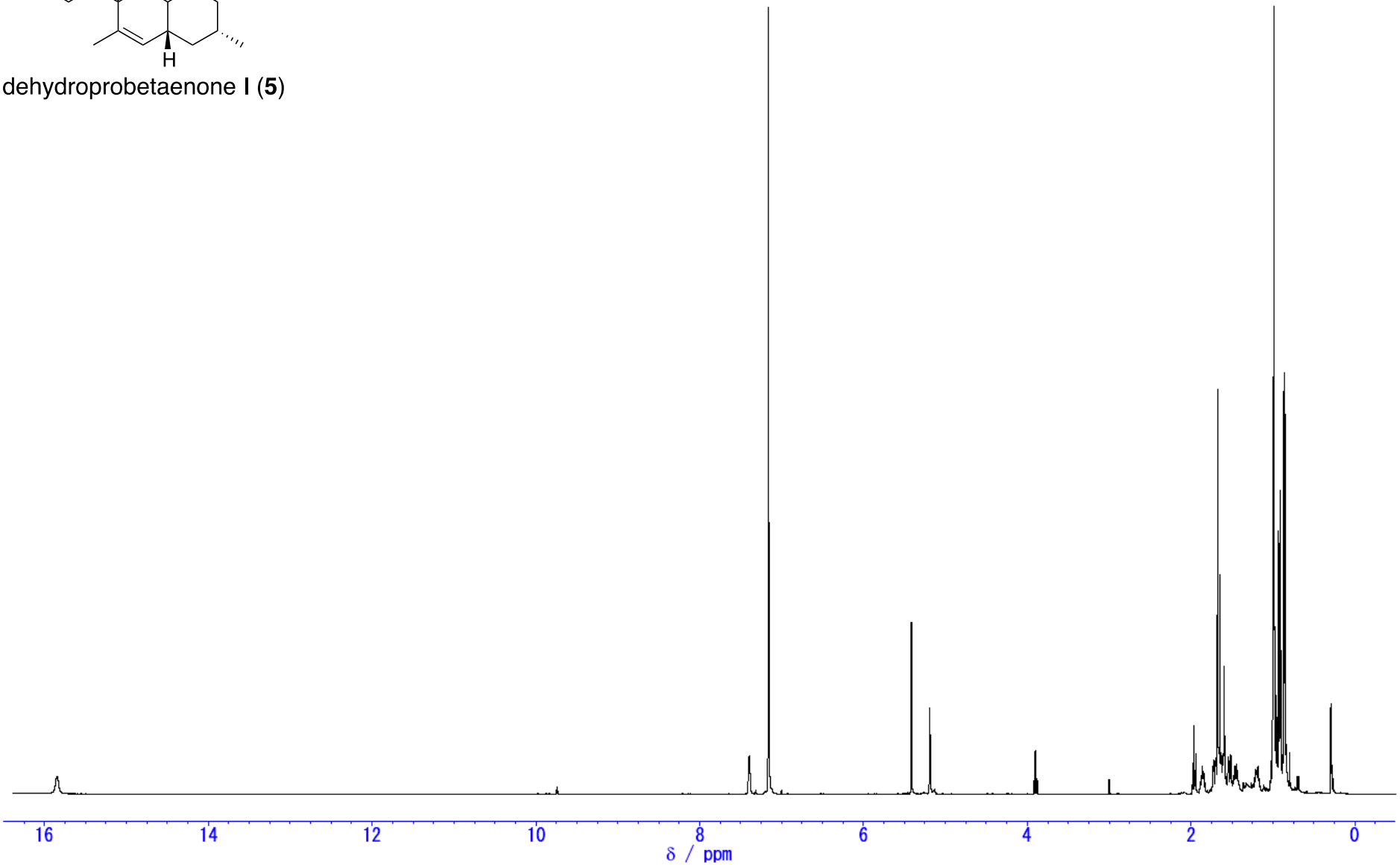
Table S3. Oligonucleotides used for construction of expression plasmids.

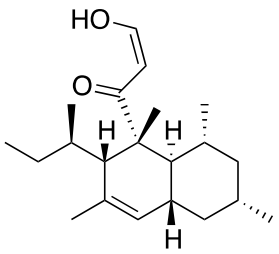
Primers

Insert	Restriction site	Sequence 5'-3'	Size Vector
bet1-Fr1	*Cla*I	F: **CAAGCTCCGAATTCGAATCG**TTATGTCATCATCAGC R: **CGCTAGCTCAAATCG**ATGGCATCTTCGTCC	2.3 kb pUARA2
bet1-Fr2	*Cla*I	F: **GGGGACGAAGATGCCATCG**ATGAGATCGCGGC R: **CGCTAGCTCAAATCG**ATCCGCGGGACTAGCTCTCG	3.2 kb pUARA2-*bet1* Fr1
bet1-Fr3	*Sac*II	F: **GGACGAGAGCTAGTCCC**GCGGTTGTTTACC R: **TAGCTCAAATCGATCC**TCACGCAGCCTCCGACC	3.4 kb pUARA2-*bet1* Fr1, 2
bet3	*Cla*I	F: **CTCCGAATTCGAATCG**ATATGCCCCCAACGAACG R: **GCGGCCGCTAGCTCAAATCG**ATCTATGAAGATATATTGACC	1.2 kb pUSA2
bet2	*Nhe*I	F: **AATTCGAATCGATTTGAGCTAG**CATGGATCTATTCAAGACCG R: **TAGTGCGGCCGCTAG**CTACTGCGCTTCC	1.8 kb pAdeA2
bet4	*Nhe*I	F: **TTCGAATCGATTTGAGCTAG**CATGACGCCGGCGAAAGC R: **GTCACTAGTGCGGCCGCTAG**CCTACAGGAAGGCTTTG	1.2 kb pAdeA2
orf1	*Kpn*I	F: **TCCGGAATTCGAGCTCG**GTACCATGAAATCATTCGC R: **ACTACAGATCCCCGGG**GTACCCTAAACCCTACAAAGC	1.9 kb pAdeA2-*bet2*
orf2	*Nhe*I	F: **TCGAATCGATTTGAGCTAG**CATGGTTGAACATATCGG R: **TAGTGCGGCCGCTAG**CTCAATTCGCCCAGTC	1.9 kb pUNA2
adeA	—	F: **GTGCCAAGCTTGCATG**TCGCAGATAAACATGTTGTGGC R: **CAGAACGGCAGATCCTC**GTGCGGAACTCCGAATCCTC	2.7 kb pUSA2
bet1-Fr3-S2705A-1	—	F: **GGACGAGAGCTAGTCCC**GCGGTTGTTTACCATCAAGGC R: GAGGGCTACACCGGC*TGC*GGAGATGTAGTGCAG	—
bet1-Fr3-S2705A-2	—	F: CTGCACTACATCTCC*GCA*GCCGGTGTAGCCCTC R: **TAGCTCAAATCGATCC**GCGGTCACGCATCCTCCGACC	—
bet1-Fr3-Y2737F-1	—	F: **GGACGAGAGCTAGTCCC**GCGGTTGTTTACCATCAAGGC R: CCATTTTCCACACAT*GAA*CCCGTGCGAGCCGTCG	—
bet1-Fr3-Y2737F-2	—	F: CGACGGCTCGCACGGG*TTC*ATGTGTGGAAAATGG R: **TAGCTCAAATCGATCC**GCGGTCACGCATCCTCCGACC	—
bet1-Fr3-K2741A-1	—	F: **GGACGAGAGCTAGTCCC**GCGGTTGTTTACCATCAAGGC R: GCATCTTCTCGCACACCCA*CGC*TCCACACATATACCC	—
bet1-Fr3-K2741A-2	—	F: GGGTATATGTGTGGA*GCG*TGGGTGTGCGAGAAGATGC R: **TAGCTCAAATCGATCC**GCGGTCACGCATCCTCCGACC	—



dehydroprobetaenone I (**5**)





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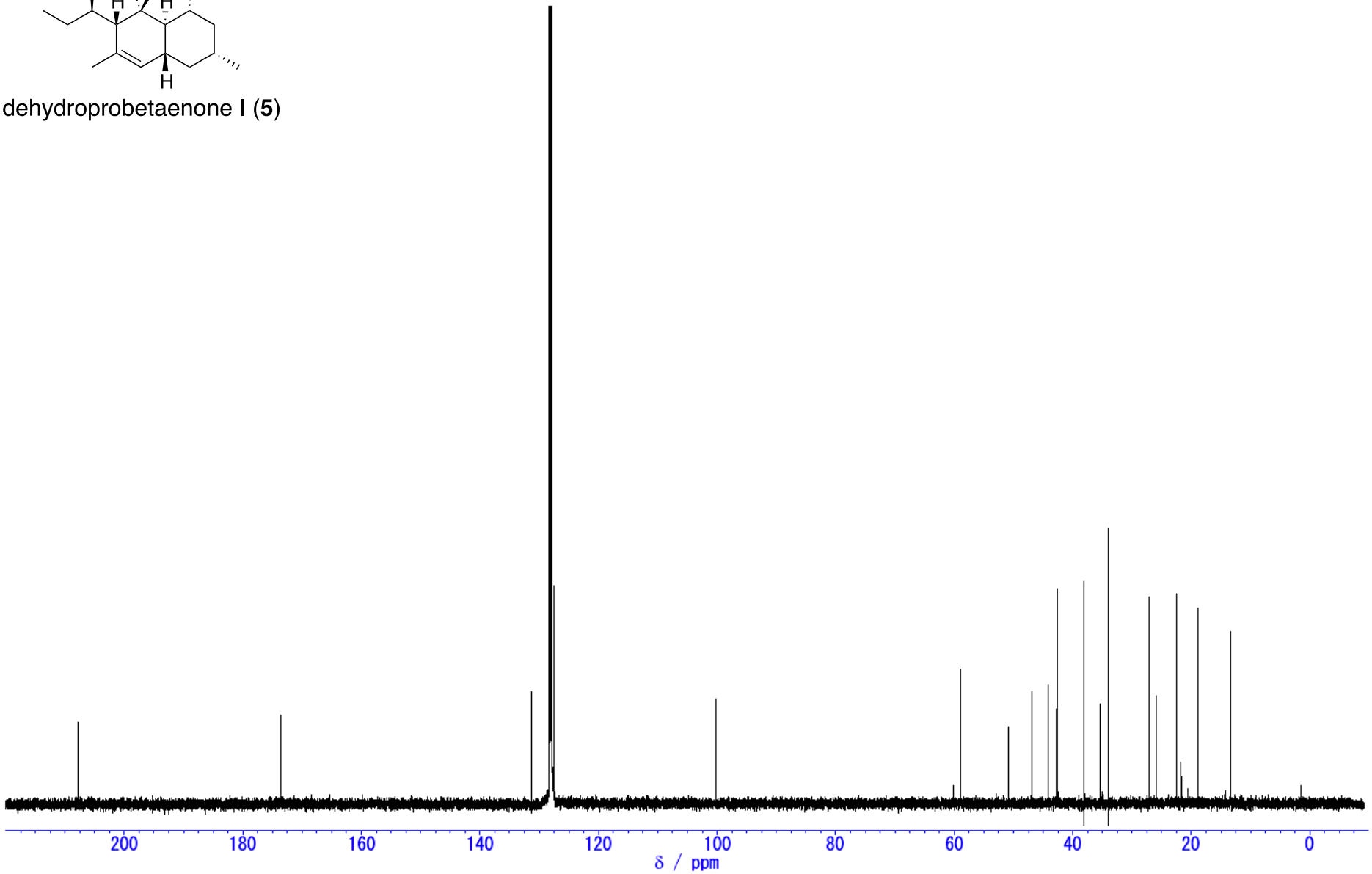
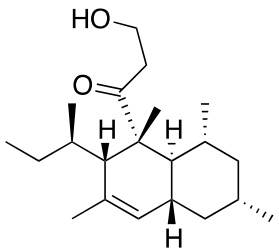
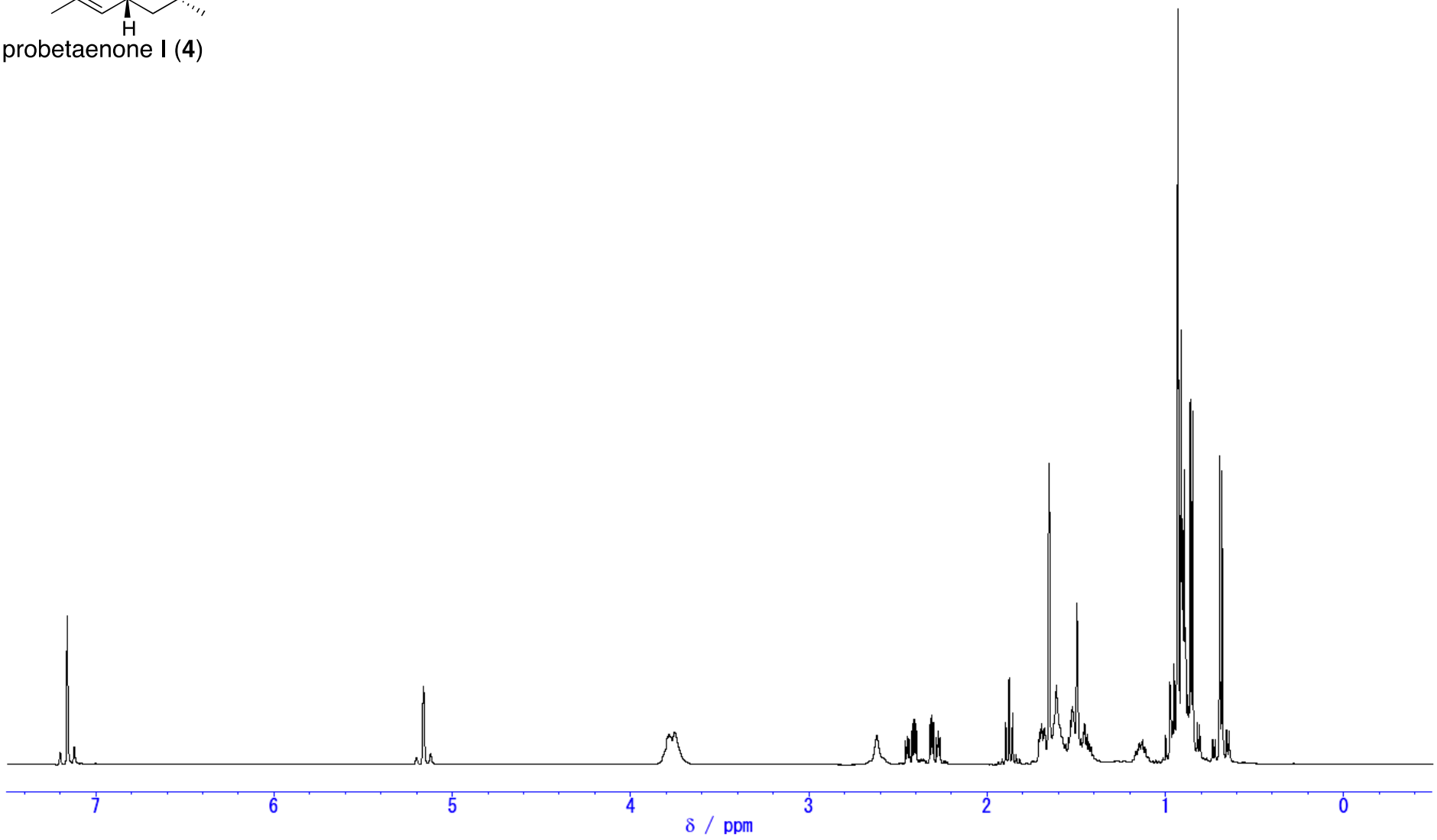
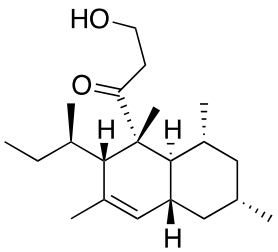




probetaenone I (**4**)





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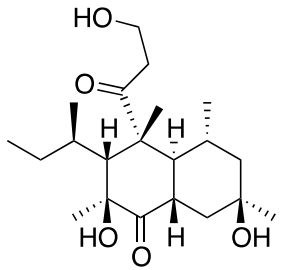
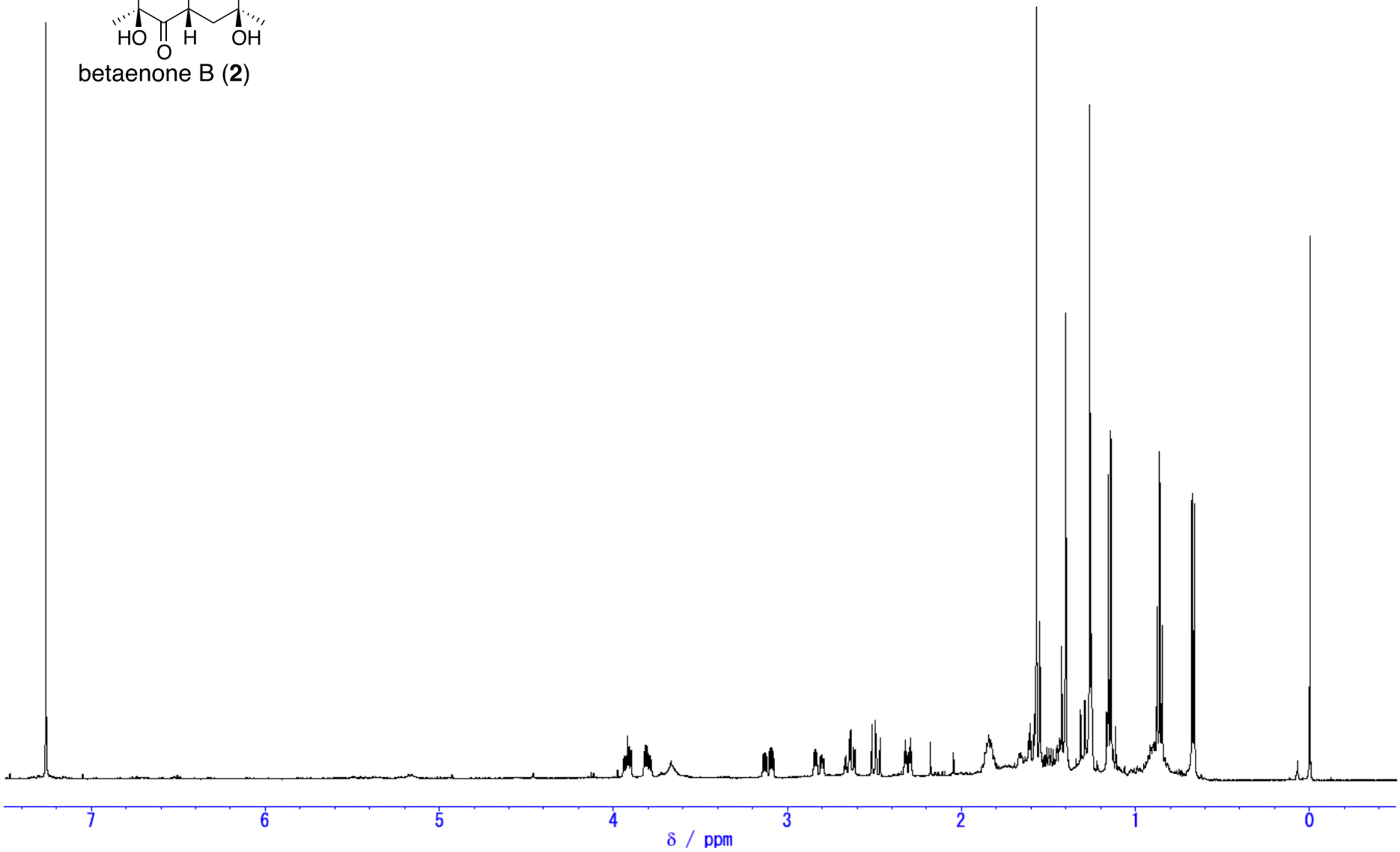
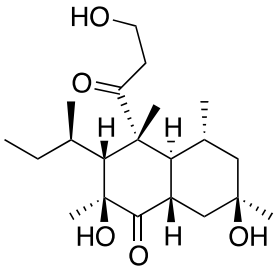


betaenone B (**2**)





betaenone B (**2**)