Supporting information

Biochemical Characterization of a Multi-Functional Mononuclear Non-Heme Iron Enzyme (PtlD) in Neopentalenoketolactone Biosynthesis

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Materials

All reagents were purchased from Sigma unless otherwise stated. All high-performance liquid chromatography (HPLC) was performed on a Shimadzu HPLC instrument, and all compounds involved were tested by monitoring absorption wavelength at 228 nm by a PDA detector (SPD-M20A). A Dikma Diamonsil C18 analytical chromatographic column (250× 4.6 mm, 5 μm) and an Agilent ZORBAX C18 semi-preparative chromatographic column (250× 9.4 mm, 5 μm) were employed in the HPLC. Compounds were analyzed by HPLC coupled with high-resolution mass spectrometry (HRMS) on a Thermo Scientific LTQ-Orbitrap instrument with a positive (or negative) ion mode. Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on Agilent 400-MR DD2 or Agilent 600-MR DD2.

Methods

PtlD protein over-expression and anaerobic purification

The codon optimized *ptlD* gene was synthesized by Genewiz according to the PtlD protein sequence (GenBank accession number WP_010984426.1) of *S. avermitilis* ATCC 31267 with a Strep-tag II (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) added at the N-terminal. The gene was cloned into the *Eco*RI/*Xho*I sites of pET28a (+) vector (Novagen) and the pET28a_*ptlD* recombinant plasmid was transformed into *E.coli* BL21 (DE3) for protein over-expression. A single colony was inoculated into 10 mL of LB media with 50 μg/mL kanamycin and incubated at 37 ℃ for 16 hrs. The seed culture was transferred into 1 L LB media with 50 μg/mL kanamycin and 0.1 mM $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ and incubated at a 37 ℃ shaker until the OD_{600} reached 0.6. The culture was cooled down and inducted by IPTG with a final concentration of 0.1 mM. Then, the culture was incubated overnight at 18 ℃ and the cells were harvested by centrifugation (4,000 ×*g*, 15 min) and stored at -80 ℃.

The following procedure was carried out in the anaerobic glove box (Coy Laboratory). 8.0 g of cells were re-suspended with 50 mL of anaerobic buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) with 100 μM ascorbic acid, 50 μM $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ and 1.0 mg/mL lysozyme, and incubated on ice for 30 min. Cell lysis was carried out by sonication and the cell debris was removed by centrifugation at 12,000 ×*g* for 40 min. The supernatant was loaded onto a Streptactin Beads 4FF column with 30 mL resin, which was equilibrated with lysis buffer. The column was washed with 50 mL of anaerobic buffer and the Strep-tagged PtlD protein was eluted with 50 mL of 2.5 mM desthiobiotin. The protein was concentrated by ultrafiltration, flash-frozen by liquid nitrogen and stored at -80 ℃ for further enzymatic assay.

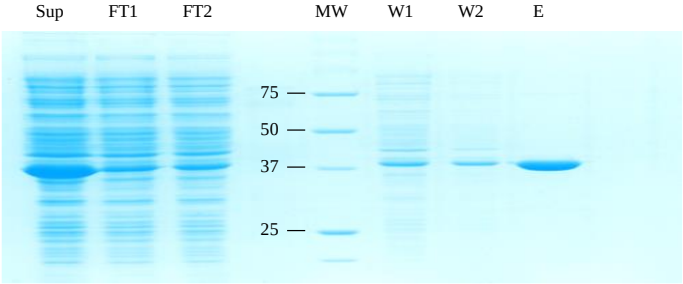


Figure S1: SDS-PAGE analysis of anaerobically purified PtlD protein. The calculated molecular

weight of Strep-tagged PtlD is 35.6 kDa. Sup, supernatant; FT1, flow through portion 1; FT2, flow through portion 2; MW, protein molecular weight marker (kDa); W1, wash portion 1; W2, wash portion 2; E, elution.

Construction of the *S. avermitilis* Δ*ptlD*/Δ*SAV_7469* double deletion mutant

We in-frame deleted the *ptlD* and *SAV_7469* genes of *S. avermitilis* ATCC 31267 to accumulate Neopentalenolactone D (**11**) according to the literature.[1-3] To conduct the homologous double cross-over, the two DNA fragments that flanked the *SAV_7469* gene were amplified by PCR with *S. avermitilis* total DNA as template. The 3193 bp up-stream homologous arm was amplified with the forward primer 7469F1 (5' ACGACGGCCAGTGCCAAGCTGTGGAGTCGCTCCTCACCATGCAA-3′) and the reverse primer 7469R2 (5′-GACCGCAGCCTCTTACCACATGAAAGACCTCGCAGGCGAAGACT -3′). The 3224 bp down-stream arm was amplified with the forward primer 7469F3 (5'-CCTGCGAGGTCTTTCATGTGGTAAGAGGCTGCGGTCCATGCACA-3') and the reverse primer 7469R4 (5'-CTATGACATGATTACGAATTTCTGCTACGTCAACGCCTTCCAGG-3'). The two amplified DNA fragments were inserted into the *Hin*dIII/*Eco*RI restrict enzyme sites of a temperature sensitive plasmid pKC1139. The recombinant plasmid was introduced into *S. avermitilis* by conjugation and the Δ*SAV_7469* mutant was screened out with a standard protocol.[4] The in-frame deletion mutant was confirmed by DNA sequencing. For the *ptlD* gene mutation, the same strategy was followed except that the conjugation host was *S. avermitilis* Δ*SAV_7469* mutant. The *ptlD* up-stream arm was amplified with the forward primer PtlDF1 (5'-CGACGGCCAGTGCCAAGCTTTGGCCACGGTGAAACACGACCT-3') and the reverse primer PtlDR2 (5'-AAAGCCCGTTTGGCGTGGACGATCTCTTCGACCTGCGTGT-3'), and the down-stream arm was amplified with the forward primer PtlDF3 (5'-ACACGCAGGTCGAAGAGATCGTCCACGCCAAACGGGCTTT-3') and the reverse primer PtlDR4 (5'-TATGACATGATTACGAATTCCGGAGCGCTTCGGTGATGAT-3'). The *S. avermitilis* Δ*ptlD*/Δ*SAV_7469* double deletion mutant was screened out and confirmed by DNA sequencing.

Purification of Neopentalenolactone D from *S. avermitilis* Δ*ptlD*/Δ*SAV_7469* mutant cells

A *S. avermitilis* Δ*ptlD*/Δ*SAV_7469* double deletion mutant was used to obtain the Neopentalenolactone D (**11**). The spores of mutant were inoculated into TSBY medium and shook at 28 ℃ for 2 days to prepare the seed cells, then 20 mL cell culture was transferred into a 2.0 L flask containing 500 mL of fermentation medium (40 g soluble starch, 20 g soy flour, 0.5 g $FeSO_4 \bullet 7H_2O$, 1.0 g K_2HPO_4, and 0.3 g KCl per liter, pH 6.5).[5] It was incubated at 28 ℃, 200 *rpm* for 6 days. The culture supernatant was collected by centrifugation and acidified with 2.5 N HCl to pH 2.5. It was extracted twice with equal volume of ethyl acetate for each time. The organic fraction was concentrated under reduced pressure and subjected to macroporous resin (Amberlite ® XAD16N) chromatography. Then, the column was eluted with five times volume of 50%, 70% and 90% methanol, respectively. Neopentalenolactone D (**11**) was eluted out with 90% methanol and concentrated for further chromatography purification, which was conducted on an Agilent ZORBAX C18 semi-preparative chromatographic column with a flow rate of 2 mL/min. Solvent A was H_2O with 0.1% formic acid, solvent B was acetonitrile with 0.1% formic acid, and the gradient was 0-3 min, 15% B, 3-17 min, from 15% B to 90% B, 17-22 min, 90% B, 22-27 min, from 90% B to 15% B, 27-30 min, 15% B. The Neopentalenolactone D (**11**) was eluted at 18.5 min.

PtlD enzymatic assay

Due to its hydrophobic nature, neopentalenolactone D (**11**) and neopentalenolactone E (**12**) was dissolved in methanol to prepare a 100 mM stock solution, respectively. Before use in PtlD-catalysis, the neopentalenolactone D (or E) methanol stock solution was diluted using the reaction buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0). In all of the activity assay, the methanol content was kept at less than 5% (v/v) to minimize its effect on PtlD activity.

1-mL reaction system contained 0.5 mM Neopentalenolactone D (**11**) (or 0.5 mM Neopentalenolactone E), 0.5 mM α-ketoglutarate (or 1.0 mM α-ketoglutarate) and 1.0 mM sodium ascorbate in air saturated Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0). The reaction was initiated by addition of anaerobically purified PtlD protein to a final concentration of 20 μM. The mixture was incubated at 25 ℃ for 2 hrs, and then the reaction was quenched by 2 mL of methanol. Protein precipitate was removed by a centrifuge at 12,000 ×*g*, 20 min and the supernatant was collected for HPLC-HRMS analysis. The mobile phase was 50% acetonitrile with 0.1% formic acid and the flow rate was 0.3 mL/min. HPLC traces were recorded by monitoring the absorption at 228 nm and mass spectra was recorded by scanning mass to charge ratio from 200 to 600 with a positive ion mode. Selected ion monitoring was employed in tandem mass spectrometry with a collision-induced dissociation at 35 eV.

A 2-mL reaction system was set up with Neopentalenolactone E (**12**) as substrate in air saturated Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) to determine the PtlD reaction time-course information. The reaction contained 0.5 mM Neopentalenolactone E (**12**), 1.0 mM α-ketoglutarate, 2.0 mM sodium ascorbate and 10 μM PtlD. The mixture was incubated at 25 ℃. A 0.2 mL aliquot of the reaction mixture was taken at each time points from 0 min to 2 hrs. Every single sample was quenched immediately by twice volume of methanol and a final concentration of 10 mM EDTA. Protein precipitate was removed and the supernatant was collected for HPLC analysis directly. To record the PtlD reaction time course by ^{1}H NMR, the reaction was run in 50 mM KPi buffer prepared with D_2O (pH 7.6). All of the co-factors and protein stocks were prepared in D_2O as well. Methanol-D_4 was used to quench the reaction at certain time points.

Isolation of PtlD reaction intermediates and products from large scale reaction

Neopentalenolactone E (**12**) was accumulated in the PtlD enzymatic reaction in which one equivalent of α- ketoglutarate was used relative to the amount of the substrate Neopentalenolactone D (**11**). To obtain Neopentalenolactone E (**12**), a 10-mL reaction was set up in a 250 mL round flask. The reaction was performed in air saturated Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0), containing 1mM Neopentalenolactone D (**11**), 1 mM α-ketoglutarate and 2 mM sodium ascorbate. The reaction was initiated by addition of anaerobically purified PtlD protein with a final concentration of 40 μM. The mixture was incubated at 25 ℃ for 2 hrs, and then the reaction was quenched by twice volume of methanol. The protein precipitate was removed by centrifuge at 12,000 ×*g*, 20 min and the supernatant was collected and concentrated under reduced pressure for further purification by HPLC with the same purification method as Neopentalenolactone D (**11**). Compound **14**, **15** and **17** were purified from the reaction in which two equivalents of α-ketoglutarate was used relative to the amount of Neopentalenolactone D (**11**). After the reaction was quenched, the supernatant was stewed at 25 ℃ for another 14 h. Then it was concentrated and injected for chromatography as described above.

Oxygen consumption assay of the PtlD reactions

The assay mixtures contained 3.4 μM enzyme, 1.0 mM α-ketoglutarate, 1.0 mM sodium ascorbate in air-saturated Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) and various amounts of Neopentalenolactone D (**11**) in a total volume of 1.0 mL. The PtlD•α-ketoglutarate•ascorbate mixture was prepared anaerobically and aliquoted in the Coy Chamber before use. The oxygen consumption rates were monitored using a Neofox oxygen electrode.

Oxygen incorporation reactions

To run the PtlD reaction in $H_2{}^{18}O$ buffer under $^{16}O_2$ gas atmosphere, a 0.2 mL reaction system was set up in air saturated Tris buffer (25 mM Tris-HCl, 50 mM NaCl, pH 8.0) prepared with $H_2{}^{18}O$. The reaction mixture contained 0.5 mM Neopentalenolactone D (**11**), 1 mM α-ketoglutarate, 1 mM sodium ascorbate and 10 μM PtlD. Substrate **11** and enzyme were prepared as highly concentrated stock solutions, which then diluted using $H_2{}^{18}O$ buffer to prepare the reaction mixture. In the final reaction mixture, $H_2{}^{18}O$ represents ~90.2% (v/v). To run the PtlD reaction in $H_2{}^{16}O$ buffer under $^{18}O_2$ gas atmosphere, the system was vacuumed to remove $^{16}O_2$. A mixture contained 1 mM Neopentalenolactone D (**11**), 2 mM α-ketoglutarate, 2 mM sodium ascorbate and 20 μM PtlD was prepared anaerobically. The reaction was initiated by mixing the PtlD•**11**•α-ketoglutarate mixture with equal volume of $^{18}O_2$ (99% purity) saturated Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0). The reaction mixture was incubated at 25 ℃ for 30 min, and then it was quenched by twice volume of methanol and a final concentration of 10 mM EDTA. The protein precipitate was removed, and the supernatant was collected for HPLC-HRMS analysis immediately. Oxygen incorporation experiment of PtlD with Neopentalenolactone E (**12**) as substrate was carried out with the same method. The HPLC mobile phase was 50% acetonitrile with 0.1% ammonia hydroxide for the negative ion mode, and 50% acetonitrile with 0.1% formic acid for the positive ion mode. High resolution mass spectra was recorded by scanning mass to charge ratio from 200 to 600.

Characterizations of Neopentalenolactone D, Neopentalenolactone E and related compounds.

Neopentalenolactone D (**11**)

^{1}H NMR (400 MHz, CD_3OD) : δ 6.77 (m, 1H, H-7), 4.52 (q, *J* = 6.4 Hz, 1H, H-9), 3.27-3.19 (m, 1H, H-5), 3.09 (m, 1H, H-8), 3.01 (dd, J = 15.2, 6.8 Hz, 1H, H-12α), 2.55 (dd, *J* = 15.2, 10.9 Hz, 1H, H-12β), 1.99 (d, *J* = 13.8 Hz,1H, H-3α), 1.71 (dd, J = 13.4, 10.2 Hz, 1H, H-1α), 1.56 (m, 1H, H-1β), 1.44 (d, *J* = 13.8 Hz, 1H, H-3β), 1.37 (d, *J* = 6.4 Hz, 3H, H-10), 1.07 (s, 3H, H-14), 1.05 (s, 3H, H-15). ^{13}C NMR (100 MHz, CD_3OD) : δ175.95 (C-11), 167.36 (C-13), 149.65 (C-7), 137.64 (C-6), 79.88 (C-9), 59.08 (C-4), 56.51 (C-8), 53.72 (C-5), 48.75 (C-3), 44.98 (C-1), 42.29 (C-2), 34.04 (C-12), 31.40 (C-14), 29.30 (C-15), 15.34 (C-10).

Neopentalenolactone E (**12**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.97 (m, 1H, H-7), 4.90 (d, *J* = 2.2 Hz, 1H, H-10α), 4.74 (d, *J* = 2.3 Hz, 1H, H-10β), 3.56 (ddt, *J* = 9.6, 6.4, 2.8 Hz, 1H, H-8), 3.24 (dtd, *J* = 6.5, 4.4, 1.7 Hz, 1H, H-5), 2.85 (dd, *J* = 14.5, 6.5 Hz, 1H, H-12α), 2.72 (dd, *J* = 14.5, 5.2 Hz, 1H, H-12β), 2.23-2.18 (m, 1H, H-3α), 1.97-1.91 (m, 1H, H-1α), 1.73 (d, *J* = 13.8 Hz, 1H, H-3β), 1.39 (dd, *J* = 12.9, 6.9 Hz, 1H, H-1β), 1.08 (s, 6H, H-14 and H-15). ^{13}C NMR (100 MHz, $CDCl_3$) : δ170.48 (C-11), 167.88 (C-13), 160.05 (C-9), 151.84 (C-7), 132.63 (C-6), 96.82 (C-10), 58.31 (C-4), 56.28 (C-8), 52.63 (C-3), 51.00 (C-5), 45.99 (C-1), 40.51 (C-2), 33.93 (C-12), 29.37 (C-14), 29.28 (C-15).

Compound (**15**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (dd, *J* = 2.4, 1.2 Hz, 1H, H-7), 3.85 (m, 1H, H-8), 3.49 (m, 1H, H-5), 2.48-2.30 (m, 3H, H-12α, H-3α, H-12β), 2.26 (s, 3H, H-10), 1.81 (dd, *J* = 13.2, 9.2 Hz, 1H, H-1α), 1.72 (d, *J* = 13.9 Hz, 1H, H-3β), 1.46 (dd, *J* = 13.2, 5.2 Hz, 1H, H-1β), 1.04 (s, 3H, H-14), 0.86 (s, 3H, H-15). ^{13}C NMR (100 MHz, $CDCl_3$): δ 210.63 (C-9), 178.22 (C-11), 169.59 (C-13), 151.18 (C-7), 133.39 (C-6), 71.31 (C-4), 51.44 (C-8), 51.27 (C-3), 49.81 (C-5), 44.40 (C-1), 39.53 (C-2), 35.98 (C-12), 30.32 (C-14), 29.25 (C-15), 28.30 (C-10).

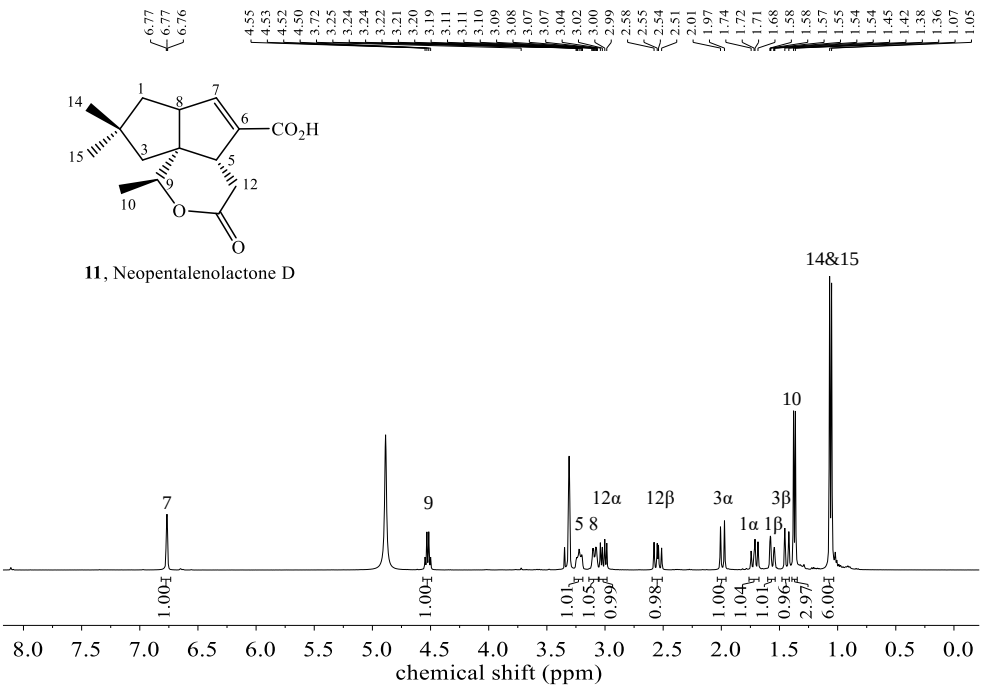
Neopentalenoketolactone (**14**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.88 (m, 1H, H-7), 4.51 (d, *J* = 16.0 Hz, H-10α), 4.35 (d, *J* = 16.0 Hz, H-10β), 3.97-3.89 (m, 1H, H-8), 3.56-3.48 (m, 1H, H-5), 2.62 (dd, *J* = 16.8, 5.5 Hz, 1H, H-12α), 2.32 (m, 2H, H-3α and H-12β), 1.86-1.76 (m, 2H, H-1α and H-3β), 1.49 (dd, *J* = 13.3, 5.1 Hz, 1H, H-1β), 1.06 (s, 3H, H-14), 0.89 (s, 3H, H-15). ^{13}C NMR (100 MHz, $CDCl_3$): δ204.13 (C-9), δ178.22 (C-11), δ169.44 (C-13), 151.32 (C-7), 133.03 (C-6), 69.91 (C-4), 52.59 (C-8), 50.95 (C-3), 50.34 (C-5), 48.10 (C-10), 44.21 (C-1), 39.66 (C-2), 35.33 (C-12), 30.20 (C-14), 29.55 (C-15).

Compound (**17**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.87 (dd, *J* = 2.4, 1.3 Hz, 1H, H-7), 4.47 (d, J = 18.9 Hz, H-10α), 4.38 (d, J = 18.9 Hz, H-10β), 3.84 (ddt, *J* = 8.0, 5.2, 2.5 Hz, 1H, H-8), 3.63 (s, 3H, H-16), 3.52 (dt, *J* = 6.7, 2.5 Hz, 1H, H-5), 2.67 (dd, *J* = 16.6, 4.3 Hz, 1H, H-12α), 2.35-2.21 (m, 2H, H-3α and H-12β), 1.84-1.71 (m, 2H, H-1α and H-3β), 1.46 (dd, *J* = 13.3, 5.4 Hz, 1H, H-1β), 1.05 (s, 3H, H-14), 0.85 (s, 3H, H-15). ^{13}C NMR (100 MHz, $CDCl_3$): δ 212.99 (C-9), 172.61 (C-11), 168.97 (C-13), 151.11 (C-7), 133.12 (C-6), 67.64 (C-4), 67.46 (C-10), 52.59 (C-8), 51.92 (C-16), 51.37 (C-5), 50.53 (C-3), 44.45 (C-1), 39.65 (C-2), 34.86 (C-12), 30.06 (C-14), 29.46 (C-15).

a



δ 6.77 (m, 1H, H-7), 4.52 (q, *J* = 6.4 Hz, 1H, H-9), 3.27-3.19 (m, 1H, H-5), 3.09 (m, 1H, H-8), 3.01 (dd, J = 15.2, 6.8 Hz, 1H, H-12α), 2.55 (dd, *J* = 15.2, 10.9 Hz, 1H, H-12β), 1.99 (d, *J* = 13.8 Hz,1H, H-3α), 1.71 (dd, J = 13.4, 10.2 Hz, 1H, H-1α), 1.56 (m, 1H, H-1β), 1.44 (d, *J* = 13.8 Hz, 1H, H-3β), 1.37 (d, *J* = 6.4 Hz, 3H, H-10), 1.07 (s, 3H, H-14), 1.05 (s, 3H, H-15).

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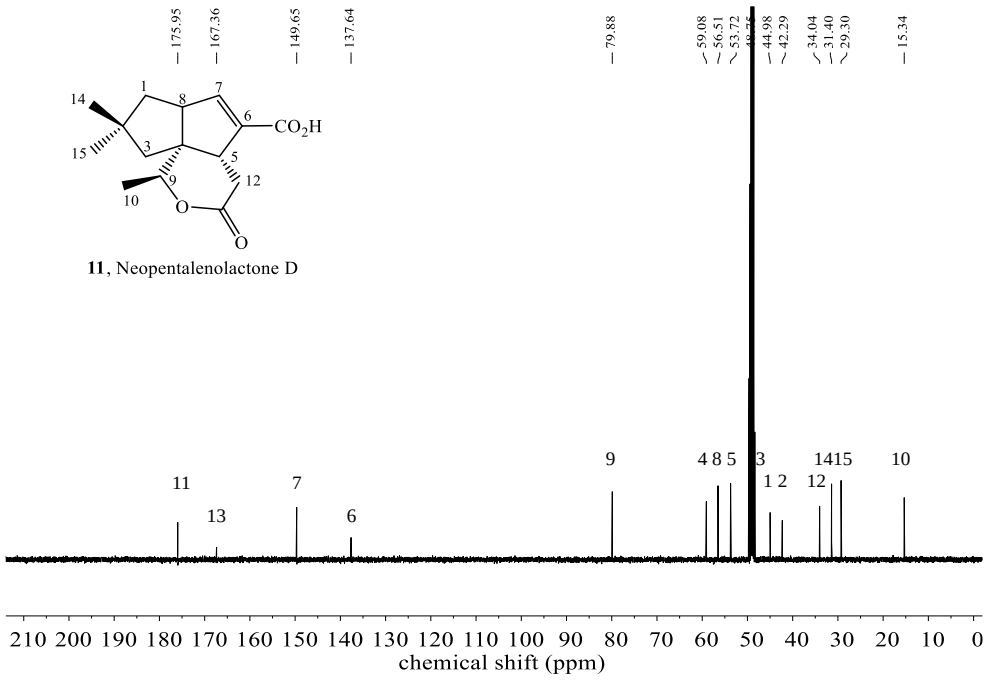

δ175.95 (C-11), 167.36 (C-13), 149.65 (C-7), 137.64 (C-6), 79.88 (C-9), 59.08 (C-4), 56.51 (C-8), 53.72 (C-5), 48.75 (C-3), 44.98 (C-1), 42.29 (C-2), 34.04 (C-12), 31.40 (C-14), 29.30 (C-15), 15.34 (C-10).

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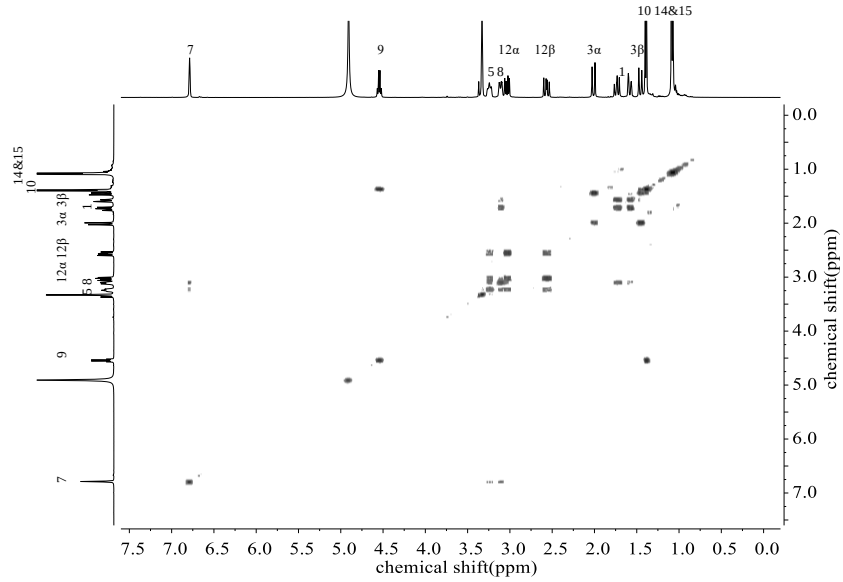
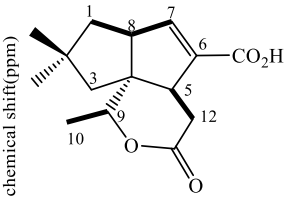
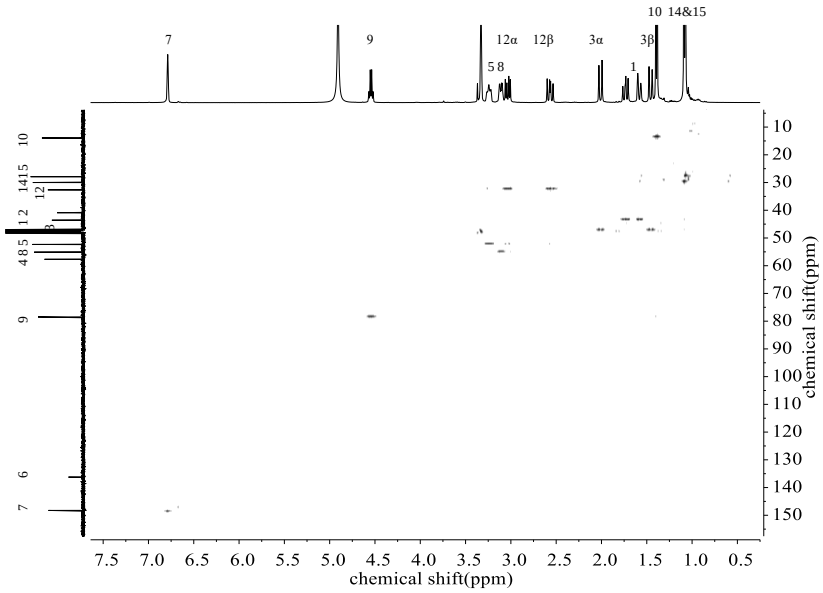
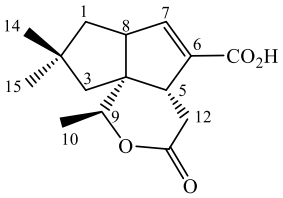




11, Neopentalenolactone D

d





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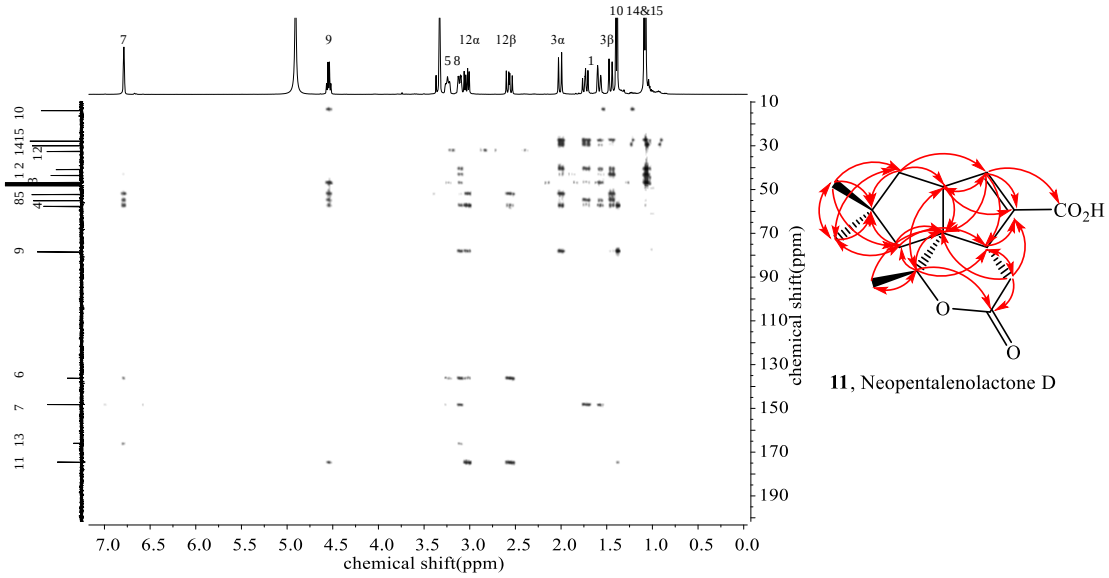

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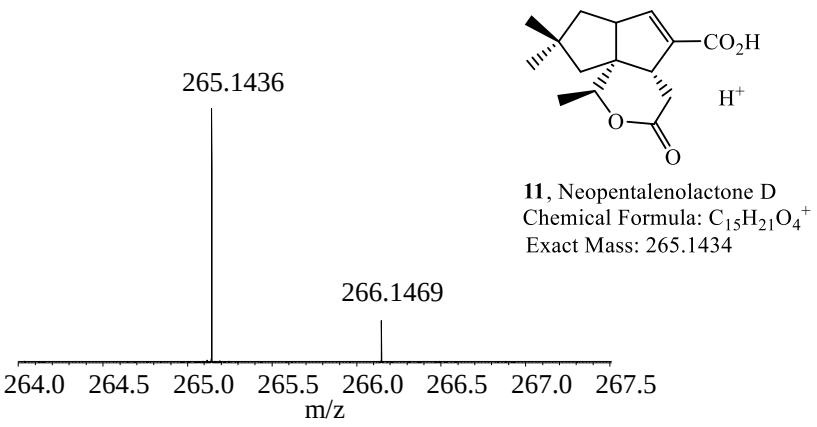
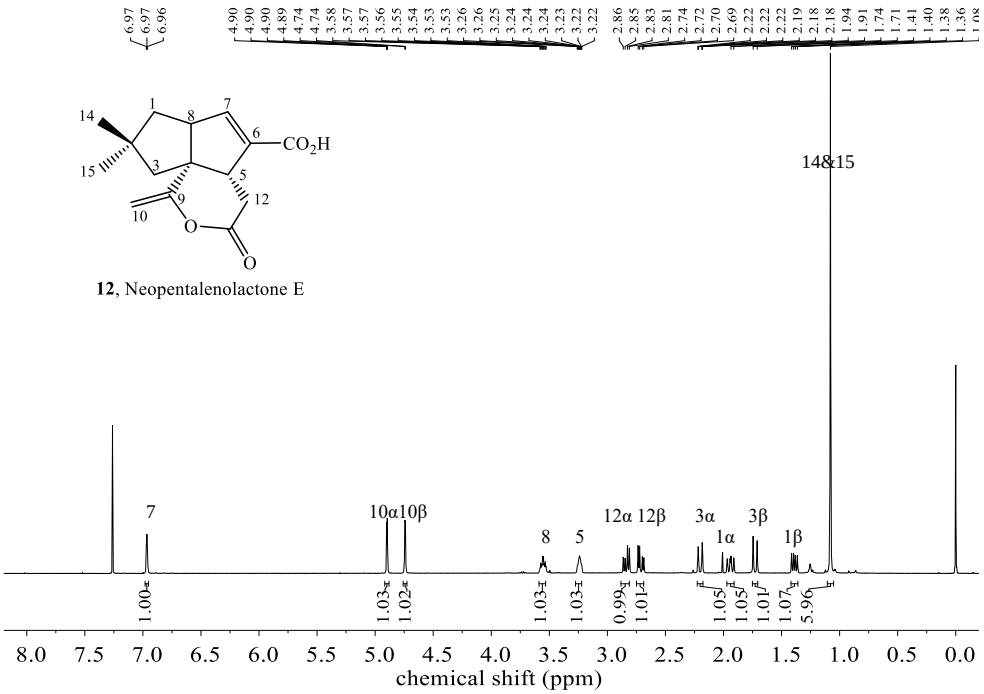


Figure S2. Neopentalenolactone D (**11**) characterizations. a, ^{1}H NMR spectrum of Neopentalenolactone D (**11**) (400 MHz, CD_3OD). b, ^{13}C NMR spectrum of Neopentalenolactone D (**11**) (100 MHz, CD_3OD). c, ^{1}H-^{1}H COSY spectrum of Neopentalenolactone D (**11**) (400 MHz, CD_3OD). d, ^{1}H-^{13}C HSQC spectrum of Neopentalenolactone D (**11**) (400 MHz ,100 MHz, CD_3OD). e, ^{1}H-^{13}C HMBC spectrum of Neopentalenolactone D (**11**) (400 MHz, 100 MHz, CD_3OD). f, High resolution ESI-MS of Neopentalenolactone D (**11**). The calculated exact mass of Neopentalenolactone D (**11**) $[M+H]^+$ is 265.1434 and found at 265.1436.

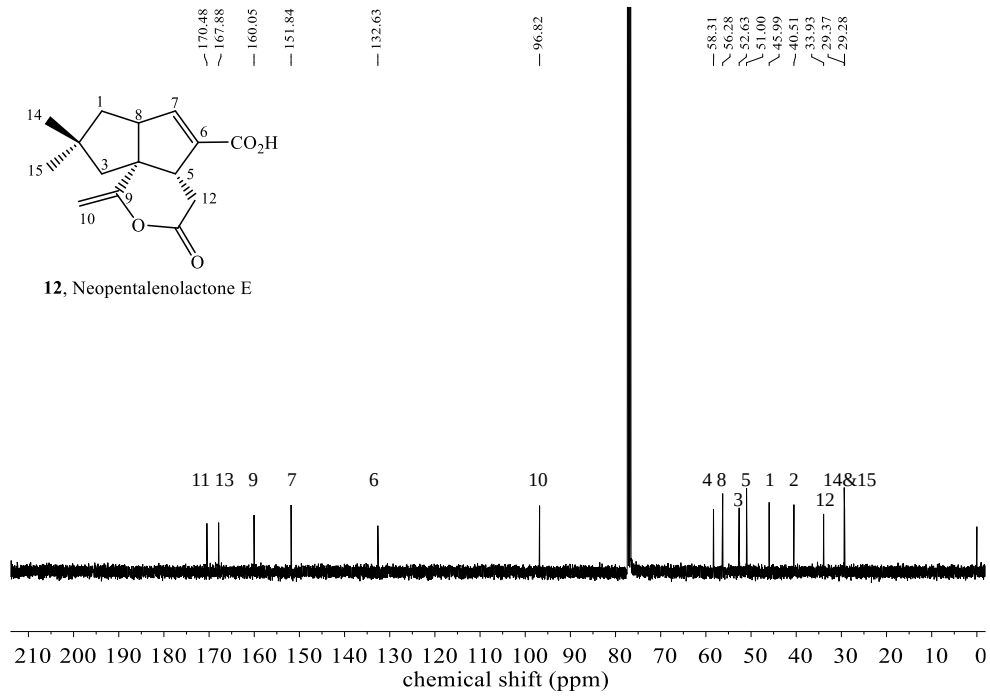
a



12, Neopentalenolactone E

δ 6.97 (m, 1H, H-7), 4.90 (d, *J* = 2.2 Hz, 1H, H-10α), 4.74 (d, *J* = 2.3 Hz, 1H, H-10β), 3.56 (ddt, *J* = 9.6, 6.4, 2.8 Hz, 1H, H-8), 3.24 (dtd, *J* = 6.5, 4.4, 1.7 Hz, 1H, H-5), 2.85 (dd, *J* = 14.5, 6.5 Hz, 1H, H-12α), 2.72 (dd, *J* = 14.5, 5.2 Hz, 1H, H-12β), 2.23-2.18 (m, 1H, H-3α), 1.97-1.91 (m, 1H, H-1α), 1.73 (d, *J* = 13.8 Hz, 1H, H-3β), 1.39 (dd, *J* = 12.9, 6.9 Hz, 1H, H-1β), 1.08 (s, 6H, H-14 and H-15).

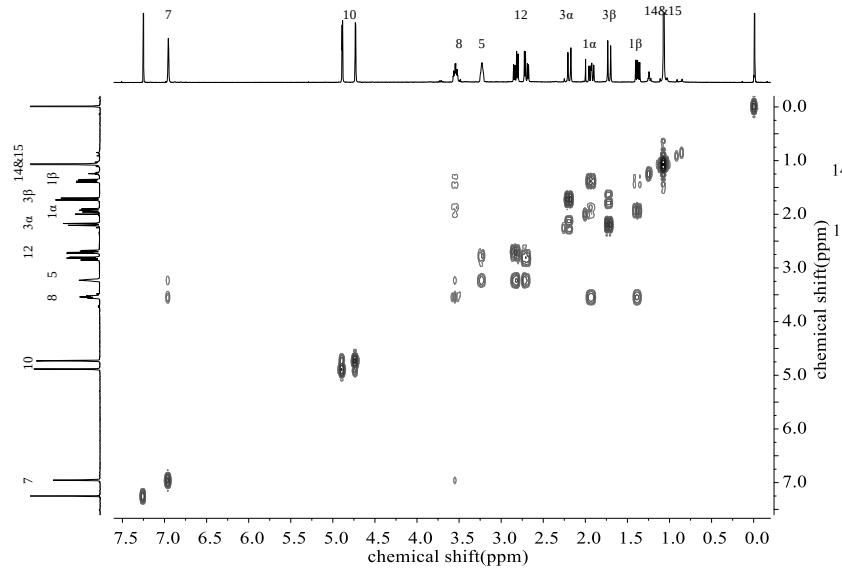
b

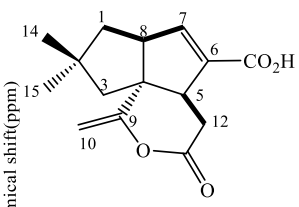


12, Neopentalenolactone E

δ170.48 (C-11), 167.88 (C-13), 160.05 (C-9), 151.84 (C-7), 132.63 (C-6), 96.82 (C-10), 58.31 (C-4), 56.28 (C-8), 52.63 (C-3), 51.00 (C-5), 45.99 (C-1), 40.51 (C-2), 33.93 (C-12), 29.37 (C-14), 29.28 (C-15).

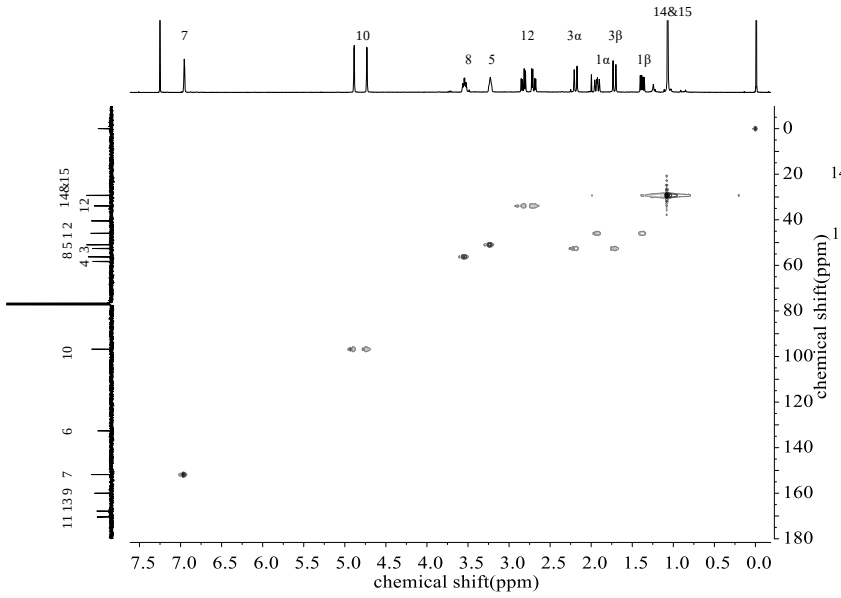
c

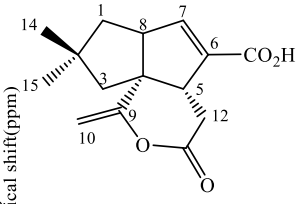




12, Neopentalenolactone E

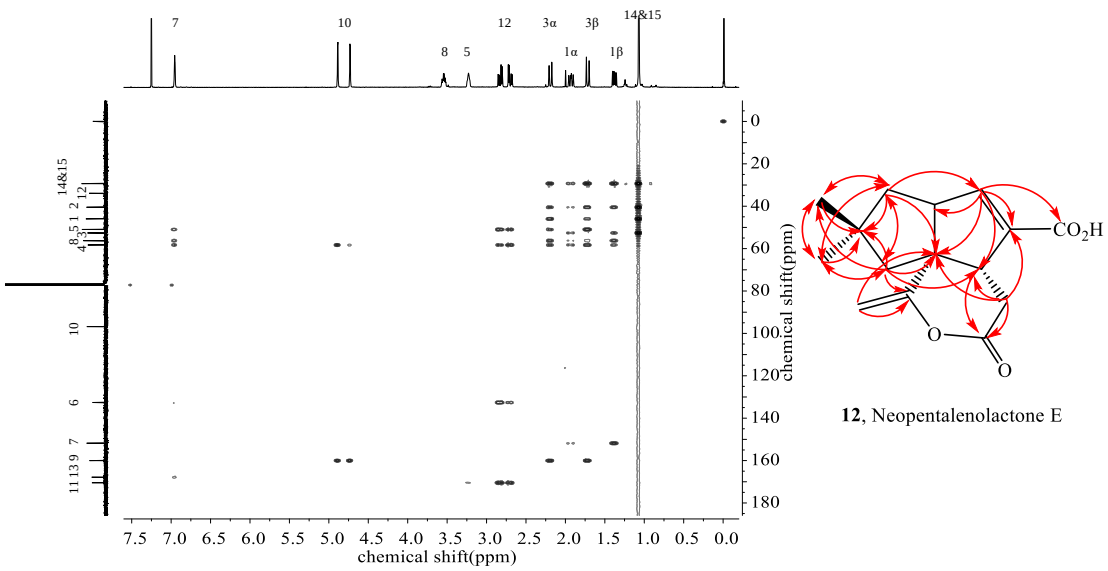
d





12, Neopentalenolactone E

e



f

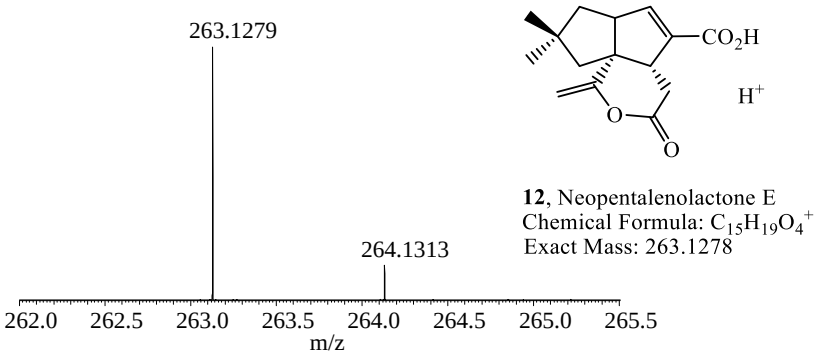
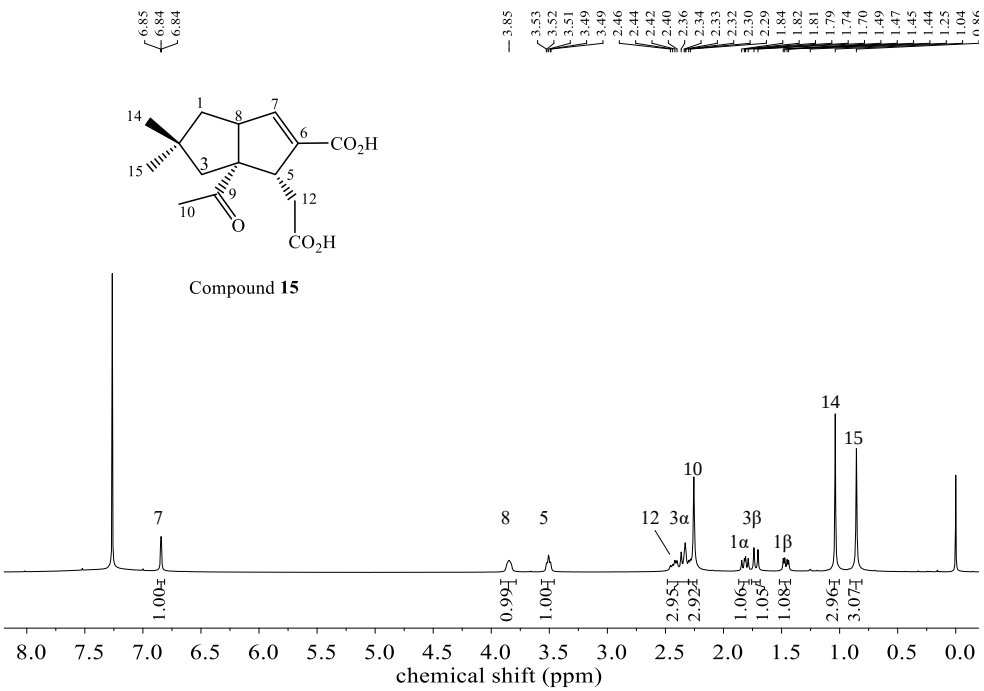


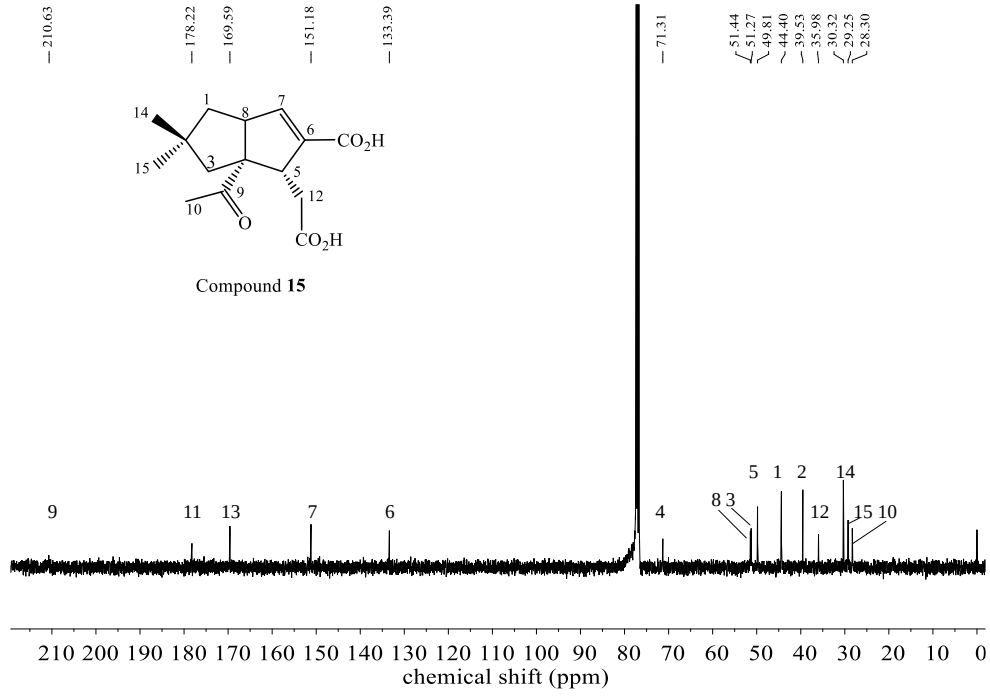
Figure S3. Neopentalenolactone E (**12**) characterizations. a, ^{1}H NMR spectrum of Neopentalenolactone E (**12**) (400 MHz, $CDCl_3$). b, ^{13}C NMR spectrum of Neopentalenolactone E (**12**) (100 MHz, $CDCl_3$). c,^{1}H-^{1}H COSY spectrum of Neopentalenolactone E (**12**) (400 MHz, $CDCl_3$). d, ^{1}H-^{13}C HSQC spectrum of Neopentalenolactone E (**12**) (400 MHz, 100 MHz, $CDCl_3$). e, ^{1}H-^{13}C HMBC spectrum of Neopentalenolactone E (**12**) (400 MHz, 100 MHz, $CDCl_3$). f, High resolution ESI-MS of Neopentalenolactone E (**12**). The calculated exact mass of Neopentalenolactone E (**12**) $[M+H]^+$ is 263.1278 and found at 263.1279.

a



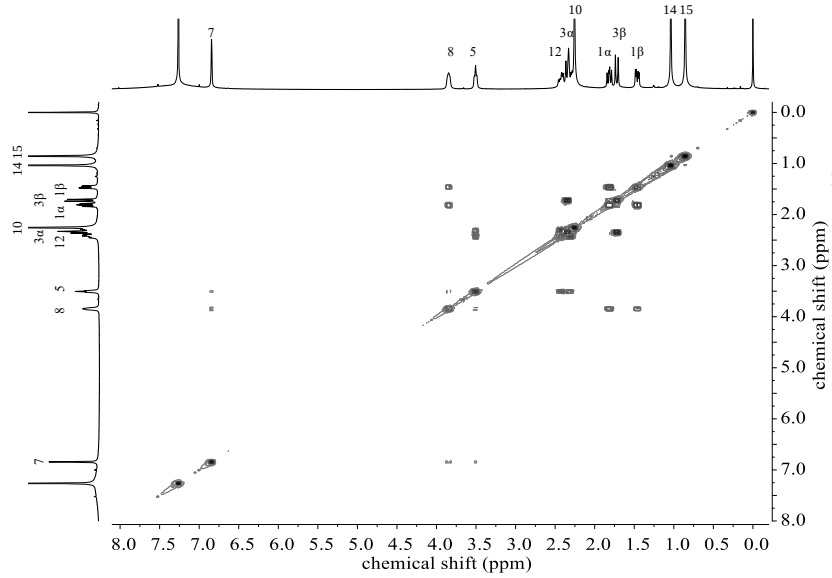
δ 6.84 (dd, *J* = 2.4, 1.2 Hz, 1H, H-7), 3.85 (m, 1H, H-8), 3.49 (m, 1H, H-5), 2.48-2.30 (m, 3H, H-12α, H-3α, H-12β), 2.26 (s, 3H, H-10), 1.81 (dd, *J* = 13.2, 9.2 Hz, 1H, H-1α), 1.72 (d, *J* = 13.9 Hz, 1H, H-3β), 1.46 (dd, *J* = 13.2, 5.2 Hz, 1H, H-1β), 1.04 (s, 3H, H-14), 0.86 (s, 3H, H-15).

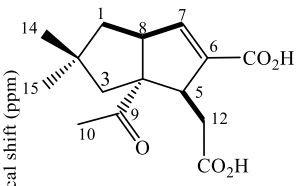
b



δ 210.63 (C-9), 178.22 (C-11), 169.59 (C-13), 151.18 (C-7), 133.39 (C-6), 71.31 (C-4), 51.44 (C-8), 51.27 (C-3), 49.81 (C-5), 44.40 (C-1), 39.53 (C-2), 35.98 (C-12), 30.32 (C-14), 29.25 (C-15), 28.30 (C-10).

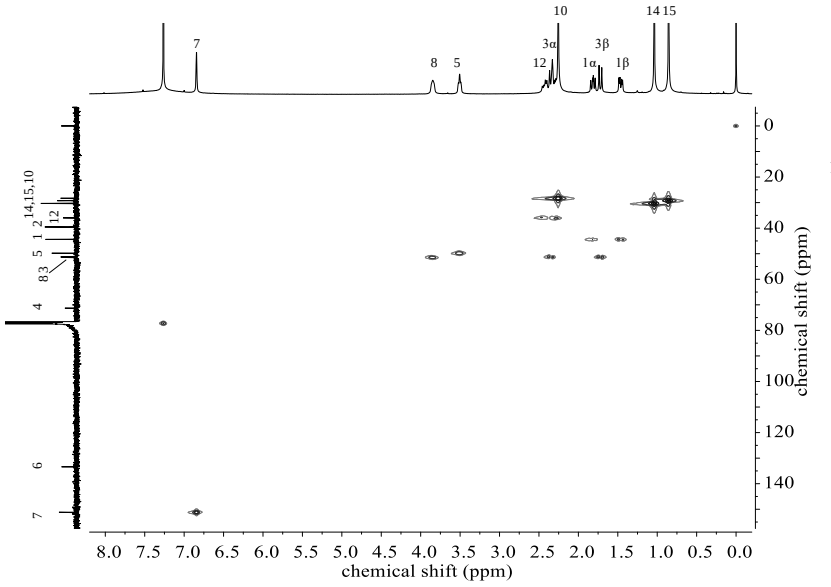
c

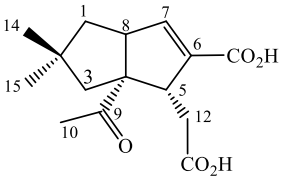




Compound **15**

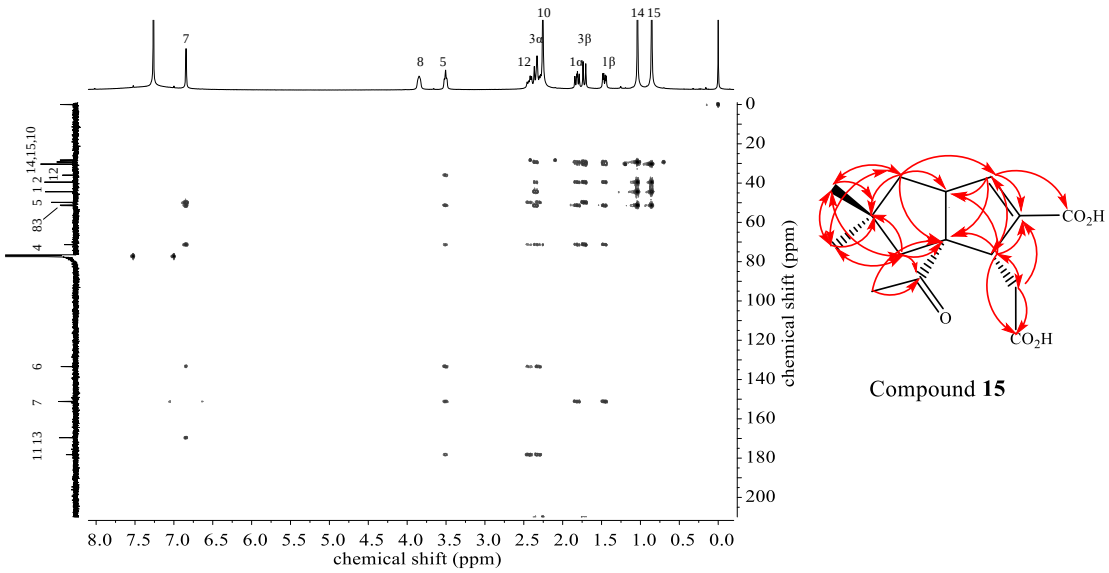
d





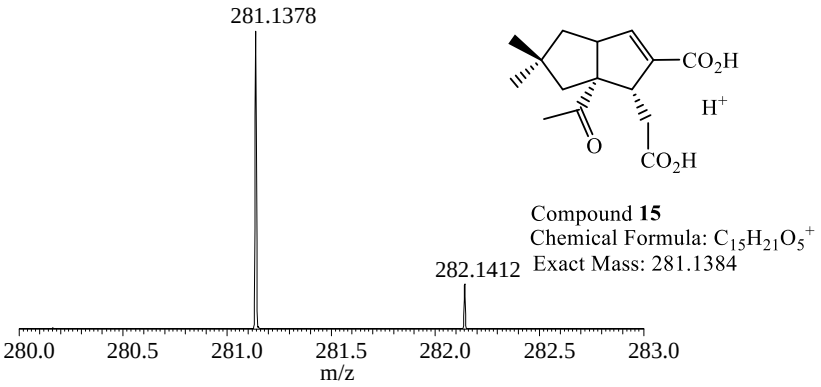
Compound **15**

e



Compound **15**

f



Compound **15**
Chemical Formula: $C_{15}H_{21}O_5^+$
Exact Mass: 281.1384

Figure S4. Compound **15** characterizations. a, ^{1}H NMR spectrum of compound **15** (400 MHz, $CDCl_3$). b, ^{13}C NMR spectrum of compound **15** (100 MHz, $CDCl_3$). c,^{1}H-^{1}H COSY spectrum of compound **15** (400 MHz, $CDCl_3$). d, ^{1}H-^{13}C HSQC spectrum of compound **15** (400 MHz, 100 MHz, $CDCl_3$). e, ^{1}H-^{13}C HMBC spectrum of compound **15** (400 MHz, 100 MHz, $CDCl_3$). f, High resolution ESI-MS of compound **15**. The calculated exact mass of compound **15** $[M+H]^+$ is 281.1384 and found at 281.1378.

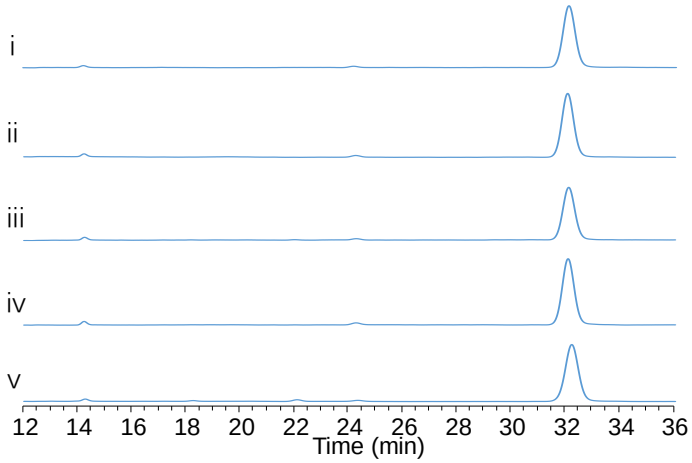


Figure S5. HPLC profiles of PtlD reactions. i), Neopentalenolactone E (**12**) standard. ii), 0.5 mM Neopentalenolactone E (**12**), 1.0 mM α-ketoglutarate, 1.0 mM sodium ascorbate, 10 mM EDTA and 20 μM anaerobically purified PtlD in air saturated Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0). iii), 0.5 mM Neopentalenolactone E (**12**), 1.0 mM sodium ascorbate and 20 μM anaerobically purified PtlD in air saturated Tris buffer. iv), 0.5 mM Neopentalenolactone E (**12**) and 20 μM aerobically purified PtlD in air saturated Tris buffer. v), 0.5 mM Neopentalenolactone E (**12**), 1.0 mM α-ketoglutarate, 1.0 mM sodium ascorbate and 20 μM anaerobically purified PtlD in anaerobic Tris buffer, reaction was carried out in anaerobic glove box. The mixture was incubated at 25 ℃ for 2 h, and then the reaction was quenched by twice volume of methanol. Protein precipitate was removed by centrifuge at 12,000 ×*g*, 20 min and the supernatant was collected for HPLC-HRMS analysis.

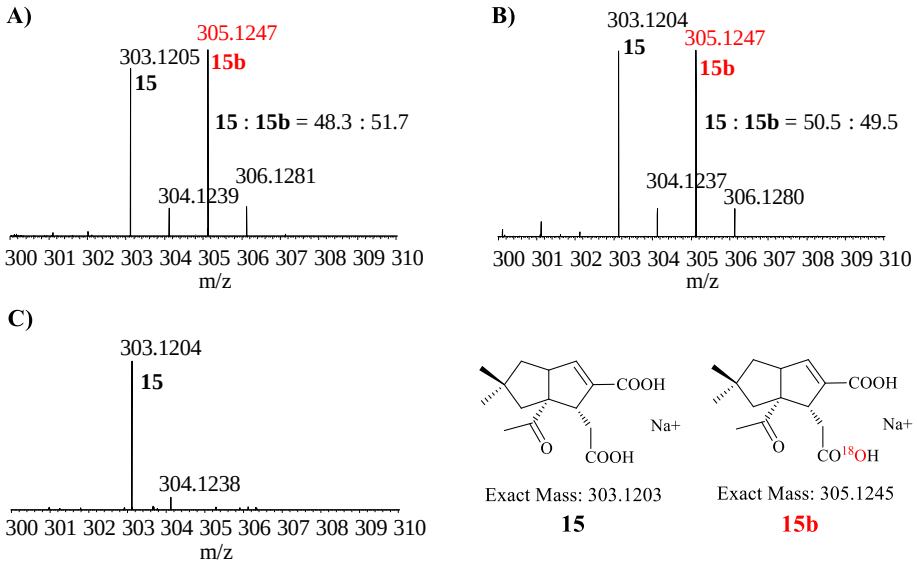
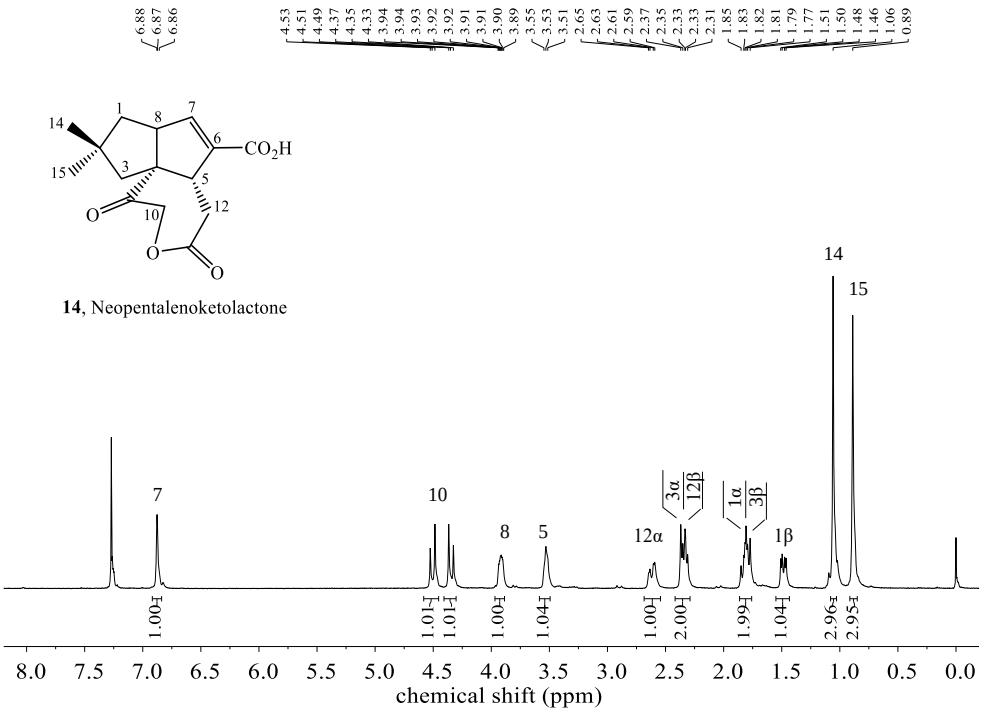


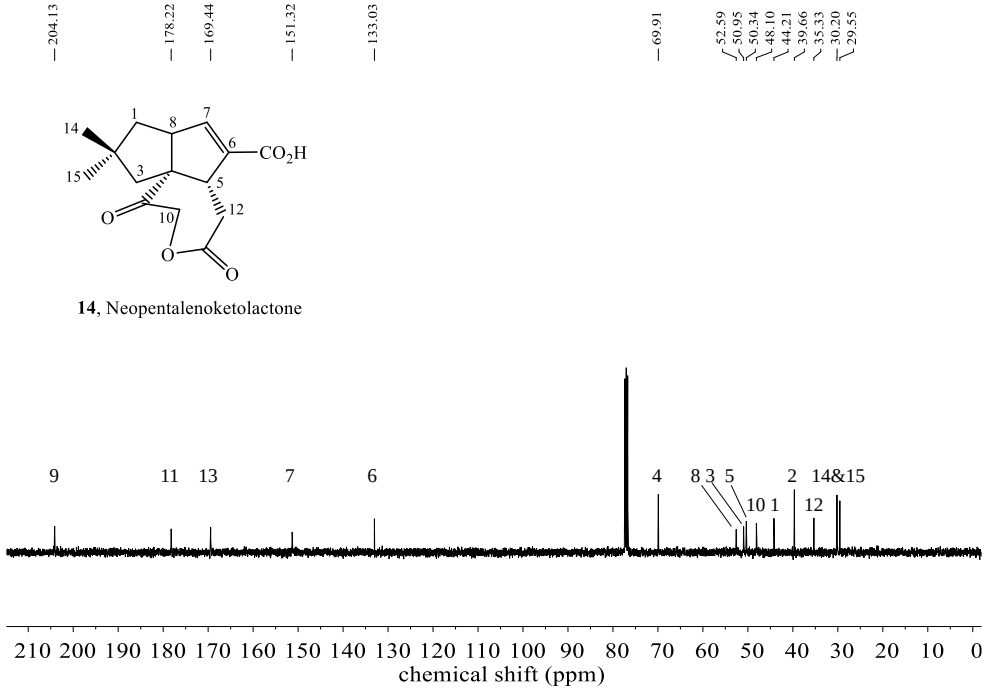
Figure S6. Oxygen incorporation reactions analyzed by high resolution mass spectrometry. PtlD-reaction with compound **11** as substrate under two conditions: A) under $^{18}O_2$ gas atmosphere; B) in $H_2{}^{18}O$ buffer. C) Compound **15** dissolved in $H_2{}^{18}O$ buffer with a final concentration of 0.5 mM was employed as a control system to check if the keto-group of compound **15** could exchange its oxygen with $H_2{}^{18}O$ under our experimental conditions (25 °C, 2 h). No [^{18}O]-incorporation was detected here. $^{18}O_2$ gas purity is 99%. $H_2{}^{18}O$ represents 90.2% (v/v) in the final reaction mixture.

a



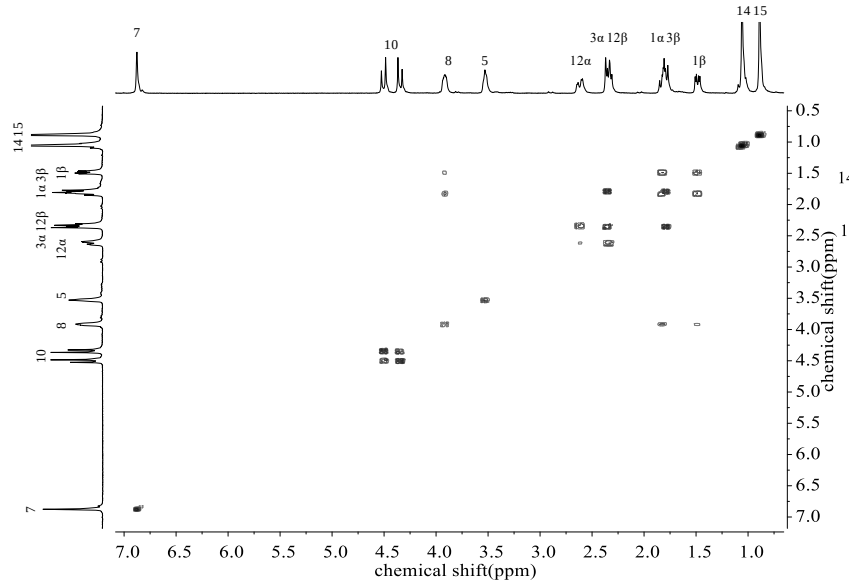
δ 6.88 (m, 1H, H-7), 4.51 (d, *J* = 16.0 Hz, H-10α), 4.35 (d, *J* = 16.0 Hz, H-10β), 3.97-3.89 (m, 1H, H-8), 3.56-3.48 (m, 1H, H-5), 2.62 (dd, *J* = 16.8, 5.5 Hz, 1H, H-12α), 2.32 (m, 2H, H-3α and H-12β), 1.86-1.76 (m, 2H, H-1α and H-3β), 1.49 (dd, *J* = 13.3, 5.1 Hz, 1H, H-1β), 1.06 (s, 3H, H-14), 0.89 (s, 3H, H-15).

b



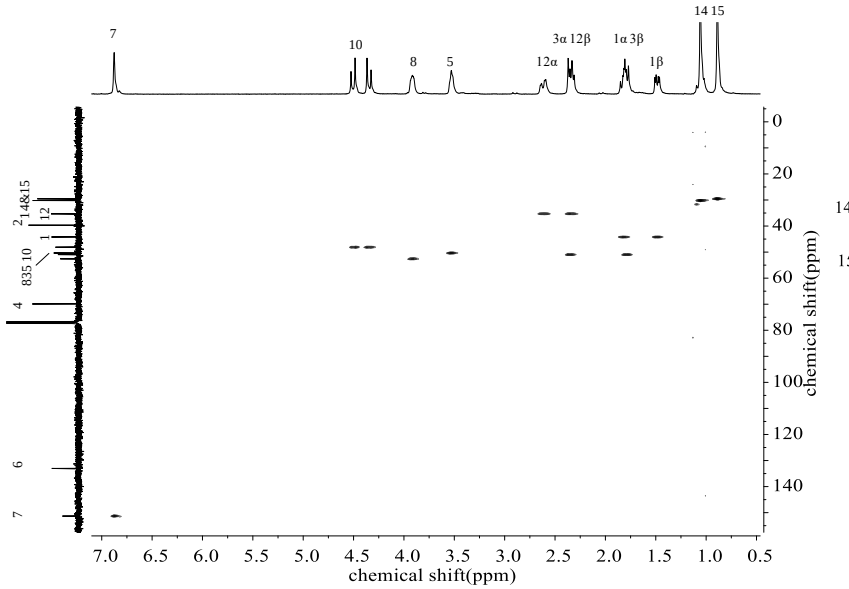
δ204.13 (C-9), δ178.22 (C-11), δ169.44 (C-13), 151.32 (C-7), 133.03 (C-6), 69.91 (C-4), 52.59 (C-8), 50.95 (C-3), 50.34 (C-5), 48.10 (C-10), 44.21 (C-1), 39.66 (C-2), 35.33 (C-12), 30.20 (C-14), 29.55 (C-15).

c



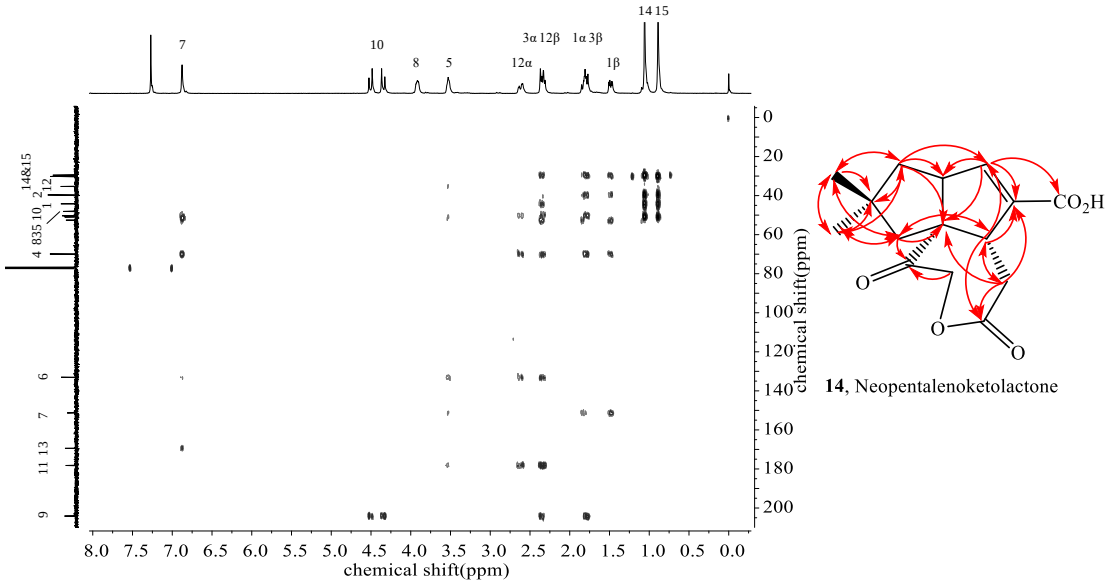
14, Neopentalenoketolactone

d



14, Neopentalenoketolactone

e



f

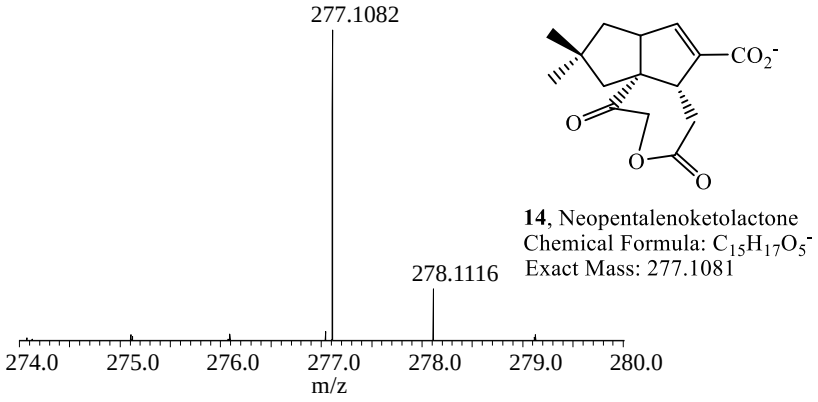
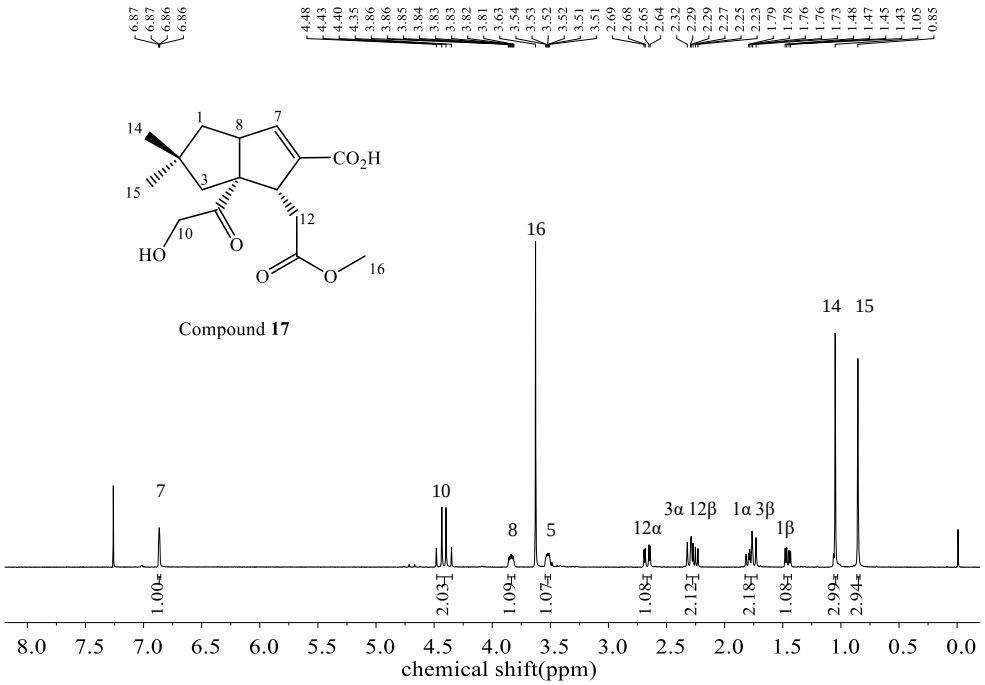


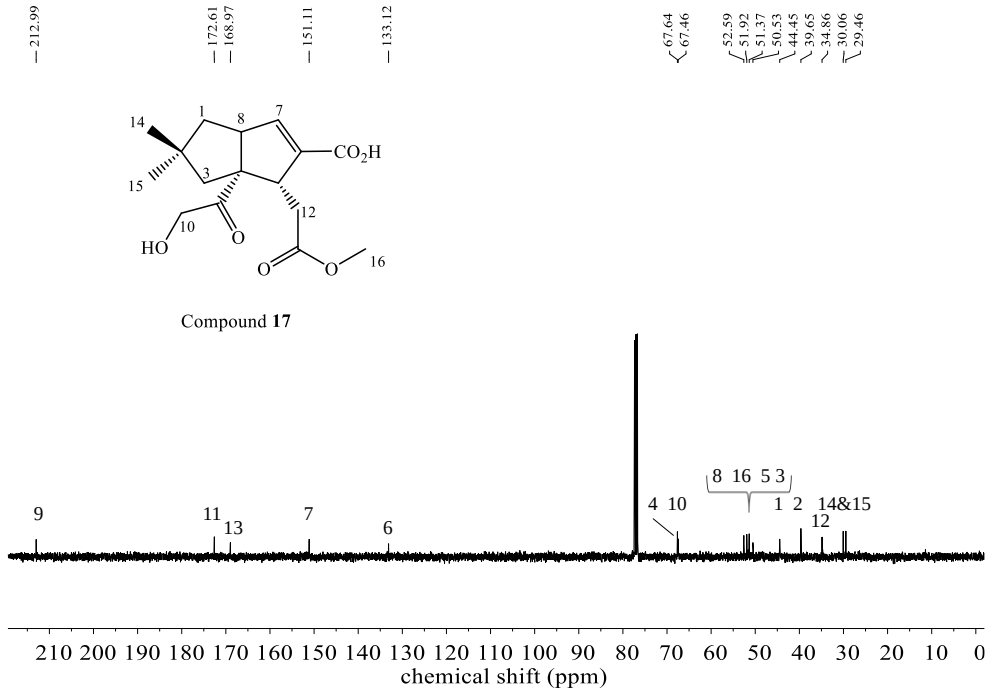
Figure S7. Neopentalenoketolactone (**14**) characterizations. a, ^{1}H NMR spectrum of Neopentalenoketolactone (**14**) (400 MHz, $CDCl_3$). b, ^{13}C NMR spectrum of Neopentalenoketolactone (**14**) (100 MHz, $CDCl_3$). c, ^{1}H-^{1}H COSY spectrum of Neopentalenoketolactone (**14**) (400 MHz, $CDCl_3$). d, ^{1}H-^{13}C HSQC spectrum of Neopentalenoketolactone (**14**) (400 MHz, 100 MHz, $CDCl_3$). e, ^{1}H-^{13}C HMBC spectrum of Neopentalenoketolactone (**14**) (400 MHz, 100 MHz, $CDCl_3$). f, High resolution ESI-MS of Neopentalenoketolactone (**14**), the calculated exact mass of Neopentalenoketolactone (**14**) $[M-H]^-$ is 277.1081 and found at 277.1082.

a



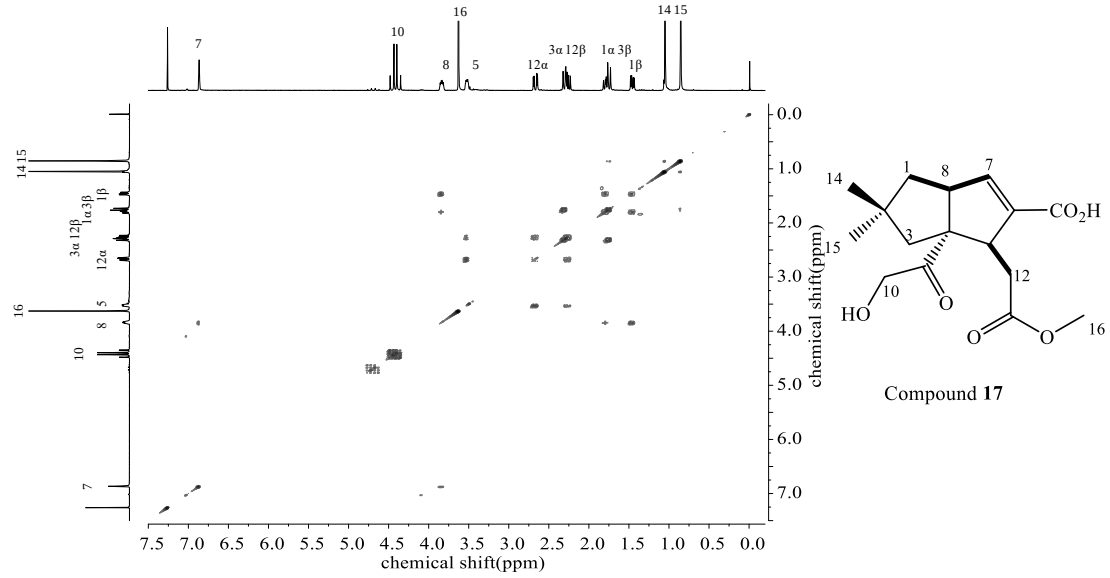
δ 6.87 (dd, *J* = 2.4, 1.3 Hz, 1H, H-7), 4.47 (d, J = 18.9 Hz, H-10α), 4.38 (d, J = 18.9 Hz, H-10β), 3.84 (ddt, *J* = 8.0, 5.2, 2.5 Hz, 1H, H-8), 3.63 (s, 3H, H-16), 3.52 (dt, *J* = 6.7, 2.5 Hz, 1H, H-5), 2.67 (dd, *J* = 16.6, 4.3 Hz, 1H, H-12α), 2.35-2.21 (m, 2H, H-3α and H-12β), 1.84-1.71 (m, 2H, H-1α and H-3β), 1.46 (dd, *J* = 13.3, 5.4 Hz, 1H, H-1β), 1.05 (s, 3H, H-14), 0.85 (s, 3H, H-15).

b



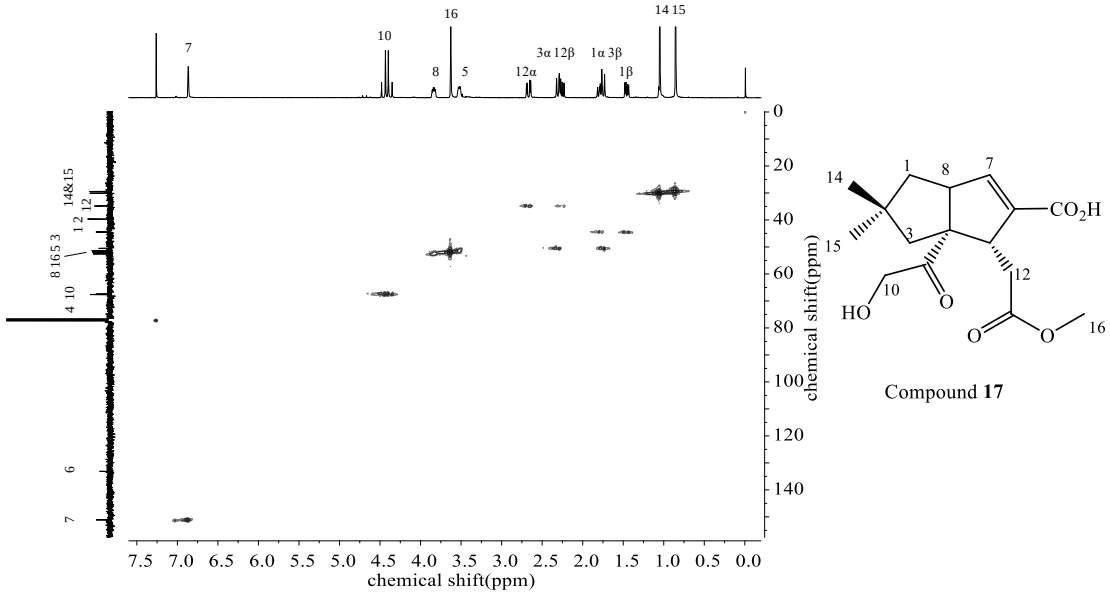
δ 212.99 (C-9), 172.61 (C-11), 168.97 (C-13), 151.11 (C-7), 133.12 (C-6), 67.64 (C-4), 67.46 (C-10), 52.59 (C-8), 51.92 (C-16), 51.37 (C-5), 50.53 (C-3), 44.45 (C-1), 39.65 (C-2), 34.86 (C-12), 30.06 (C-14), 29.46 (C-15).

c



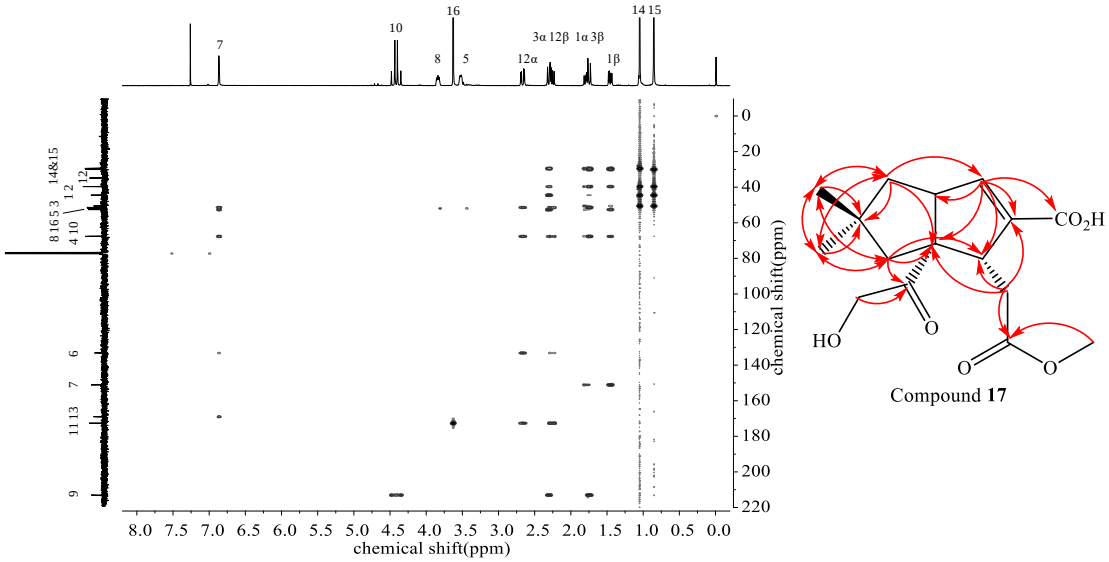
Compound **17**

d



Compound **17**

e



f

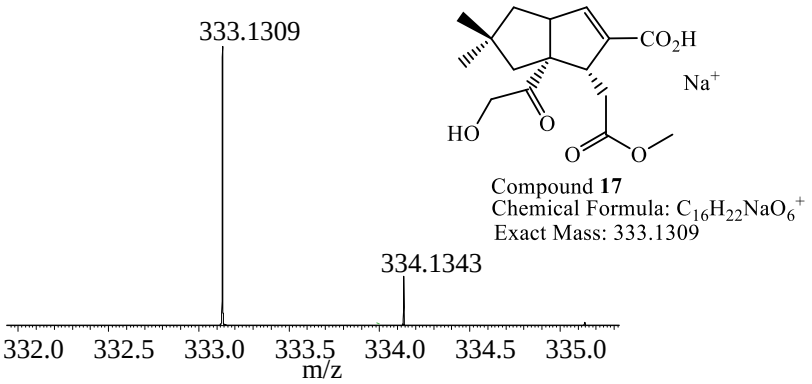
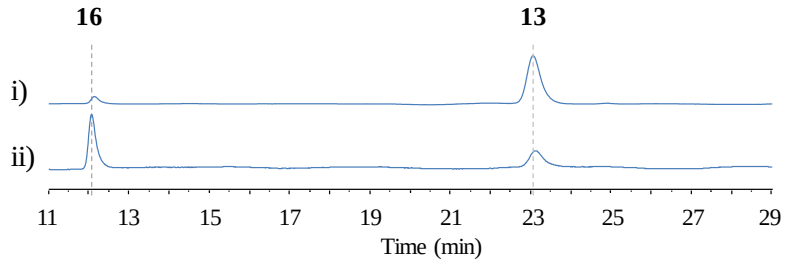
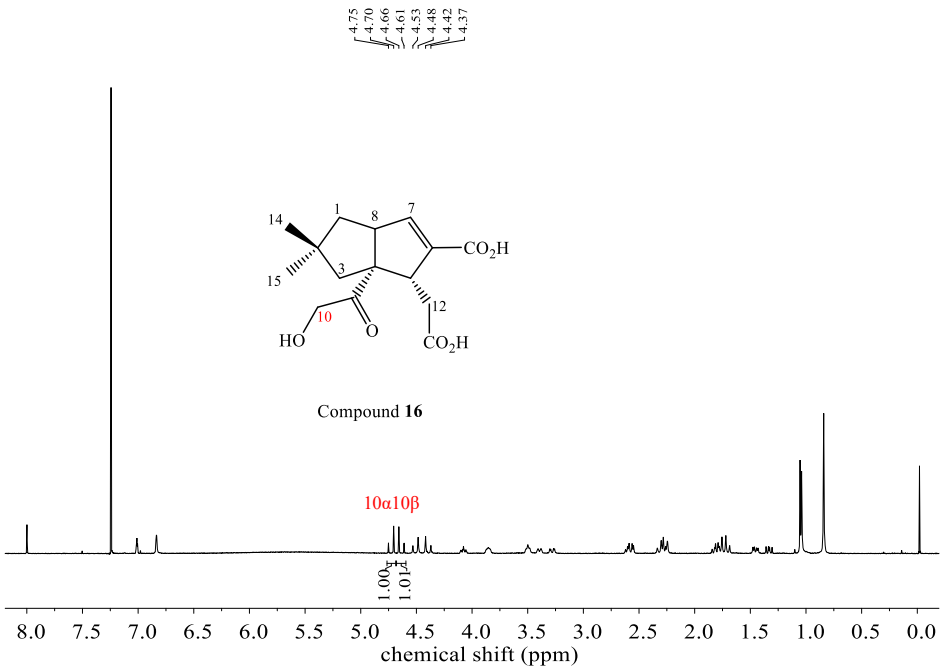


Figure S8. Compound **17** characterizations. a, ^{1}H NMR spectrum of compound **17** (400 MHz, $CDCl_3$). b, ^{13}C NMR spectrum of compound **17** (100 MHz, $CDCl_3$). c,^{1}H-^{1}H COSY spectrum of compound **17** (400 MHz, $CDCl_3$). d, ^{1}H-^{13}C HMQC spectrum of compound **17** (400 MHz, 100 MHz, $CDCl_3$). e, ^{1}H-^{13}C HMBC spectrum of compound **17** (400 MHz, 100 MHz, $CDCl_3$). f, High resolution ESI-MS of compound **17**. The calculated exact mass of compound **17** $[M+Na]^+$ is 333.1309 and found at 333.1309.

a



b



c

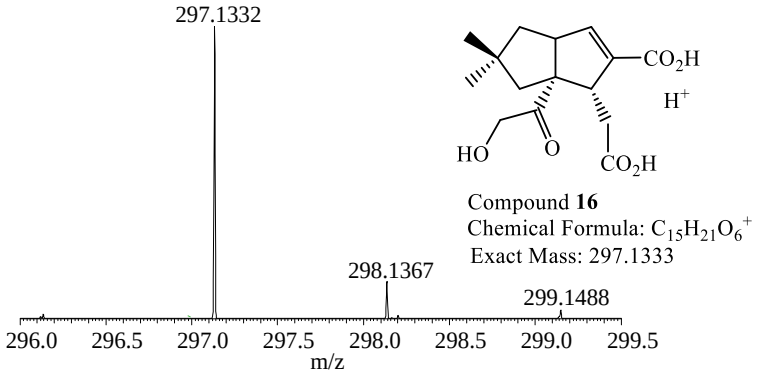


Figure S9. Compound **13** decomposed product characterizations. a**,** HPLC trace of compound **13** detected at i) 0 h and ii) 4 h after purification, respectively. b, ^{1}H NMR spectrum of the decomposed product of compound **13** (600 MHz, $CDCl_3$). The two sets of doublets (δ 4.75-4.7 *ppm*, d, J = 18.7 Hz and δ 4.66-4.61 *ppm*, d, J = 18.7 Hz) are consistent with the ^{1}H NMR spectrum of compound **16** (H-10α and 10β) described in the previous literature.[6] c, High resolution ESI-MS of compound **16**. The calculated exact mass of compound **16** $[M+H]^+$ is 297.1333 and found at 297.1332.

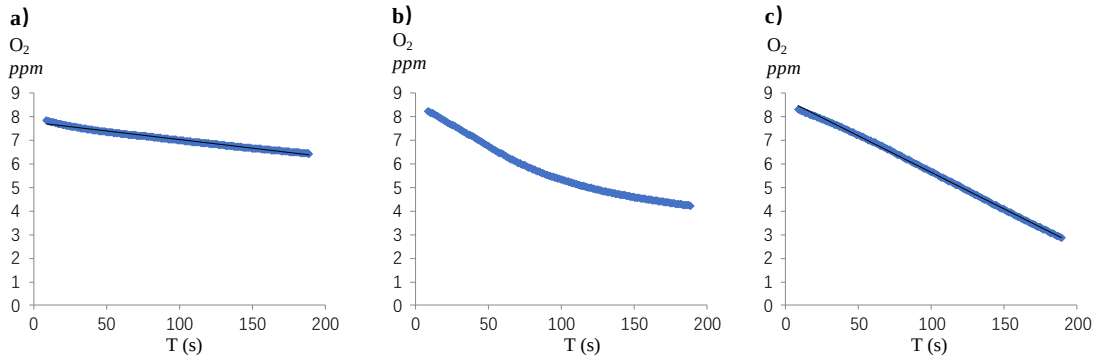


Figure S10. Oxygen consumption assay of the PtlD reactions. a, Neopentalenolactone D (**11**) final concentration was 0 µM. y = -0.0072x + 7.7531, R^2 = 0.9908 (10-190 s). b, Neopentalenolactone D (**11**) final concentration was 50 µM. From 10-60 s, y = -0.0361x + 8.5716 (R^2 = 0.9999), k_{obs} = 16.1 min^{-1}. From 120-190 s, y = -0.0108x + 6.2449 (R^2 = 0.9916), k_{obs} = 2 min^{-1}. c, Neopentalenolactone D (**11**) final concentration was 100 µM. y = -0.0309x + 8.722, R^2 = 0.9992 (10-190 s).

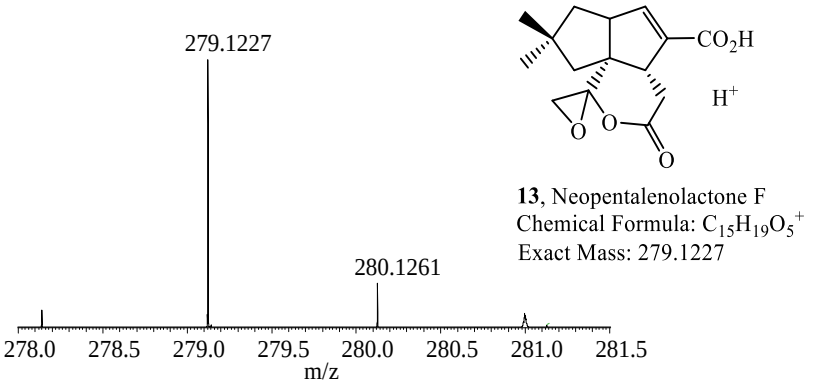


Figure S11. High resolution ESI-MS of Neopentalenolactone F (**13**). The calculated exact mass of Neopentalenolactone F $[M+H]^+$ is 279.1227 and found at 279.1227.

References

(1) Omura, S.; Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Takahashi, C.; Shinose, M.; Takahashi, Y.; Horikawa, H.; Nakazawa, H.; Osonoe, T.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proc. Natl. Acad. Sci. USA* **2001**, *98* (21), 12215-12220.

(2) Zhu, D.; Seo, M. J.; Ikeda, H.; Cane, D. E. Genome mining in *Streptomyces*. Discovery of an unprecedented P450-catalyzed oxidative rearrangement that is the final step in the biosynthesis of pentalenolactone. *J. Am. Chem. Soc.* **2011**, *133* (7), 2128-2131.

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