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Functional analyses of cytochrome P450 genes responsible for the early steps of brassicicene C biosynthesis

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ABSTRACT

We previously revealed that Orf8 and Orf6, which were identified in the brassicicene C biosynthetic gene cluster in *Alternaria brassicicola* strain ATCC96836, were fusicoccadiene (FD) synthase and 16-*O*-methyltransferase, respectively. In the present Letter, the early biosynthetic steps after the formation of FD were investigated. Plasmids carrying the FD synthase gene, one (or two) of five cytochrome P450 genes (*orf1*, *orf2*, *orf5*, *orf7*, and *orf11*) identified in the cluster and a cytochrome P450 reductase gene cloned from strain ATCC96836 were constructed and introduced into *Saccharomyces cerevisiae*. Based on the structures of the compounds produced by the transformants, Orf1 is suggested to be an 8β-hydroxylation enzyme that yields FD 8β-ol (**4**), followed by 16-hydroxylation by Orf7 to produce FD 8β16-diol (**5**).

Fusicoccin (FC) A[1,2] (**1**) and cotylenin (CN) A[3–5] (**2**) (Fig. 1) are structurally related diterpene glycosides produced by the plant-pathogenic fungus *Phomopsis amygdali* and soil-isolated fungus *Cladosporium* sp. 501-7W, respectively. Both compounds show phytohormone-like activities via activation of plasma membrane H^+-ATPase.[6] Crystallographic analysis of a ternary complex comprised of a plant 14-3-3 protein, FC and a phosphopeptide derived from the C-terminus of H^+-ATPase revealed that FC stabilizes the interaction between 14-3-3 protein and H^+-ATPase to form the ternary complex, resulting in continuous activation of H^+-ATPase.[7] However, it has been demonstrated that only CN induces the differentiation of human myeloid leukemia cells[8–10] and acts synergistically with IFN-α to induce apoptosis in a wide array of cancer cells.[11,12] These different biological activities of FC and CN suggest that FC/CN analogs are attractive tools for investigating the physiological roles of 14-3-3 proteins in intracellular signal transductions. Since complete understanding of FC/CN biosynthesis will facilitate biological and biochemical productions of novel FC/CN analogs, characterization and functional analyses of the biosynthetic gene cluster for FC/CN and structurally related compounds are of great importance.

A biosynthetic pathway for FC was proposed based on analyses of biosynthetic intermediates and feeding experiments with labeled compounds, as shown in Figure 2A.[1,2,13–15] Recently, we identified fusicoccadiene (FD) synthase (PaFS) from *P. amygdali*, which is a unique chimeric enzyme possessing both a geranylgeranyl diphosphate (GGDP) synthase domain and a diterpene cyclase domain (Fig. 2B).[16] It was also revealed that some of the genes (*dioxygenase*, *cytochrome P450*, *short-chain dehydrogenase/reductase* (SDR), and *mannosidase*) that are probably involved in FC biosynthesis are clustered near *PaFS* (Fig. 2B).[16] However, the other genes involved, such as glycosyltransferase, acetyltransferase, and prenyltransferase, were not identified around *PaFS*. These findings indicate that the FC biosynthetic genes are located in at least two loci, and this makes it difficult to study the whole biosynthetic pathway of FC. Moreover, *Cladosporium* sp. 501-7W, the CN producer, has lost its proliferation activity and we have missed the opportunity to clone the CN biosynthetic gene cluster. Therefore, we previously searched for additional FC/CN-like producers and found that *Alternaria brassicicola* ATCC96836, whose partial genome sequences have been released,[17] has an ortholog (*orf8*) of *PaFS*. The gene was clustered with five cytochrome P450 genes (*orf1*, *orf2*, *orf5*, *orf7*, and *orf11*), one SDR gene (*orf3*), one acetyltransferase gene (*orf4*), one methyltransferase gene (*orf6*), one dioxygenase gene (*orf9*) and one hypothetical gene (*orf10*) (Fig. 3B).[18] We also confirmed that strain ATCC96836 produced brassicicene (BC) C (**3**) (Fig. 1) and that Orf8 and Orf6 encoded fusicoccadiene (FD)

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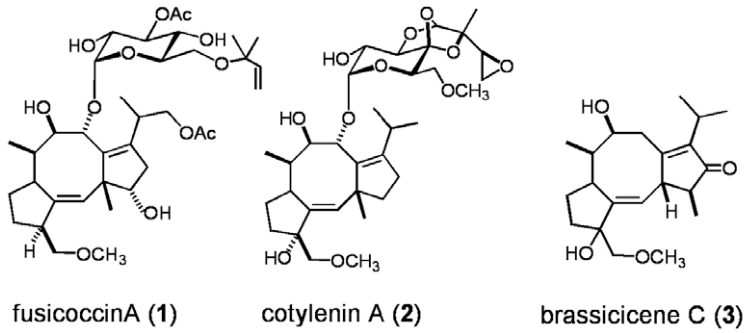

Figure 1. Structures of fusicoccin A (**1**), cotylenin A (**2**) and brassicicene C (**3**).

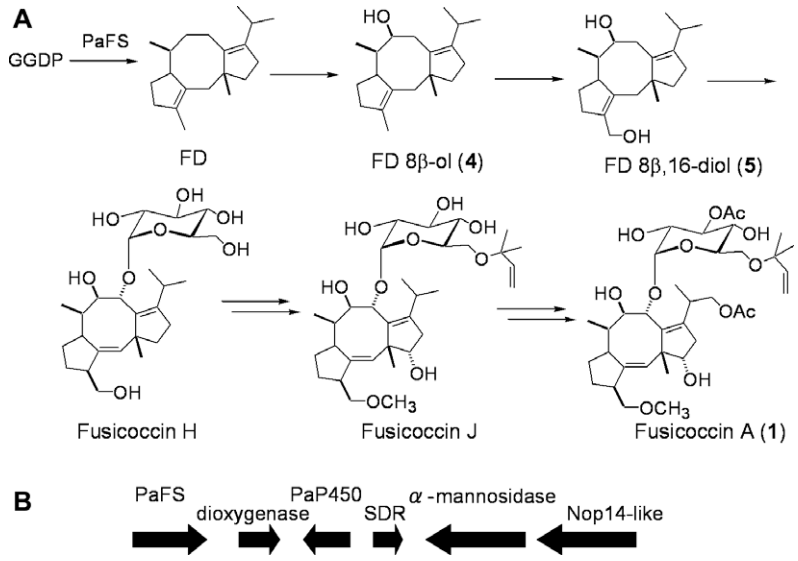

Figure 2. (A) Proposed biosynthetic pathway of FC A and FC J. (B) Putative biosynthetic gene cluster of FC A.

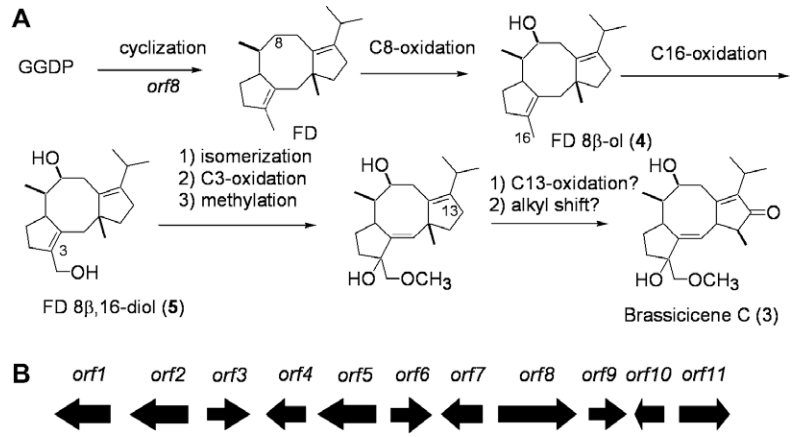

Figure 3. (A) Proposed biosynthetic pathway and (B) biosynthetic gene cluster of brassicicene C.

synthase and 16-*O*-methyltransferase, respectively, using recombinant enzymes.[18]

As described above, FC and CN showed different biological activities toward human myeloid leukemia cells. Considering the structural differences between FC and CN, modified sugar moieties and/or hydroxyl groups introduced into different positions would have critical roles in the acquisition of these specific biological activities. As a first step toward understanding the hydroxylation enzymes involved in the FC/CN/BC biosyntheses, we characterized the cytochrome P450 genes essential for the biosynthesis of FD 8β,16-diol, which would be the common intermediate of FC/CN/BC biosyntheses (Figs. 1, 2A and 3A), using the five cytochrome P450 genes identified in the BC biosynthetic gene cluster.

Each of the DNAs carrying *orf1*, *orf2*, *orf5*, *orf7*, and *orf11* was amplified by PCR using gene-specific primers containing appropriate restriction sites and first-strand cDNAs[19] prepared from total RNA isolated from strain ATCC96836 as a template. For functional analyses of cytochrome P450 enzymes, a cytochrome P450

reductase is essential. Therefore, we searched the genome database of strain ATCC96836 for a cytochrome P450 reductase gene (*AbP450Red*) and amplified it by PCR. We initially tried to express these genes as recombinant enzymes in *Escherichia coli*. Since the P450s from strain ATCC96836 have a hydrophobic membrane-anchor region in the N-terminus, we constructed dozens of plasmids to express the recombinants without this region. However, almost all of the recombinants formed inclusion bodies, even after several attempts to increase the amounts of soluble enzymes by decreasing the amount of IPTG added for the induction and by prolonging the cultivation at a low temperature. Subsequently, we used *Saccharomyces cerevisiae* YPH500 as a host for heterologous expression. One of the cytochrome P450 genes and *AbP450Red* were inserted into pESC-URA to construct pESC-URA-*orf1* (*orf2*, *orf5*, *orf7*, or *orf11*)-*AbP450Red*. In these plasmids, the *P450* and *AbP450Red* genes were expressed under the control of the galactose promoters Gal10 and Gal1, respectively.[20] The constructed plasmids were introduced into *S. cerevisiae* YPH500 (*his*$^-$, *leu*$^-$, *trp*$^-$, *ura*$^-$, *ade*$^-$, *and lys*$^-$). After cultivation of the transformants in the presence of galactose, microsomal fractions were prepared and incubated with FD. However, we did not detect any products. A differential CO-reduced cytochrome P450 spectral analysis indicated that all the cytochrome P450 genes were expressed as inactive forms with an absorption maximum at 420 nm. However, a slight shift of the absorption maximum from 420 to 390 nm, which results from the substrate–heme binding, was observed for one of the cytochrome P450 genes, suggesting that functional analyses might become possible if the substrate could be supplied into microsomes, by analyzing the structure of the products that accumulated in the microsomes. We first attempted to detect the accumulation of FD in *S. cerevisiae* YPH500 strain harboring the pESC-TRP-*orf8* plasmid that carried the FD synthase gene.[21] GC–MS analysis[22] of a pentane extract of the transformant clearly showed the production of FD. Since co-expression experiments with two different plasmids for production of secondary metabolites in yeast cell have been reported,[23,24] we next introduced pESC-URA-*orf1* (*orf2*, *orf5*, *orf7* or *orf11*)-*AbP450Red* into the transformant harboring pESC-TRP-*orf8* and searched for a compound that accumulated in the transformant.[25] Consequently, one major peak (17.6 min) was detected in a culture extract of the pESC-URA-*orf1*-*AbP450Red*-introduced transformant by GC–MS analysis together with three minor peaks (13.9, 14.1, and 17.2 min), which were also detected in transformants harboring the other cytochrome P450 genes (*orf2*, *orf5*, *orf7*, or *orf11*) as previously observed.[16] The retention time and MS fragment patterns of the major peak were confirmed to be identical to those of authentic FD 8β-ol by GC–MS analysis[22] (Fig. 4), suggesting that Orf1 is an 8β-hydroxylation enzyme that yields FD 8β-ol (**4**).

We could not apply the same method to identify the next cytochrome P450 gene that hydroxylates FD 8β-ol because of the low productivity of FD 8β-ol. To overcome this problem, the promoter region of pESC-TRP-*orf8* was replaced with an alcohol dehydrogenase (ADH) promoter as described previously.[26] The productivity of the transformant harboring pESC-TRP-*orf8*-ADH[27] was 80-fold (approximately 5 mg/L) higher than that of the transformant harboring pESC-TRP-*orf8*. Moreover, by introducing pESC-URA-*orf1*-*AbP450Red* into the transformant, approximately 120-fold more FD 8β-ol (approximately 1 mg/L) was accumulated. We also replaced the promoters of pESC-URA-*orf1*-*AbP450Red* with the promoter of ADH[28] but no enhanced productivities were observed. Therefore, the transformant harboring pESC-TRP-*orf8*-ADH and pESC-URA-*orf1*-*AbP450Red* was used as the host for the next experiment.

The *orf2*, *orf5*, *orf7*, and *orf11* genes were individually cloned into pESC-LEU by the same method used for the constructions with the pESC-URA plasmid.[29] The constructed plasmids were introduced into the host carrying pESC-TRP-*orf8*-ADH and pESC-URA-*orf1*-*AbP450Red*, and followed by screening for new metabolites. Finally, a new peak was detected in the culture extract of a transformant harboring pESC-LEU-*orf7* in addition to pESC-URA-*orf1*-*AbP450Red* and pESC-TRP-*orf8*-ADH by HPLC analysis (Fig. 5).[30] To determine the structure of the compound, a large-scale cultivation (26 L) was carried out. The compound was extracted with pentane and the pentane layer was dried over sodium sulfate before concentration in vacuo. The thus obtained sample (1.2 g) was resolved in a small volume of ethanol and subjected to successive preparative HPLC[31] to give purified compounds (0.5 mg, colorless oil). Based on NMR (Supplementary Fig. 1) and MS data (Supplementary Fig. 2), the new compound was confirmed to be FD 8β,16-diol (**5**) by comparison with an authentic FD 8 β,16-diol (Supplementary Fig. 3).[32] Therefore, Orf7 is suggested to be a 16-hydroxylation enzyme that yields FD 8β,16-diol (**5**).

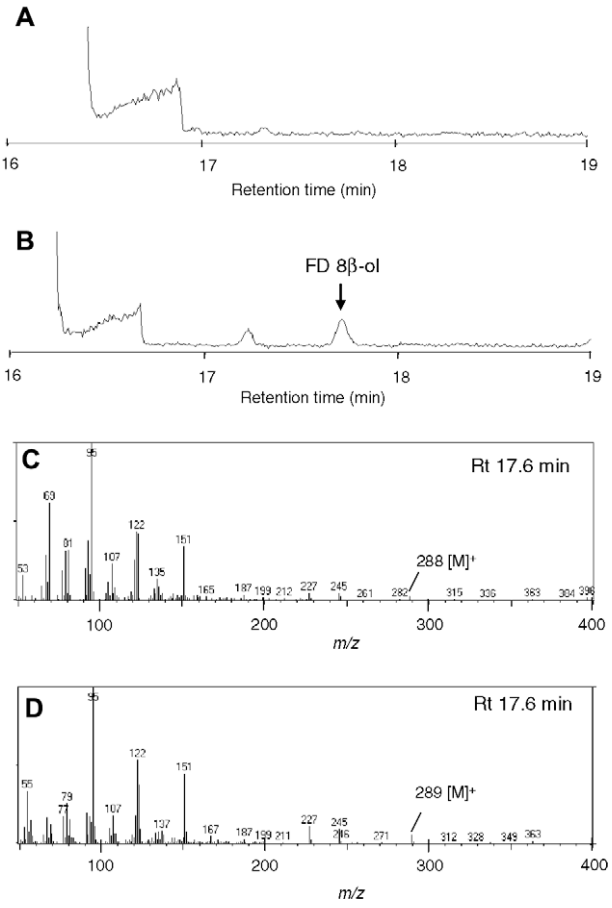

Figure 4. GC–MS analyses of pentane extracts of transformants. (A) pESC-URA with pESC-TRP-*orf8*. (B) pESC-URA-*orf1* + *AbP450Red* with pESC-TRP-*orf8*. Mass spectrum of (C) the major peak and (D) the authentic [19-^{2}H] FD 8β-ol.[13]

Next, we tried to identify the cytochrome P450 that accepts FD 8β,16-diol as a substrate by the same method. The *orf2*, *orf5*, and *orf11* genes were individually cloned into pESC-HIS and introduced into the FD 8β,16-diol-producing host. However, we were unable to detect a new metabolite because of the very low productivity of FD 8β,16-diol. Another method, such as feeding of synthetic FD 8β,16-diol to the host, appears to be necessary.

In conclusion, we have revealed that Orf1 is an 8β-hydroxylation enzyme that yields FD 8β-ol (**4**), followed by successive 16-hydroxylation by Orf7 to produce FD 8β,16-diol (**5**). These results and previous experiments strongly suggest that this gene cluster is involved in brassicicene C biosynthesis. Since the *orf7* gene shows significant similarity (47% amino acid identity) to the *PaP450* gene (Fig. 2), PaP450 may be involved in C-16 hydroxyl-

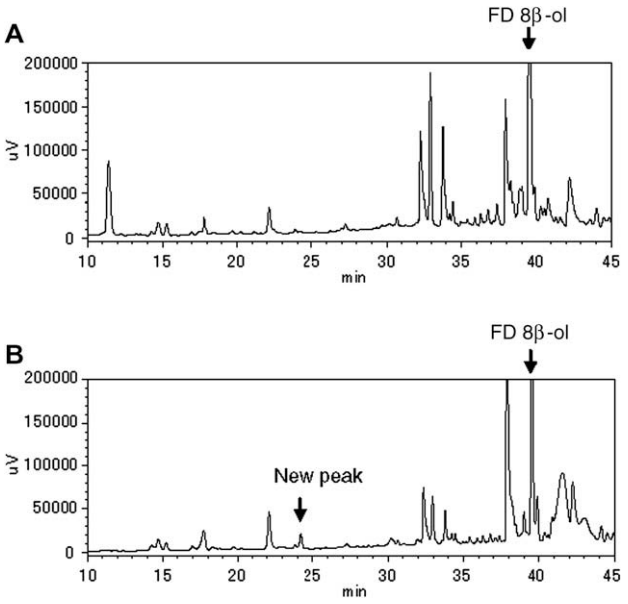

Figure 5. HPLC profiles of pentane extracts of transformants. (A) pESC-URA-*orf1* + *Ab450Red* with pESC-TRP-*orf8*-ADH. (B) pESC-LEU-*orf7* and pESC-URA-*orf1* + *AbP450Red* with pESC-TRP-*orf8*-ADH.

ation during FC biosynthesis. Functional analyses of PaP450 and other enzymes in the cluster of *A. brassicicola* ATCC96836 are now in progress and will be reported in the near future.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.026.

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19. The cDNA sequences of *orf1*, *orf2*, *orf5*, *orf7*, *orf11* and *AbP450Red* from *A. brassicicola* ATCC 96836 was deposited at GenBank under Accession No. AB506078, AB506079, AB506080, AB506081, AB506082 and AB506083, respectively.
20. The *orf1*, *orf2*, *orf5*, *orf7*, *orf11*, and *AbP450Red* genes were amplified by PCR with first-strand cDNA as a template.[18] The following primers were used: *AbP450Red*, 5′-ACGCGTCGACATGGCACAACTCGATACCTTGGACA-3′ and 5′-CGGGGTACCTCATGACCAGACGTCCTCTTGGTAT-3′; *orf1*, 5′-ATAAGAAT GCGGCCGCATGGAGATGGCTACAACCTTTACA-3′ and 5′-CGCGGATCCAGATCTACTCCTTGTTTTTTCTAACGATT-3′; *orf2*, 5′-ATAAGAATGCGGCCGCATGGAATCTGCTCAAATACATTCCA-3′ and 5′-CGCGGATCCAGATCTAGGCGGTCTCCTTCGCTACCTTTGT-3′; *orf5*, 5′-ATAAGAATGCGGCCGCATGCTCGAAGGCACGCTTCAGGACT-3′ and 5′-CCGGAATTCAGATCTACTCATACAGTGCCTCAATTCGGA-3′; *orf7*, 5′-ATAAGAATGCGGCCGCATGGCTTCCATACTATGGACAACT-3′ and 5′-CCGGAATTCAGATCTATTTCGTTCTCGGAGCGAAACGCA-3′; *orf11*, 5′-ATAAGAATGCGGCCGCATGATTTTTCTTGCTCCCTTCGAAT-3′ and 5′-CGCGGATCCAGATCTTAGGTGCTCGATATAGGAGAATA-3′. The *orf1*, *orf5*, and *orf7* genes were cloned in the *Not*I-*Bgl*II site of pESC-URA to construct pESC-URA-*orf1*, pESC-URA-*orf5* and pESC-URA-*orf7*, respectively. The *AbP450Red* gene was then inserted into the *Sal*I-*Kpn*I sites of these plasmids to yield pESC-URA-*orf1* + *AbP450Red*, pESC-URA-*orf5* + *AbP450-Red* and pESC-URA-*orf7* + *AbP450Red*, respectively. To construct pESC-URA-*orf2* + *AbP450Red* and pESC-URA-*orf11* + *AbP450Red*, the *AbP450Red* gene was first inserted into pESC-URA, followed by insertion of the *orf2* and *orf11* genes into the *Not*I-*Bgl*II sites.
21. The previously obtained *orf8* gene[18] was cloned into the *Bam*HI-*Sal*I sites of pESC-TRP (Stratagene) to construct pESC-TRP-*orf8*.
22. GC–MS analyses were carried out using a QP5000 mass spectrometer (Shimadzu) connected to a GC-17A gas chromatograph equipped with a HP1-MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The column temperature program was described previously.[16] Mass spectra were recorded at 70 eV, scanning from 50 to 500.
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25. Procedures used for maintenance and transformation of *S. cerevisiae* were essentially the same as those described in the manufacturer's protocols. SGI medium consisted of 0.1% Bacto casamino acids, 0.7% yeast nitrogen base without amino acids, and 2% glucose. Transformed yeast cells were inoculated into the liquid SGI medium and cultured at 28 °C for 30 h on a reciprocal shaker (120 strokes/min). Then, 2 mL of culture was inoculated in 100 mL of the same medium and was cultivated for 48 h at the same condition. After the check of glucose assumption by urinalysis reagent strip, 2% galactose was fed to the culture and cultivation was continued for 60–108 h. The culture broth was extracted with equal volume of acetone and 1.5 volume of pentane by stirring for 1.5 h at room temperature. The pentane extract was then concentrated in vacuo and subjected to GC–MS and LC–MS analysis.
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27. The ADH1 promoter and ADH1 terminator fragments were amplified by PCR with pYE352-URA-ADH1[26] as a template and the following primers; 5′-CGGGATCCGCTAGCCGTGGAATATTTCGGATATCCTT-3′ and 5′-CCGCTCGAGCTAGCACTAGTGGACGGATTACAACAGGTATTGT-3′. The amplified products were cloned into the *Nhe*I-*Spe*I sites of pESC-TRP to construct pESC-TRP-ADH. Then, the *orf8* fragment was inserted into the *Eco*RI-*Sal*I sites of pESC-TRP-ADH to yield pESC-TRP-*orf8*-ADH.
28. The *orf1* gene with the ADH1 promoter and terminator was cloned into the *Nhe*I-*Spe*I sites of pESC-URA to construct pESC-URA-*orf1*-ADH. Then, the *AbP450Red* gene with ADH1 promoter and terminator was inserted into the *Nhe*I site of pESC-URA-*orf1*-ADH to yield pESC-URA-*orf1*-ADH + *AbP450Red*-ADH.
29. The *orf2*, *orf5*, *orf7* and *orf11*genes were cloned into the *Not*I-*Bgl*II sites of pESC-LEU to construct pESC-LEU-*orf2*, pESC-LEU-*orf5*, pESC-LEU-*orf7*, and pESC-LEU-*orf11*, respectively.
30. The pentane extracts were analyzed under the following conditions: column; Mightysil RP-18 (250 × 4.6 mm, Merck), column temperature; 40 °C, detection at 205 nm, flow rate; 1.0 mL/min, 40% acetonitrile for 0–5 min, a linear gradient from 40% to 100% for an additional 40 min.
31. The pentane extracts were fractionated with preparative HPLC under the following conditions: column; Mightysil RP-18 (250 × 20 mm, Merck), column temperature; 40 °C, detection at 205 nm, flow rate; 5.0 mL/min, 40% acetonitrile for 0–5 min, a linear gradient from 40% to 100% for an additional 40 min. The fractionated materials (2.7 mg) were further purified with the same column by an isocratic elution of 40% acetonitrile to obtain the purified FD 8β,16-diol.
32. Colorless prisms (recrystallized from *n*-hexane/Et_2O), mp 167.7–169.2 °C, $[\alpha]_D^{27}$ = +45.57 (*c* 0.18, CH_3OH). ^1H NMR ($CDCl_3$, 400 MHz; Because of the conformational mobility of **5**, NMR signals are heavily broadened. Therefore, only diagnostic signals are recorded here.): 0.91 (3H, s), 0.92 (6H, d, *J* = 6.8 Hz), 1.00 (3H, br d, *J* = 6.8 Hz), 2.13 (1H, dd, *J* = 13.1, 12.1 Hz), 2.37 (1H, dm, *J* = 13.1 Hz), 3.82 (1H, dt, *J* = 11.8, 4.4 Hz), and 4.18 (2H, br s). Supplementary Fig. 1b. ^1H NMR (C_6D_6, 70 °C, 400 MHz; Because of the conformational mobility of **5**, NMR signals are heavily broadened. Therefore, only diagnostic signals are recorded here.) δ 0.90 (3H, d, *J* = 7.0 Hz), 0.92 (3H, s), 0.97 (3H, d, *J* = 6.8 Hz), 0.99 (3H, d, *J* = 7.3 Hz), 2.64 (1H, sept, *J* = 6.8 Hz), 3.75 (1H, dt, *J* = 11.6, 4.4 Hz), 4.04 (1H, dd, *J* = 12.6, 8.6 Hz), and 4.05 (1H, br d, *J* = 12.6 Hz). HR-ESI-MS: *m/z* calcd for $C_{20}H_{32}O_2Na$ $[M+Na]^+$ 327.2300, found 327.2278.