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Dammaradiene synthase, a squalene cyclase, from *Dryopteris crassirhizoma* Nakai ☆

Junichi Shinozaki [a,b], Masaaki Shibuya [a], Kazuo Masuda [b], Yutaka Ebizuka [a,*]

[a] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b] *Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan*

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ABSTRACT

Ferns produce a variety of cyclic triterpene hydrocarbons in large amount. Squalene cyclases (SCs) are responsible enzymes for formation of cyclic triterpene hydrocarbon skeletons. Although more than ten bacterial SCs have been cloned and four of them characterized for their enzymatic products, the only example of a fern SC is ACH, from *Adiantum capillus-veneris*, which produces hydroxyhopane. To obtain a deeper understanding of the molecular evolution of SCs and the origin of the structural diversity of fern triterpenes, further cloning and characterization of SCs have been pursued. In this study, a SC cDNA, DCD, was cloned from *Dryopteris crassirhizoma* by homology-based RT-PCR. DCD contains a 2058-bp open reading frame that encodes a 685 amino acid polypeptide exhibiting 66% identity to the previously identified fern SC, ACH, and 35–40% identity to bacterial SCs. Heterologous expression of DCD in yeast established it to be a dammaradiene synthase affording dammara-18(28),21-diene, a tetracyclic triterpene hydrocarbon. Although neither this compound nor any derived metabolites have been previously reported from *D. crassirhizoma*, re-investigation of the leaflets demonstrated the presence of dammara-18(28),21-diene. DCD represents the first SC that produces a tetracyclic triterpene hydrocarbon.

1. Introduction

Ferns are the most primitive vascular plants and re-produce by spores. More than 10,000 species of ferns exist on earth inhabiting areas ranging from tropical to cold temperate regions and from lowland to alpine zones. Ferns produce unique secondary metabolites that are not found in higher plants, drawing a great attention as a rich source of natural products.

Triterpenes are a large and structurally diverse group of natural products derived from C_{30} acyclic precursors. More than 200 triterpene derivatives have been reported from some 90 species of ferns. Among them, diplopterol (22-hydroxyhopane, **1**), diploptene (hop-22(29)-ene, **2**) and fernene (fern-9(11)-ene, **3**) are the most common triterpenes in ferns, which are found in almost all ferns (Ageta et al., 1998). A structural feature of triterpenes of ferns is the absence of an oxygen functionality at C-3. Triterpenes of higher plants, in contrast, possess an oxygen functionality at C-3, as they are formed from a common substrate, (3*S*)-2,3-oxidosqualene (**4**), by oxidosqualene cyclases (OSCs) and the epoxide oxygen is retained at C-3 in the products (Fig. 1, Phillips et al., 2006). Absence of an oxygen functionality at the C-3 of triterpenes of ferns suggests their direct derivation from squalene (**5**) by squalene cyclases (SCs). In fact, ACH, a hydroxyhopane (**1**) synthase, has been cloned from *Adiantum capillus-veneris* and characterized as the first SC from ferns (Fig. 1, Shinozaki et al., 2008).

Some bacteria are known to produce 3-deoxy triterpenes of the hopane skeleton and their metabolites (Ourisson and Rohmer, 1992). Prokaryotic SCs have been cloned from various bacterial species, namely *Alicyclobacillus acidocaldarius* (Ochs et al., 1992), *Zymomonas mobilis* (Reipen et al., 1995), *Bradyrhizobium japonicum* (Perzl et al., 1997) and *Methylococcus capsulatus* (Tippelt et al., 1998). The first cloned SC (ACH) from a fern shed light on the molecular evolution of SCs and OSCs. Phylogenetic analysis clearly indicated that the fern SC, ACH, is more closely related to bacterial SCs than eukaryotic OSCs (Shinozaki et al., 2008). To obtain further insight into the molecular evolution of SCs and the origin of structural diversity of fern triterpenes, cDNA cloning of SCs from *Dryopteris crassirhizoma* Nakai was attempted in this study.

D. crassirhizoma Nakai (Dryopteridaceae) is widely distributed in the middle to northern Japan, and taxonomically close to an European species *D. filix-mas* L. Powdered and dried rhizomes of various *Dryopteris* ferns were formerly used as remedies for helminthiasis caused by *Diphyllobothrium latum* (Murakami and Tanaka, 1988). Helmintic activity could be attributable to acylphloroglucinol derivatives, such as albaspidins (Murakami and Tanaka, 1988). Our extensive studies on chemical constituents of *D. crassirhizoma* collected in Japan resulted in the isolation of twelve triterpenes, in addition to acylphloroglucinols, from the dried and fresh leaflets

☆ Nucleotide sequence data are available in the DDBJ/EMBL/GenBank nucleotide sequence databases under the Accession No. AB429303 (DCD).

* Corresponding author. Tel.: +81 3 5841 4740; fax: +81 3 5841 4744.
E-mail address: yebiz@mol.f.u-tokyo.ac.jp (Y. Ebizuka).

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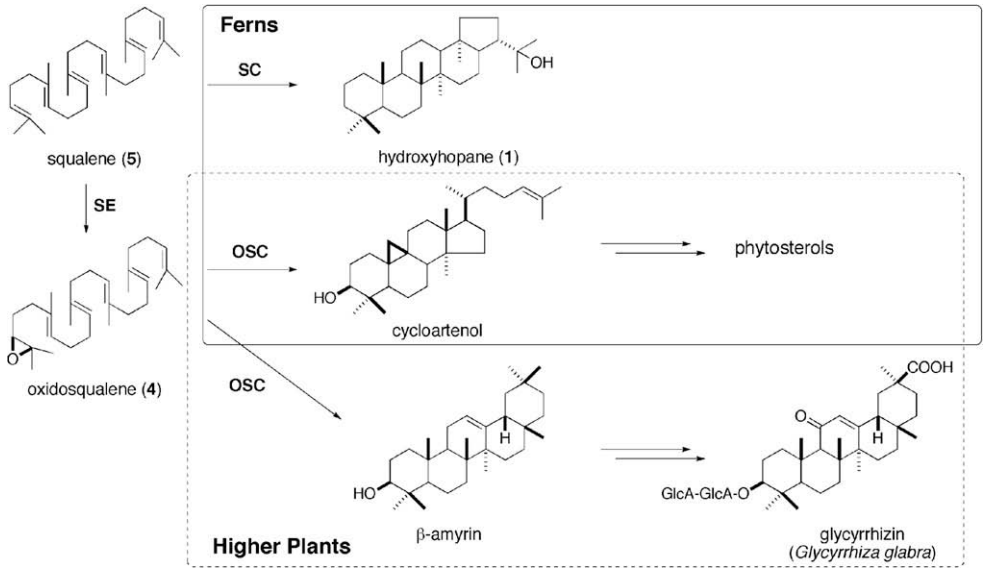

Fig. 1. Biosynthesis of triterpenes in ferns and higher plants. SC, squalene cyclase; OSC, oxidosqualene cyclase; SE, squalene epoxidase.

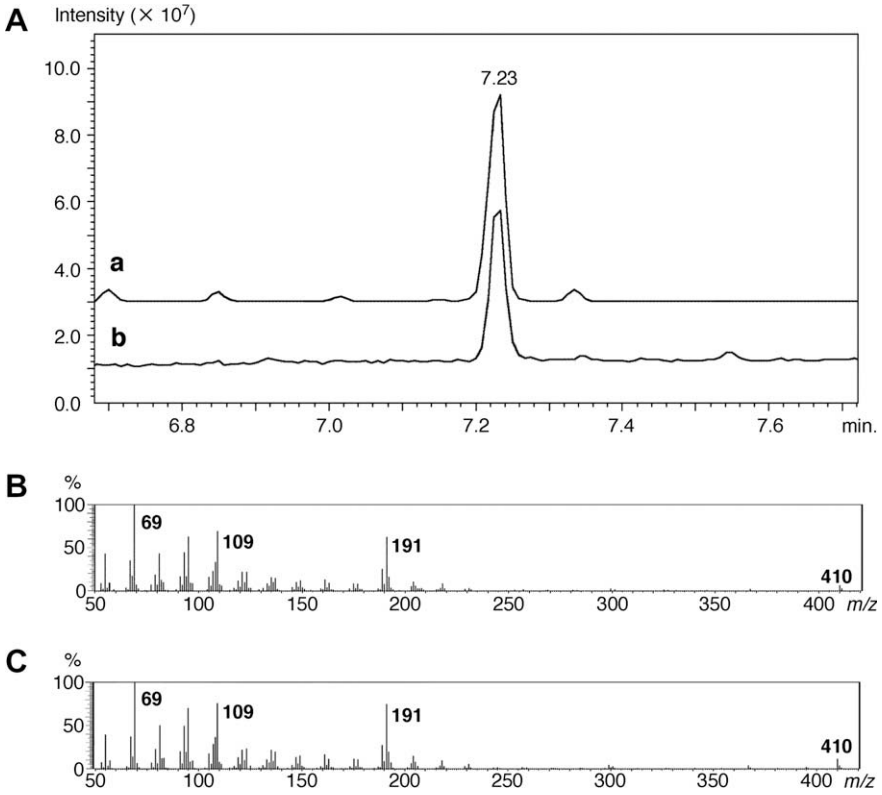

Fig. 2. GC–MS analysis of DCD product. Gas chromatograms of authentic dammara-18(28),21-diene (**6**, a) and DCD product (b) with total ion monitoring are shown in (A). MS spectra of DCD product and authentic dammara-18(28),21-diene (**6**) are shown (B) and (C), respectively.

(Ageta et al., 1963, 1975; Shiojima et al., 1994). All of these triterpenes have either hopane or migrated hopane skeletons. *D. crassirhizoma*, therefore, is a promising source material for cloning of the SC genes encoding hopane and migrated hopane synthases.

In this study, we cloned a SC cDNA from *D. crassirhizoma* and characterized it to encode a novel SC, dammaradiene synthase, affording dammara-18(28),21-diene (**6**) (The numbering system used for the tetracyclic triterpene is according to that of Masuda et al. (1983)). Although dammara-18(28),21-diene (**6**) and/or its metabolites have not been identified in our previous studies, re-investigation of the hydrocarbon constituents of the leaflets led to isolation of dammara-18(28),21-diene (**6**) from this fern.

2. Results and discussion

2.1. Cloning of SC cDNA from D. crassirhizoma

A *SC* homologue, designated as DCD, was obtained from *D. crassirhizoma* by RT-PCR using degenerate primers based on the

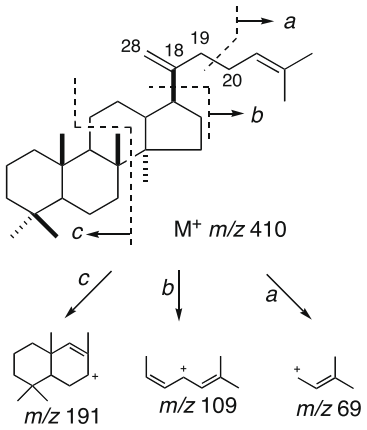

Fig. 3. MS fragmentations of dammara-18(28),21-diene (**6**).

conserved sequences of known bacterial SCs as described in our previous paper (Shinozaki et al., 2008). DCD has an open reading frame (ORF) of 2058-bp nucleotides that encodes a 685 amino acid polypeptide. The deduced amino acid sequence showed 66% identity to that of the ACH (a hydroxyhopane synthase) from *A. capillus-veneris*, 35–40% identity to those of SCs from bacteria and less than 20% identity to those of the known OSCs including ACX (a cycloartenol synthase) from *A. capillus-veneris*. The DXDD motif, which is highly conserved among bacterial SCs and responsible for protonation of the terminal double bond of squalene to initiate cyclization, is also found in the DCD sequence (Wendt et al., 1997; Sato and Hoshino, 1999).

2.2. Functional analysis of DCD by expression in yeast

An ORF of *DCD* amplified by PCR was introduced to pYES2 (Invitrogen) to construct an expression plasmid pYES2-DCD. With this plasmid, a *Saccharomyces cerevisiae* strain INV*sc*2 (Invitrogen) was transformed. The transformant was cultured and expression of recombinant DCD protein was induced as described previously (Shinozaki et al., 2008). Cells were then harvested and extracted with *n*-hexane after alkaline treatment. The crude hexane extract was subjected to Bond Elut®-Si (Varian) to yield a cyclic triterpene hydrocarbon fraction. The resulting

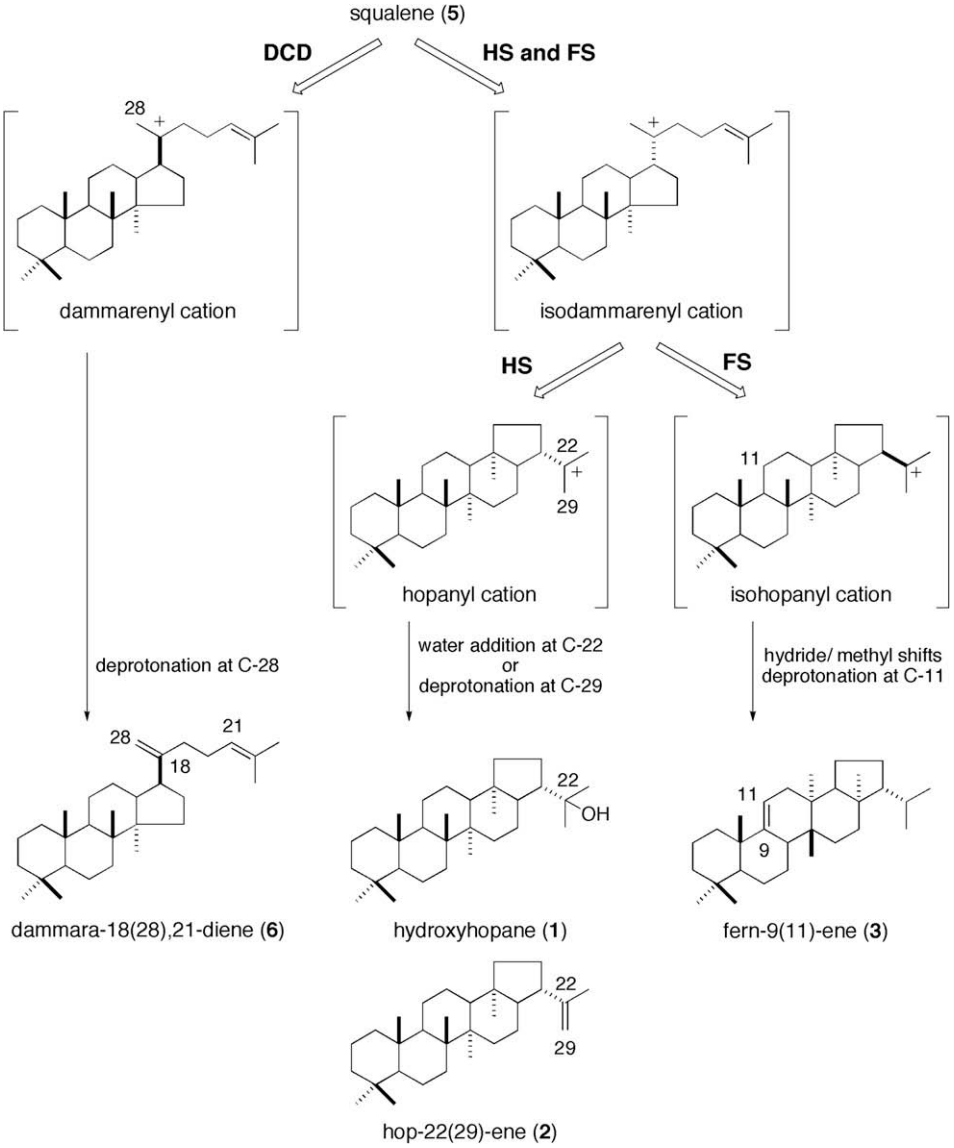

Fig. 4. Proposed biosynthetic pathway to triterpenes in *D. crassirhizoma*. DCD, dammaradiene synthase; HS, putative hopene/hydroxyhopane synthase; FS, putative fernene synthase.

fraction was analyzed by GC–MS (Fig. 2). GC chromatogram showed a peak at 7.23 min which is absent in a control transformant harboring pYES2. No other significant product peak was detected. EI-MS analysis of the peak at 7.23 min showed strong ions at *m/z* 191, *m/z* 109 and *m/z* 69. These are well known as characteristic fragmentation ions of the dammarane skeleton with two double bonds in its side-chain (Masuda et al., 1983). The fragment ion at *m/z* 191 derives from A/B ring, whereas those at *m/z* 109 and *m*/z 69 result from the side-chain (Fig. 3) (Masuda et al., 1983). These results suggest that the DCD product has a dammaradiene structure with two double bonds in the side-chain. The retention time and fragmentation pattern of the DCD product were identical to those of authentic **6** (Fig. 2). Therefore, it is unambiguously established that the DCD product is **6,** and thus that DCD is a cDNA encoding a novel SC, a dammaradiene synthase.

Interestingly, **6** was included in the multiple products, ranging from bi- to penta-cyclic triterpenes, of a bacterial SHC mutant which carries mutations of I261A and Y609F (Hoshino et al., 2000; Füll and Poralla, 2000). Formation of dammaradiene **6** and hopanes, namely hop-22(29)-ene (**2**) and hydroxyhopane (**1**), is shown in Fig. 4. The key intermediate for formation of **6** is a dammarenyl cation with a β-oriented side-chain, whereas that for formation of **1** and **2** is an isodammarenyl cation with the side-chain in an α-orientation. Based on the X-ray crystal structure together with the results of site-directed mutagenesis, Hoshino and Sato (2002) proposed that an appropriate bulky residue at position 261 in SHC directs the destiny of the side-chain orientation. A single mutant I261V of SHC, however, afforded no abortive cyclization products (Hoshino et al., 2000), suggesting that the formation of the dammarane skeleton needs additional mutations. Isoleucine at 261 of SHC corresponds to valine at 285 of DCD and tyrosine at 609 of SHC is conserved in DCD. Thus, amino acid residues other than tyrosine 609 must take part in producing the dammarane skeleton. We assume that phenylalanine at 454 of DCD, which corresponds to tyrosine at 420 of SHC, might be a candidate residue, since this tyrosine, together with isoleucine at 261, is proposed to locate on an upper side of C-ring of hopanyl cation in SHC active site (Hoshino and Sato, 2002). However, this needs to be established.

DCD is the second SC cloned from ferns next to ACH from *A. capillus-veneris* (Shinozaki et al., 2008). Both SCs afforded a single product in contrast to bacterial SHCs which yield multi-products, mostly **1** and **2** (Pale-Grosdemange et al., 1998). Although the SCs obtained from ferns are still only two, they seem to possess a high product-specificity unlike bacterial SHCs.

2.3. Isolation of dammara-18(28),21-diene (**6**) from leaflets of *D. crassirhizoma*

In this study, DCD of *D. crassirhizoma* was characterized to yield a tetracyclic triterpene product, dammara-18(28),21-diene (**6**). This result was rather unexpected, since no tetracyclic triterpene has been reported from this fern. cDNA cloning of DCD from *D. crassirhizoma* implies that this fern produces dammara-18(28),21-diene (**6**) and must contain **6** itself and/or its metabolites. To clarify the presence of **6** in *D. crassirhizoma*, we re-investigated the triterpene hydrocarbon constituents of this fern (Shiojima et al., 1990). Careful silica gel column chromatography and repeated reversed phase HPLC of the hexane extract of dried leaflets yielded a tiny amount of **6** (0.00011%), whose structure was verified by ^{1}H NMR spectroscopic analysis. Although **6** is not a new natural triterpene, these results clearly demonstrated that functional analysis of the gene products could lead to identification of yet unknown natural compounds. Indeed, functional analysis of OSC homologues cloned from the genome sequence of *Arabidopsis thaliana* led to the identification of new triterpenes such as thalianol (Fazio et al., 2004), arabidiol (Xiang et al., 2006), marneral (Xiong et al., 2006) and *seco*-amyrins (Shibuya et al., 2007).

3. Conclusions

All SCs thus far cloned, 4 from bacteria (Ochs et al., 1992; Reipen et al., 1995; Perzl et al., 1997; Tippelt et al., 1998) and one from a fern (Shinozaki et al., 2008), afforded cyclic hydrocarbons with a pentacyclic hopane skeleton. No SC yielding tetracyclic triterpenes has been cloned from any organism. In this study, we have cloned and characterized a novel SC affording the tetracyclic triterpene dammaradiene (**6**) from the fern, *D. crassirhizoma*. Ferns produce a variety of triterpenes ranging from mono- to penta-cyclic skeletons. They include derivatives of lupane and oleanane, which are popularly found among triterpenes of higher plants (Ageta et al., 1998, and cited therein). Further cloning of novel SCs from ferns is expected to provide a deeper insight not only into molecular evolution of SCs but also into the origin of structural diversity exhibited by natural triterpenes.

Characterization of a novel SC in this study as dammara-18(28),21-diene (**6**) led us to re-investigate hydrocarbon constituents of the leaflets to isolate **6** from this fern. This is a good example to show potentials of functional analysis of the biosynthetic gene products for identification and isolation of new natural compounds.

4. Experimental

4.1. General experimental procedures

Synthetic oligonucleotides for PCR primers were obtained from Nihon Bioservice (Saitama, Japan). The PCR reaction products were separated by agarose gel electrophoresis, purified by a Wizard® PCR Preps DNA Purification System (Promega), and ligated into the pT7 Blue T-Vector (Novagen). Resulting plasmids were propagated in *Escherichia coli* DH5a (Takara Biochemicals), and isolated by GFX™ Micro Plasmid Prep Kit (GE Healthcare). Sequencing was carried out by ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Dammara-18(28),21-diene (**6**) isolated from rhizomes of *Pyrrosia lingua* (Polypodiaceae) (Yamashita et al., 1998) served as an authentic standard. Other chemicals were of analytical grade. Each procedure using kits was carried out according to the manufacturers' instructions.

4.2. Plant

For RNA preparations, leaflets of *D. crassirhizoma* Nakai were collected at the medicinal botanical garden of Showa Pharmaceutical University. They were immediately frozen using liquid N_2 and stored at −80 °C. For product isolation, leaflets of *D. crassirhizoma* Nakai (1.2 kg) were collected at the City of Chino, Nagano Prefecture.

4.3. RNA and cDNA preparations

Total RNA was extracted from leaflets of *D. crassirhizoma* using RNAqueous™–Midi (Ambion). Poly $(A)^+$ RNA was purified from the total RNA using a mRNA Purification Kit (Amersham Biosciences). A single-stranded cDNA pool was prepared by SuperScript™II (Invitrogen) with 50 mM of RACE32 (5′-GACTCGAGTCGACATCGATTT TTTTTTTTTT-3′) primer in a total volume of 20 ml. A double-stranded cDNA mixture was also synthesized using the Marathon™ cDNA Amplification Kit (Clontech) for 5′-RACE.

4.4. cDNA cloning of SC

Following the conditions reported in our previous paper (Shinozaki et al., 2008), nested PCR was carried out with the primers, SC-306S-1 (5′-GCIWSIATHWSICCIRTITGGGAYAC-3′), SC-306S-2 (5′-SCITGYBTIWSICCIRTITGGGAYAC-3′), SC-358S (5′-SCIGGI GGITGG GCITT-3′), SC-494A (5′-WARTTIRYICCCCAICKICCRWACCA-3′) and SC-537A (5′-KYYTCICCCCAICCICCRTC-3′) to obtain a 450-bp cDNA fragment. After subcloning and plasmid purification, 21 plasmids were sequenced. All of them were identical each other, and its full length cDNA was named as DCD. 3′–RACE was performed with the primers of DCD-424S (5′-GCATTGGATCAACTCTACCCCTTTTAGC-3′) and DCD-468S (5′-ATTTTCTGCCCCCGGAGTCGATTGCCAG–3′), and 5′-RACE with the primers of DCD-481A (5′-TATACCAAGCCTC TGGCAATCGACTCCG-3′) and DCD-431A (5′-AGGGGTAGAGTT GATCCAATGCTGGTGG-3′), following the reported procedure with some minor modifications, gave the sequence of the full length DCD.

4.5. Expression of DCD in yeast

A full length cDNA was obtained by nested PCR with the following primers: DCD-5′ (5′-CCTTTCCCACACGCTACTGGGTTAC-3′) and DCD-3′ (5′-CCAAACTCAGGCTGCTTGCTTGCAG-3′) for 1st PCR, and N-DCD-Bam (5′-ATCTTGGATCCAAAATGCTGCCATACAATCAAGAT-3′, the *Bam*HI site is underlined) and C-DCD-Xho (5′-GATGCCT CGAGTTATGGAATTGGAGGCTTGAT-3′, the *Xho*I site is underlined), as previously reported (Shinozaki et al., 2008). The resultant PCR product was digested with *Bam*HI and *Xho*I and ligated into the site of pYES2 (Invitrogen) to construct the plasmid pYES2-DCD. *S. cerevisiae* strain INV*Sc*2 (Invitrogen) was transformed with the plasmid, following the previous paper (Kawano et al., 2002). The *in vivo* squalene cyclization assay using the resulting transformant was the same as previously reported (Shinozaki et al., 2008). The crude hexane extract was subjected to Bond Elut®-Si (500 mg, 3 ml, Varian) eluting with *n*-hexane to yield a cyclic triterpene hydrocarbon fraction.

4.6. GC–EIMS analysis of DCD product

GC–EIMS analysis was performed on GCMS-QP2010 (Shimadzu) equipped with Rtx-5MS column (30 m × 0.25 mm, Restek) under the following conditions: electron impact at 70 eV, temperature program being at 250 °C for 2 min, 20 °C min^{-1} to 330 °C and 330 °C for 5 min.

Dammara-18(28),21-diene (**6**): t_R. 7.23 min. *m/z* (rel. int.): 410 $[M]^+$ (5%), 191 (fragment from A/B ring, 62%), 109 (fragment from side-chain, 69%) and 69 (fragment from side-chain, 100%).

4.7. Isolation of dammara-18(28),21-diene (*6*) from leaflets of D. crassirhizoma

Leaflets (1.2 kg) of *D. crassirhizoma* were extracted with *n*-hexane. The hexane extract (12 g) was applied to silica gel CC eluting with *n*-hexane to yield nine fractions according to the TLC and GC profiles. One of these fractions (57 mg) was repeatedly purified by reversed phase HPLC using a Senshu PAK ODS-3251D column (diameter 8 mm, length 250 mm, Senshu Scientific) with MeOH–$CHCl_3$ (8:2) to give **6** (1.4 mg, t_R = 20 min). The structure of **6** was determined by ^{1}H NMR spectroscopic analysis, using tetramethylsilane as an internal standard (BRUKER AV600).

^{1}H NMR spectral data (600 MHz, $CDCl_3$): *d* 0.807 (3 H, *s*, H-24), 0.844 (3 H, *s*, H-25), 0.852 (3 H, *s*, H-23), 0.878 (3 H, *s*, H-27), 0.973 (3 H, *s*, H-26), 1.621 (3 H, *s*, H-30), 1.695 (3 H, *s*, H-29), 4.705 (1 H, *s*, H-28), 4.741 (1 H, *s*, H-28), 5.136 (1 H, *t*, *J* = 6.6 Hz, H-21). The assignments of H-23 and H-25 are interchangeable. Values are identical to those reported by Yamashita et al. (1998).

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