Organic LETTERS

Letter

pubs.acs.org/OrgLett

Identification of Serratane Synthase Gene from the Fern *Lycopodium clavatum*

Yusuke Saga,[†] Takeshi Araki,[†] Hiroshi Araya,[†] Kazuki Saito,[‡] Mami Yamazaki,[‡] Hideyuki Suzuki,[§] and Tetsuo Kushiro*[,†]

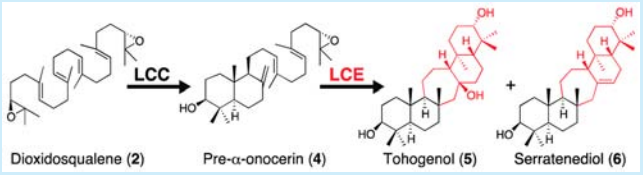
[†]Graduate School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan

[‡]Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Chuo-ku, Chiba 260-8675, Japan

[§]Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan

Supporting Information

ABSTRACT: Ferns are known to produce onoceroids including onoceranes and serratanes having unusual structures among triterpenes. From the fern *Lycopodium clavatum*, a novel onoceroid synthase gene was cloned that showed high sequence identity with a previously identified α-onocerin synthase. Functional analysis by coexpression with pre-α-onocerin synthase in yeast led to the production of tohogenol and serratenediol. The result suggested that serratanes are directly biosynthesized from pre-α-onocerin and not from α-onocerin as previously assumed.



Triterpenes are a large class of natural products with diverse structures and bioactivities. To date, over 100 different types of triterpene skeletons have been reported from natural sources.[1] Triterpene skeletons derive from a common precursor, (3*S*)-2,3-oxidosqualene (**1**), via a cyclization reaction catalyzed by oxidosqualene cyclases (OSCs).[2] These skeletons include sterol precursors such as lanosterol and cycloartenol as well as precursors for various triterpenoids that are widely distributed among plants. In addition to the well-known triterpenes, there are several unusual types that are formed by irregular cyclization patterns.

The bis-cyclic triterpenes, also known as onoceroids, are biosynthesized from squalene and (3*S*,22*S*)-2,3,22,23-dioxidosqualene (**2**) by cyclization from both termini.[3−6] They include compounds having onocerane and serratane skeletons and have been isolated from several ferns including *Lycopodium clavatum*[7] and *Huperzia serrata*[8] and also from some higher plants.[9] α-Onocerin (**3**) (onocerane-type), characterized by its unique symmetrical structure that contains two decalin portions, was recently demonstrated to be biosynthesized by two highly unusual OSC-like enzymes in *L. clavatum*.[4]

In its biosynthetic processes, first the dioxidosqualene cyclase LCC forms the bicyclic polypodane compound pre-α-onocerin (**4**) with strict substrate specificity toward **2**. Subsequently, onocerin synthase LCD catalyzes the second half of the cyclization using **4** as a substrate to produce α-onocerin (Scheme 1). Discovery of such unusual OSC-like enzymes having substrate specificity toward **2** and **4** has prompted us to study their recognition mechanism as well as cyclization reaction in detail. On the other hand, however, no genetic and molecular bases for the biosynthesis of serratanes have been reported.

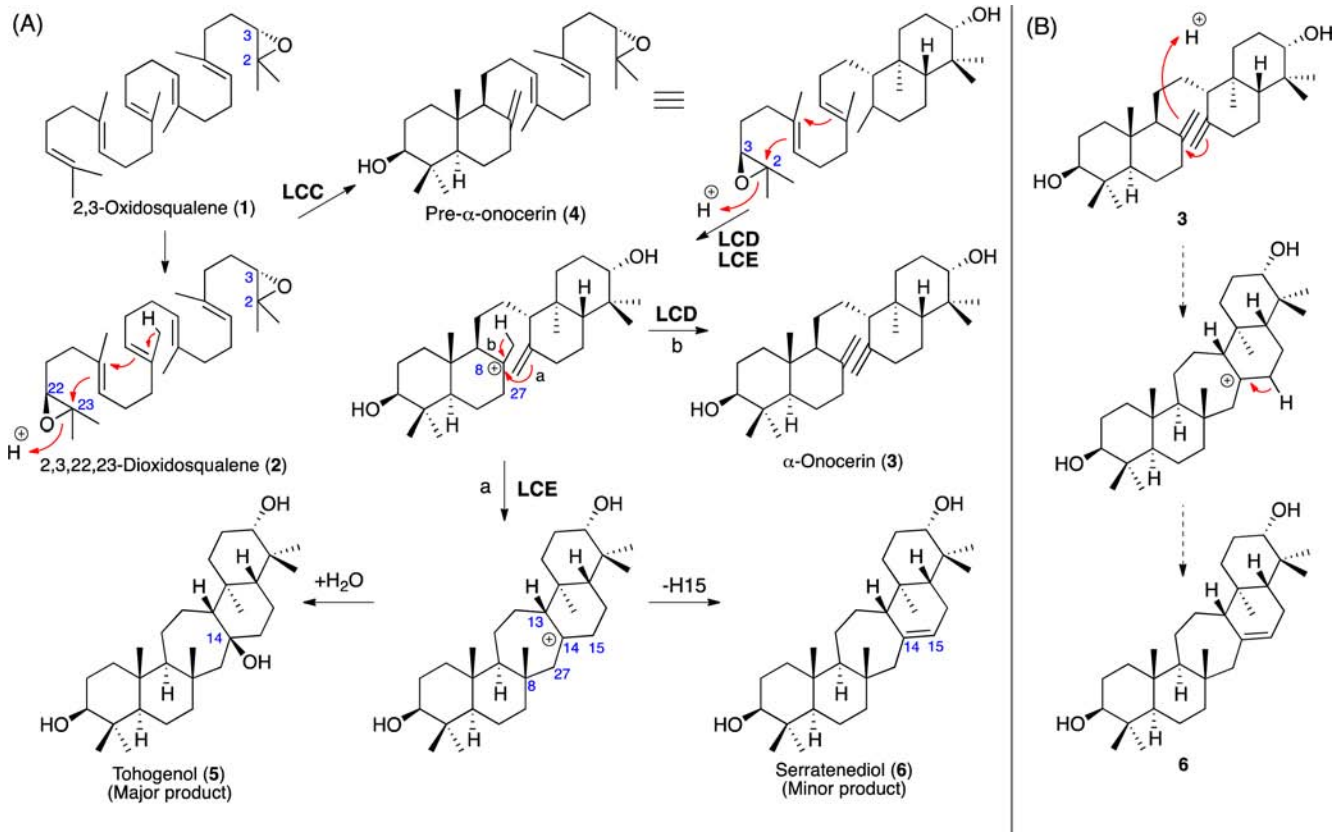
Serratane-type triterpenes possess an unusual seven-membered C-ring with seven tertiary methyl groups (Figure 1). One methyl group is incorporated into a seven membered C-ring. Tohogenol (**5**) and serratenediol (**6**) are the parent members of serratane-type triterpenes and were found in *H. serrata*.[8] Compound **6** has been shown to induce apoptosis and inhibit cell proliferation of human HL-60 leukemia cells.[10] Lycophlegmarin (**7**) has been isolated from *H. phlegmaria* and exhibited modest growth-inhibitory activity against human hepatoma cells BEL 7402.[11]

Their biosynthesis had been proposed to involve protonation of one of the *exo*-methylenes of **3** to generate a C8 tertiary cation to which a cyclization from the neighboring *exo*-methylene would furnish the seven membered C-ring.[12] Indeed, protonation of one of the *exo*-methylenes of **3** by purely chemical means afforded serratenediol.[13] Therefore, the formation of serratanes was assumed to be a secondary transformation after the formation of the onocerane skeleton. Our recent discovery of onocerin synthase gene LCD, however, has raised an alternative possibility in which serratanes could be formed directly from **4** without formation of an intermediate **3** as depicted in Scheme 1. Such a scenario may require an additional enzyme quite similar to LCD, and there is no need to assume a hypothetical protonating enzyme that acts on **3**. In this study, we searched for a novel onoceroid synthase gene from *L. clavatum* to see whether serratane formation involves tricyclization of **4** or monocyclization of **3** and identified the gene involved in the biosynthesis of serratanes for the first time.

Received: December 8, 2016
Published: January 19, 2017

DOI: 10.1021/acs.orglett.6b03659
Org. Lett. 2017, 19, 496−499

Scheme 1. Cyclization of Dioxidosqualene (2) into α-Onocerin (3), Tohogenol (5), and Serratenediol (6)[a]



[a](A) Proposed reaction mechanism of LCC, LCD, and LCE. (B) Previous proposal of serratenediol formation from **3**.

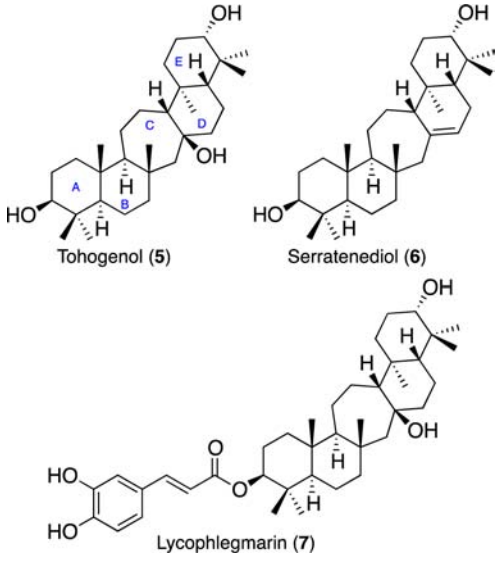


Figure 1. Structures of representative serratane-type triterpenes.

In our previous study on the biosynthesis of **3**, we performed the RNA-seq analysis of *L. clavatum*.[4] To obtain further onoceroid synthase genes, we searched the RNA-seq data for additional OSC homologues using known OSC genes from plants as a query. To our delight, we found that *L. clavatum* possesses another OSC family gene that might be responsible for an onoceroid biosynthesis. This gene, which we denoted LCE, showed moderate sequence identity (58%) to both LCD and cycloartenol synthase LCA (51%), suggesting that this may code for another onoceroid synthase. Characteristic DCTAE and QW motifs of OSCs were found as well, and similar to the LCD case, a sequence around the MWCYC motif is rather diverged from known OSCs that produce a normal type of triterpenes. The corresponding motif of β-amyrin synthase was previously shown to be the active site residues largely responsible for determining the product outcome.[14] Thus, we considered LCE as a candidate gene for onoceroid synthase and set out a functional study of this gene.

The full-length sequence of LCE was obtained by PCR from cDNAs of *L. clavatum*. The obtained cDNA was cloned into the yeast expression vector pESC-Ura under the control of the *GAL10* promoter to produce pESC-LCE. In addition, LCC and LCE were simultaneously introduced into pESC-Ura to obtain pESC-LCC and -LCE, while pESC-LCC was from our previous study.[4] These plasmids were introduced and heterologously expressed in the *Saccharomyces cerevisiae* strain GIL77, a lanosterol synthase deficient mutant that accumulates **1** within the cells, and because of a squalene epoxidase that epoxidizes another terminal olefin, **2** also accumulates in these cells.[15] After induction by galactose and the resting cultures, the transformant cells were harvested, extracted with hexane, and analyzed by TLC. While the LCC expressing transformant produced **4**, both LCC and LCE expressing transformant gave transgene specific spots, one with a R_f value similar to **3** and the other more polar on TLC, both of which were absent in the transformants expressing only LCE. These spots were further analyzed by GC−MS (Figure 2). The major product gave a molecular ion peak at m/z 460 which was 18 Da larger than that of **4** (m/z 442), implicating a structure equivalent to a

DOI: 10.1021/acs.orglett.6b03659
Org. Lett. 2017, 19, 496−499

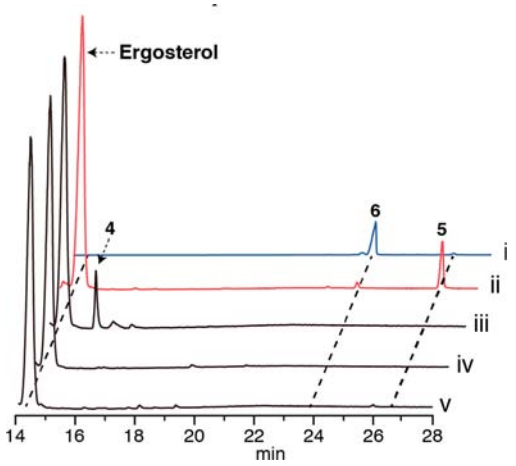


Figure 2. GC−MS analysis of each enzymatic product: (i) standard sample of **6**, (ii) hexane extracts of samples expressing both LCC and LCE, (iii) LCC, (iv) LCE, (v) empty vector. Compounds **5** and **6** were only seen in (ii).

hydrated **4**. On the other hand, the minor product gave a molecular ion peak at m/z 442 that was equivalent to **4**. In addition, the characteristic peaks at m/z 207 and 189 due to retro Diels−Alder fragmentation of the D-ring were observed for this compound.[16]

To clarify the structures of these products, from a large-scale culture (4 L), these products were purified by silica gel column to yield 10 mg of the major product and 1 mg of the minor product and then subjected to NMR analyses. The ^{1}H NMR spectrum (in $CDCl_3$) of the major product showed the presence of seven singlet methyls (δ_H 0.751 (s, 3H), 0.781 (s, 3H), 0.786 (s, 3H), 0.932 (s, 6H), 0.946 (s, 3H), 0.973 (s, 3H)) and two hydroxymethine protons (δ_H 3.195 (dd, (J = 11.5, 4.5 Hz, 1H), 3.202 (dd, J = 11.0, 5.0 Hz, 1H). The ^{13}C NMR spectrum showed 30 carbon signals in which three of them bear a hydroxyl group (δ_C 75.64, 78.76, 78.85). Two of them were methines while the other was a quaternary carbon. Among serratane-type triterpenes, **5** possesses a structure that agrees with this data. The literature data for **5** were reported with pyridine-d_5 as a solvent,[17] while the data for diacetylated **5** were reported with $CDCl_3$.[8] Therefore, our sample of the major product was acetylated and analyzed by NMR, which showed complete agreement with the literature values. We also measured ^{1}H and ^{13}C NMR in pyridine-d_5 as solvent, which showed complete agreement with the literature data.[17]

To further clarify the structure including the stereochemistry of the tertiary hydroxyl group at C14, the diacetylated sample was subjected to dehydration conditions using $SOCl_2$. Under such conditions, **5** having a β-hydroxyl group at C14 would result in production of serratenediol bearing an olefin between C14−C15, while the α-epimer would give a mixture of serratenediol and its olefinic isomer isoserratenediol having an olefin between C13−C14.[8] The configuration of the hydroxyl group may result in such a difference since *trans*-elimination toward C13 to produce isoserratenediol is only possible with the α-configuration. Our result after GC−MS analysis showed almost exclusive production of diacetylated serratenediol, which agreed well with the results from the literature and further confirmed that our major product was indeed **5**.[8]

On the other hand, the minor product showed identical retention time and a MS spectrum with an authentic sample of **6** upon GC−MS analysis. The ^{1}H NMR spectrum (in $CDCl_3$) of the minor product exhibited seven singlet methyl signals (δ_H 0.664 (s, 3H), 0.764 (s, 3H), 0.796 (s, 3H), 0.826 (s, 6H), 0.961 (s, 3H), 0.967 (s, 3H)), two hydroxymethine signals (δ_H 3.185 (dd, J = 11.2, 4.8 Hz, 1H), 3.231 (dd, J = 11.5, 4.1 Hz, 1H)), and an olefinic signal (δ_H 5.331 (m, 1H)), indicating a presence of an olefin. The ^{13}C NMR spectrum showed the presence of two carbons bearing hydroxyl groups (δ_C 78.81, 79.14) and an olefin (δ_C 122.14, 138.18). These data were in complete agreement with the literature data as well as the data for the authentic sample of **6**.[18]

Therefore, LCE was demonstrated to be a novel onoceroid synthase producing serratane-type triterpenes with **5** as the major product together with **6** as the minor product in a ∼10:1 ratio. The structures of these LCE products suggested that the LCE-catalyzed reaction of **4** was initiated by a proton attack on the epoxide moiety, followed by bicyclization to give a C8 cation, a common cationic intermediate en route to **3** (Scheme 1). Subsequent cyclization from the neighboring C14−C27 *exo*-methylene bridged the two decalin structures to construct a seven-membered C-ring and gave a 6/6/7/6/6-fused pentacyclic C14 carbocation intermediate. This cation underwent a nucleophilic addition of a water to produce **5**, or alternatively, deprotonation of H15 to yield **6**. As with the case of LCD, LCE preferably utilized **4** among other substrates such as **1** and **2** and catalyzed the formation of the fused cyclic structure on the linear side chain of **4**. These results indicated that in ferns, onocerane and serratane, two of the major onoceroid, were each individually produced by a different cyclase using **4** as a common substrate. It is noteworthy that the serratane skeleton was biosynthesized from a bicyclic triterpene such as **4**. This finding may disprove the previously accepted proposal that the serratane formation involves protonation of one of the *exo*-methylenes of **3** followed by a cyclization into a seven-membered ring. Our novel finding proposed that serratane formation did not require the formation of an intermediary **3** and was directly produced from **4**.

With both onocerin and serratane synthases in hand, we can now study the difference in the catalytic mechanism of the two enzymes. As mentioned, the two enzymes catalyze the same cyclization cascade up to the C8 cation intermediate, from which an immediate deprotonation will give rise to onocerane while an additional cyclization from the neighboring *exo*-methylene will result in the formation of serratane skeleton. What factor of these enzymes is responsible for the difference between these reaction paths remains to be elucidated. In addition, the LCD-catalyzed cyclization into **3** is terminated by deprotonation, while the LCE-catalyzed reaction is terminated by water addition. The difference of termination mechanism between LCD and LCE is intriguing. We speculate that LCE may have an additional space around C−D rings in its active site cavity, which allows a solvent water molecule to enter. Mutational studies would clarify such questions and elucidate the molecular basis for the formation of two of the most unusual triterpenes found in nature.

Our studies also pointed out that an evolutionary divergence of OSC-like genes such as LCD and LCE was responsible for a production of diverse onoceroid structures in ferns. While the LCC gene would produce the bicyclic triterpene **4**, a combination of LCC and LCD would produce onoceranes, while that of LCC and LCE would produce serratanes. Such a switch in production of these onoceroids can be controlled by an expression of either gene in the cells. Here we also

DOI: 10.1021/acs.orglett.6b03659
Org. Lett. 2017, 19, 496−499

demonstrated that such a selection of onoceroid production could be performed on a yeast platform by choosing the combination of genes to be expressed. It should be noted that an isolation of serratane-type triterpenes have not been reported from *L. clavatum* yet. Our results indicated that this fern does have the ability to produce serratanes. It would be interesting to see if other ferns that are known to produce serratanes possess a gene similar to LCE.

In conclusion, LCE was cloned and characterized as a new serratane synthase that catalyzed the conversion of **4** into **5** and **6** in *L. clavatum*. This is the first time at the gene level to demonstrate that onoceroid skeletons are individually produced by the combination of pre-α-onocerin synthase and different onoceroid synthases. Identification of onoceroid synthases is expected to contribute for future construction of triterpenoids with diverse structures with unusual carbon skeletons.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03659.

Experimental details, supplementary figures, and spectral data including EIMS and NMR (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: kushiro@meiji.ac.jp.

ORCID

Tetsuo Kushiro: 0000-0002-7598-4791

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Emeritus Ushio Sankawa and Prof. Emeritus Yutaka Ebizuka of the University of Tokyo for providing the authentic sample of **6**, Ms. Sayaka Shinpo and Dr. Michimi Nakamura from Kazusa DNA Research Institute for technical support for RNAseq analysis, and Dr. Takahisa Nakane from Showa Pharmaceutical University for measuring optical rotations. We are grateful to Meiji University for financial support.

■ REFERENCES

(1) Xu, R.; Fazio, G. C.; Matsuda, S. P. T. *Phytochemistry* **2004**, *65*, 261−291.

(2) Kushiro, T.; Ebizuka, Y. In *Comprehensive Natural Products II, Vol. 1: Natural Products Structural Diversity−I. Secondary Metabolites: Organization and Biosynthesis*; Mander, L., Liu, H. W., Eds.; Elsevier: Amsterdam, 2010; pp 673−708.

(3) Ueda, D.; Hoshino, T.; Sato, T. *J. Am. Chem. Soc.* **2013**, *135*, 18335−18338.

(4) Araki, T.; Saga, Y.; Marugami, M.; Otaka, J.; Araya, H.; Saito, K.; Yamazaki, M.; Suzuki, H.; Kushiro, T. *ChemBioChem* **2016**, *17*, 288−290.

(5) Rowan, M. G.; Dean, P. D. G.; Goodwin, T. W. *FEBS Lett.* **1971**, *12*, 229−232.

(6) Rowan, M. G.; Dean, P. D. G. *Phytochemistry* **1972**, *11*, 3111−3118.

(7) (a) Tsuda, Y.; Hatanaka, M. *J. Chem. Soc. D* **1969**, 1040−1042. (b) Tsuda, Y.; Fujimoto, T. *J. Chem. Soc. D* **1969**, 1042−1043. (c) Tsuda, Y.; Fujimoto, T. *J. Chem. Soc. D* **1970**, 260−261. (d) Ageta, H.; Iwata, K.; Ootake, Y. *Chem. Pharm. Bull.* **1962**, *10*, 637.

(8) (a) Inubushi, Y.; Sano, T.; Tsuda, Y. *Tetrahedron Lett.* **1964**, *5*, 1303−1310. (b) Sano, T.; Tsuda, Y.; Inubushi, Y. *Tetrahedron* **1970**, *26*, 2981−2986.

(9) (a) Barton, D. H. R.; Overton, K. H. *J. Chem. Soc.* **1955**, 2639−2652. (b) Rowe, J. W. *Tetrahedron Lett.* **1964**, *5*, 2347−2353. (c) Rowe, J. W.; Bower, C. L. *Tetrahedron Lett.* **1965**, *6*, 2745−2750.

(10) Ham, Y.-M.; Yoon, W.-J.; Park, S.-Y.; Jung, Y.-H.; Kim, D.; Jeon, Y.-J.; Wijesinghe, W. A. J. P.; Kang, S.-M.; Kim, K.-N. *Food Chem. Toxicol.* **2012**, *50*, 2629−2634.

(11) Shi, H.; Li, Z.-Y.; Guo, Y.-W. *Nat. Prod. Res.* **2006**, *19*, 777−781.

(12) Zhang, J.; Corey, E. J. *Org. Lett.* **2001**, *3*, 3215−3216.

(13) Tsuda, Y.; Sano, T.; Kawaguchi, K.; Inubushi, Y. *Tetrahedron Lett.* **1964**, *5*, 1279−1284.

(14) Kushiro, T.; Shibuya, M.; Masuda, K.; Ebizuka, Y. *J. Am. Chem. Soc.* **2000**, *122*, 6816−6824.

(15) Gollub, E. G.; Liu, K.-P.; Dayan, J.; Adlersberg, M.; Sprinson, D. B. *J. Biol. Chem.* **1977**, *252*, 2846−2854.

(16) Kutney, J. P.; Eigendorf, G.; Rogers, I. H. *Tetrahedron* **1969**, *25*, 3753−3766.

(17) Sun, Z.-H.; Li, W.; Tang, G.-H.; Yin, S. *J. Asian Nat. Prod. Res.* **2016**, DOI: 10.1080/10286020.2016.1208182.

(18) Fang, J.-M.; Tsai, W.-Y.; Cheng, Y.-S. *Phytochemistry* **1991**, *30*, 1333−1336.

DOI: 10.1021/acs.orglett.6b03659
Org. Lett. 2017, 19, 496−499