JOC The Journal of Organic Chemistry

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β-Amyrin Biosynthesis: Promiscuity for Steric Bulk at Position 23 in the Oxidosqualene Substrate and the Significance of Hydrophobic Interaction between the Methyl Group at Position 30 and the Binding Site

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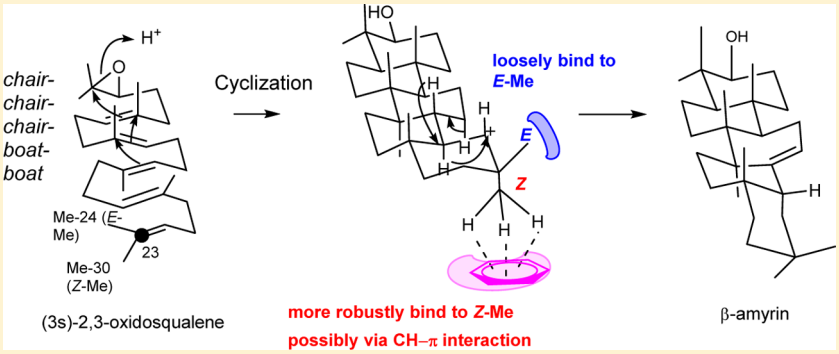
Supporting Information



ABSTRACT: To examine how the sterics at the 23 position of (3*S*)-2,3-oxidosqualene **1** influence the polycyclization cascade in β-amyrin biosynthesis, substrate analogues substituted with an ethyl group (**10**, **11**), a hydrogen atom (**12**, **13**), or a propyl residue (**14**) at the 23 position were incubated with β-amyrin synthase. The bulkier ethyl group was accepted as a substrate, leading to formation of the β-amyrin skeleton (**42**, **43**) without truncation of the multiple cyclization reactions. Analogue **13**, possessing a hydrogen atom and an ethyl group at the 23*E* and 23*Z* positions, respectively, was also converted into the β-amyrin skeleton **45**. However, the analogue lacking an ethyl group at the 23*Z* position (**12**) underwent almost no conversion, strongly indicating that an alkyl group must exist at the *Z* position. The cyclization of the analogue with a propyl substituent at the *Z* position (**14**) was poor. Analogue **15** possessing CH_2OH at the 23*E* position afforded a new compound **47** in a high yield as a result of trapping of the final oleanyl cation. Conversely, **16** with 23*Z*-CH_2OH afforded novel compounds **48**−**50** in low yields, which resulted from the intermediary dammarenyl and baccharenyl cations. Therefore, the hydrophobic interaction between the 23*Z*-alkyl group and its binding site (possibly via CH/π interaction) is critical for adopting the correct chair−chair−chair−boat−boat conformation and for the full cyclization cascade.

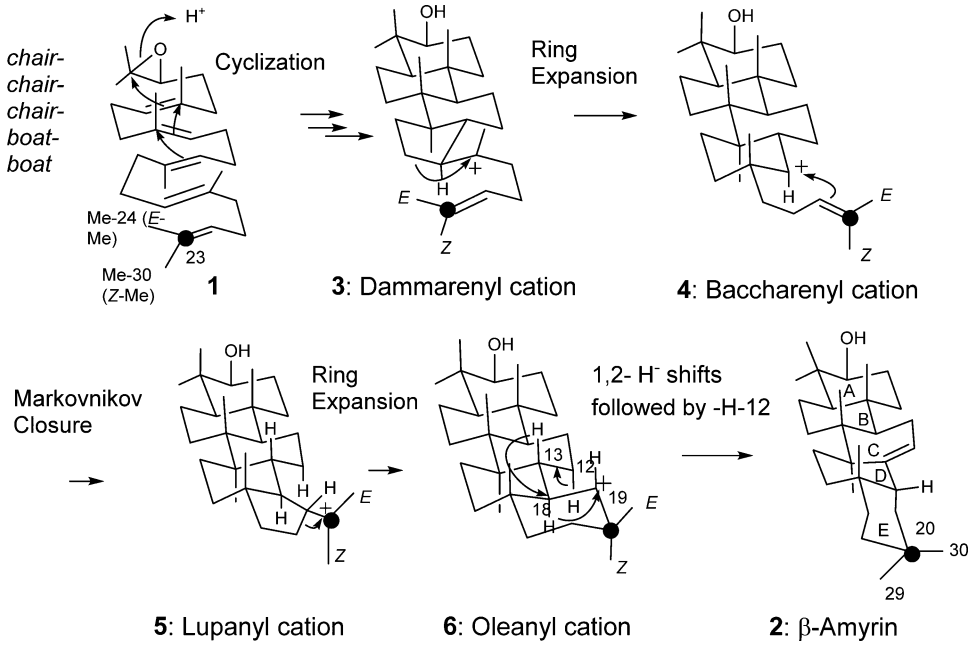
INTRODUCTION

The polycyclization cascades of squalene and (3*S*)-2,3-oxidosqualene **1** have been attractive to organic chemists and biochemists for more than 70 years since Ruzicka and co-workers proposed the "biogenetic isoprene rule".[1] The reactions proceed with complete regiospecificity and stereospecificity to yield sterols and triterpenes[2−7] with remarkable structural diversity; more than 100 different carbon frameworks that exhibit important biological activities can be produced.[8] β-Amyrin **2** consists of a pentacyclic scaffold with eight chiral centers and is widely distributed among plants. Recently, we succeeded in the complete purification of β-amyrin synthase from *Euphorbia tirucalli* (EtAS) and in characterizing its enzymatic properties.[9] Substrate **1** is folded into a chair−chair−chair−boat−boat conformation by β-amyrin synthase (Scheme 1).[1,10−15] Proton attack on the epoxide ring initiates the polycyclization reaction to yield the dammarenyl cation **3** with the 6/6/6/5-fused A/B/C/D tetracyclic ring system, which undergoes ring expansion to create the 6/6/6/6-fused tetracyclic baccharenyl cation **4**. Further cyclization occurs to give the 6/6/6/6/5-fused pentacyclic lupanyl cation **5**, which undergoes further ring expansion to provide the 6/6/6/6/6-fused pentacyclic oleanyl cation **6**. Deprotonation of the H-12α of **6** confers the final product **2**. The enzymatic reactions of substrate analogues have provided deep insights into how the polycyclization reaction is affected by specific modifications, such as alteration of the folding conformation, leading to different stereochemistry, truncation of the ring-forming cascade, or different cation-quenching modes. Recently, we reported the effect of steric bulk at the C-19 and C-23 positions of **1** on the cyclization cascade.[16,17] In addition to

Received: May 31, 2016
Published: July 15, 2016

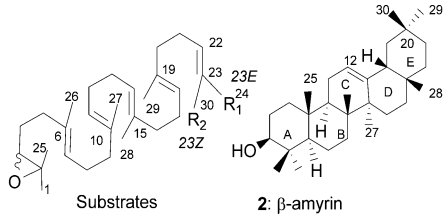
DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657−6671

Scheme 1. Cyclization Pathway of (3*S*)-2,3-Oxidosqualene 1 into *β*-Amyrin 2[a]



[a]The Me-24 and Me-30 groups of compound **1** are regiospecifically converted into the Me-30 and Me-29 groups of compound **2**, respectively. The filled circle shows the destiny of the C-23 atom of compound **1** during the formation of compound **2**; atom C-23 of compound **1** is transformed into atom C-20 of compound **2**.

bisnoroxidosqualene **7**, we further examined the cyclization reactions of nor-analogues **8** and **9**, which lack the methyl-24 and methyl-30 groups, respectively (Figure 1).[17] We discovered



1: $R_1 = R_2 =$ Me: 2,3-oxidosqualene (C_{30})
7: $R_1 = R_2 =$ H: 24,30-bisnoroxidosqualene (C_{28})
8: R_1 = H, R_2 = Me: 24-noroxidosqualene (C_{29})
9: R_1 = Me, R_2 = H: 30-noroxidosqualene (C_{29})
10: R_1 = Et, R_2 = Me: (23*E*)-ethyl-oxidosqualene (C_{31}, Et at 23*E*)
11: R_1 = Me, R_2 = Et: (23*Z*)-ethyl-oxidosqualene (C_{31}, Et at 23*Z*)
12: R_1 = Et, R_2 = H: (23*E*)-ethyl-30-noroxidosqualene (C_{30}: Et at 23*E*, H at 23*Z*)
13: R_1 = H, R_2 = Et: (23*Z*)-ethyl-24-noroxidosqualene (C_{30}: H at 23*E*, Et at 23*Z*)
14: R_1 = H, R_2 = Pr: (23*Z*)-propyl-24-noroxidosqualene (C_{32}: H at 23*E*, Pr at 23*Z*)
15: $R_1 = CH_2OH$, R_2 = Me: (23*E*)-hydroxymethyl-oxidosqualene (C_{30}: CH_2OH at 23*E*)
16: R_1 = Me, $R_2 = CH_2OH$: (23*Z*)-hydroxymethyl-oxidosqualene (C_{30}: CH_2OH at 23*Z*)

Figure 1. Structures of substrate **1** and its analogues **7**–**16** and *β*-amyrin **2**.

that the Me-30 of **1** is critical to accurately folding **1** into a chair–chair–chair–boat–boat structure that leads to the pentacyclic scaffold **2**. Me-24 and Me-30 of **1** were regiospecifically converted into Me-30 and Me-29 of **2**, respectively, during *β*-amyrin biosynthesis.[17]

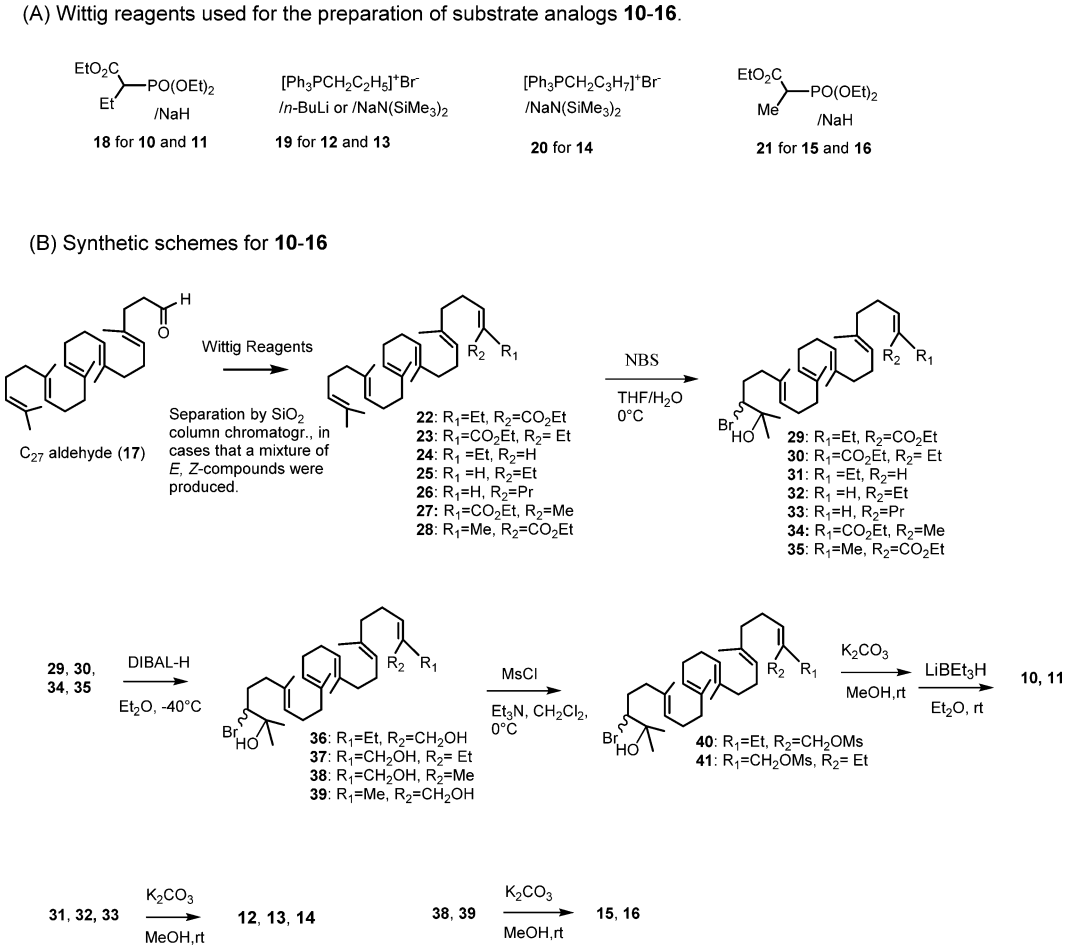
In this paper, we describe how the sterics at position 23 of **1** influence the outcome of the polycyclization reaction. (23*E*)-Ethyloxidosqualene **10** and (23*Z*)-ethyloxidosqualene **11** with the bulkier ethyl group were accepted as a substrate in a considerably high yield, leading to formation of the *β*-amyrin skeleton without truncation of the multiple cyclization reactions. Comparing the cyclization yields between (23*E*)-ethyl-30-noroxidosqualene **12** and (23*Z*)-ethyl-24-noroxidosqualene **13** clearly demonstrated that the absence of the *Z*-configured alkyl group resulted in nearly no cyclization. The larger propyl-substituted analogue **14** afforded a substantially decreased conversion yield, even though the alkyl group is *Z*-configured in **14**. These results indicate that a methyl group is the appropriate steric bulk and that the alkyl group must be *Z*-configured. Little difference in the cyclization yields between **11** and **13** further suggests that the role of the *E*-Me is minimal. Furthermore, we report here that substrate **15** with *E*-CH_2OH (a hydrophilic group) and *Z*-Me groups at the 23 position produced only the oleanyl cation **6**-trapped product **47**. By contrast, substrate **16** with a *Z*-CH_2OH group and *E*-Me gave three products: the dammarenyl cation **3**-trapped products **48** and **49**, and the baccharenyl cation **4**-trapped product **50**. These results indicate that *Z*-Me at the terminus strongly associates with the binding site possibly via CH/π interaction, leading to the ordered architecture of the chair–chair–chair–boat–boat conformation, which further leads to the formation of final cation **6**. A lack of *Z*-Me (**16**) resulted in a disordered folding conformation; thus, trapping of the prematurely cyclized cations **3** and **4** occurred.

RESULTS AND DISCUSSION

Syntheses of Analogues 10–16. Scheme 2 outlines the overall synthetic scheme. The Wittig reagents **18**–**21** used for the preparation of these analogues are shown in Scheme 2A. The starting material **17** with a C_{27}-aldehyde was subjected to Wittig reactions using each of the phosphorus ylides obtained by base treatment. Aldehyde **17** was prepared from squalene.[17] Scheme 2B depicts the subsequent reaction steps. Synthetic intermediates **22** and **23** were obtained from **18** using NaH as a base; **24** and **25** were obtained from **19** in the presence of *n*-

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657–6671

Scheme 2. (A) Wittig Reagents Used in This Study and (B) Synthetic Schemes of Analogues 10−16 from 17[a]



[a]Reagents: NBS, *N*-bromosuccinimide; DIBAL-H, diisobutylaluminum hydride; MsCl, methanesulfonyl chloride; $LiBEt_3H$, lithium triethylborohydride (super hydride).

BuLi and $NaN(SiMe_3)_2$, respectively, where the latter base promoted selective formation of the *Z*-isomer. Product **26** was obtained from **20** using $NaN(SiMe_3)_2$; **27** and **28** were obtained from **21** using NaH as base, yielding a mixture of *E*- and *Z*-isomers. In cases where a mixture of *E*- and *Z*-isomers was produced, separation of the isomers was attained using SiO_2 column chromatography. Next, **22**−**28** were converted to the bromohydrin derivatives **29**−**35** through a reaction with *N*-bromosuccinimide (NBS) in a H_2O/THF solution. The ethyl ester groups of **29**, **30**, **34**, and **35** were reduced by DIBAL-H reagents to give the corresponding alcohols **36**−**39**. Alcohols **36** and **37** were converted into the corresponding mesylates **40** and **41**, respectively. The mesylates were treated with K_2CO_3 to give the corresponding epoxides, which were then demesylated using the hydride reagent $LiBEt_3H$, yielding the desired analogues **10** and **11**. The epoxide analogues **12**−**14** were prepared from **31**−**33**, respectively, by treatment with K_2CO_3. Using a similar treatment, the desired analogues **15** and **16** were obtained from **38** and **39**, respectively.

Incubation of Substrates 10−16 with β-Amyrin Synthase EtAS and Product Profiles. A reaction mixture (2.5 mL, pH 7.4, 100 mM potassium buffer) consisting of TritonX-100 (0.1%, w/v), **1** [200 μg of a (3*R*,*S*)-racemic mixture], BSA (1 mg/mL), DTT (1 mM), and purified His-tagged EtAS (5 μg) was incubated at 30 °C for 12 h.[9] To this reaction mixture was added 7.5 mL of 15% KOH/MeOH, and the resulting mixture was heated to 70−80 °C for 30 min. The lipophilic materials were extracted with hexane, and after removing TritonX-100 included in the extract by a SiO_2 column with hexane/EtOAc (100:10), the hexane extract was evaporated to dryness. Next, 150 μL of hexane was added to the residue, and 1.0 μL of the solution was subjected to GC analysis.

Product profiles are shown in Figure S3 (Supporting Information). Substrates **10**, **11**, and **13** afforded substantial amounts of products **42**, **43**, and **45**, respectively (Figure S3B,C,E). Figure 2 indicates the relative cyclization yields of analogues **10**−**16** compared to that of **1**. The cyclization yields of (3*S*)-**10**, **11**, and **13** were ca. 40−50% in a comparison to that (100%) estimated from genuine substrate (3*S*)-**1** (Figure 2). By contrast, the conversion yield of substrate **12** appeared null (Figure S3D), although a negligible amount of product **44** was detected from the product fraction enriched by partial purification with a SiO_2 column. Other products (peaks A−E and G) were also detected in negligibly small amounts (see Figure S7.1.1 in Supporting Information). This result suggests that many products were produced from **12**, although the quantity of each product was extremely small. A non-negligible amount of product **46** [ca. 4% of the incubation of (3*S*)-**1**] was indicated in the GC trace from the incubation of **14** (Figure S3F). These findings indicate that only one product was detected in a substantial amount from the experiments with **10**,

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J. Org. Chem. 2016, 81, 6657−6671

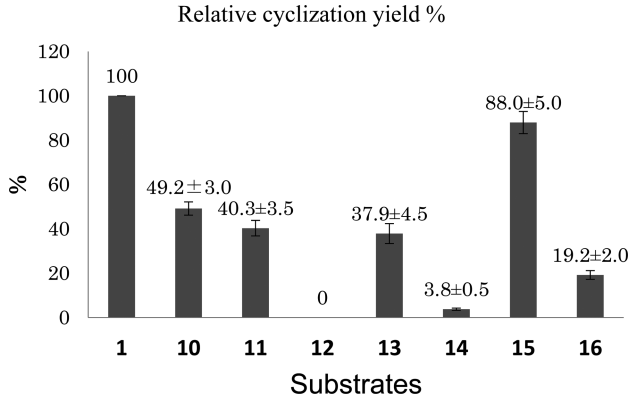

Figure 2. Relative cyclization yields of substrate analogues **10**–**16** against that of **1**. Error bars represent the deviation among three experiments.

11, **13**, and **14**, whereas little was detected for substrate **12**. Substrates **10**–**14** are lipophilic; by contrast, substrates **15** and **16** have a hydrophilic hydroxyl group. From the incubation mixture of **15**, only one product, **47**, was obtained in high yield (88% of that of substrate **1**), whereas three products—**48**, **49**, and **50**—were detected from the reaction with **16** in a total yield of ca. 19% of that of (3*S*)-**1**. The **48**/**49**/**50** product ratio was 2.9:2.1:1.

Structural Determinations of the Enzymatic Products 42–46. EIMS of *β*-amyrin **2** is shown in the Supporting Information (Figure S4.1). The characteristic fragment ions, *m*/*z* 218 (100%) and 203 (60%), were observed; the fragment structures are depicted in Figure S4.2.[13,18,19] As shown in Figure S5.1, a fragment pattern similar to **2** was observed in the EIMS of product **42**: *m*/*z* 232 (100%), 217 (25%), 203 (75%), and 440 (M^+, 3%), indicating that **42** possesses the *β*-amyrin scaffold. The proposed EIMS fragment structures are shown in Figure S5.2. To isolate **42** in an amount sufficient for structural determination by NMR, we incubated 5 mg of substrate **10** with 1.45 mg of EtAS for 24 h and purified the product by SiO_2 column chromatography using hexane/EtOAc (100:10) as the eluent, which afforded pure **42** (2.1 mg, isolation yield). The NMR spectra, including ^1H, ^{13}C, ^1H–^1H COSY, HOHAHA, NOESY, HSQC, and HMBC spectra, are shown in the Supporting Information (Figures S5.3–S5.9). DEPT 45, 90, and 135 spectra were also measured to differentiate methyl, methylene, methine, and quaternary carbons. One double bond was observed: δ_H 5.17 ppm (br s, 1H) in the ^1H NMR (600 MHz, $CDCl_3$) δ_C 121.7 ppm (d), 145.3 ppm (s) in ^{13}C NMR (150 MHz, $CDCl_3$). In substrate **10**, five olefinic Me groups are involved: δ_H 1.67 (s, 3H), 1.73 (s, 9H), 1.81 (s, 3H). Furthermore, two singlet Me groups (δ_H 1.23, s, 3H; 1.27, s, 3H for Me-1 and Me-25) were found on the epoxide ring, and one triplet Me (δ_H 1.073, t, J = 7.6 Hz, 3H for Me-31) exists on the ethyl group (23*E*-Et) in **10**. Product **42** had no olefinic methyl group, and clear HMBC cross-peaks were observed between Me-31 (δ_H 0.764, t, J = 7.4 Hz, 3H) and C-20 (δ_C 33.3, s), between Me-29 (δ_H 0.781, s, 3H) and C-20 and between H-30 (δ_H 1.28, m, 1H; 1.35, m, 1H) and C-20. These findings indicate that full cyclization occurred. The detailed HMBC analyses (Figure S5.10) further allowed us to propose a *β*-amyrin skeleton for product **42**. Complete assignments of the chemical shifts were attained by detailed analyses of the HMBC contours (Figure S5.9) from the singlet Me groups and by analyses of the COSY and HOHAHA spectra (Figures S5.5 and S5.6), as shown in Figure 3 and Figure S5.10. Definitive NOE data for H-18 (δ_H 1.89, m, 1H)/H-30 clearly indicated that the stereochemistry at C-20 was in the *S*-configuration.

This finding leads to an important conclusion that *E*-Et and *Z*-Me at C-23 of **10** are stereospecifically directed toward *β*- and *α*-orientation at the C-20 position, respectively. This conclusion is in a good agreement with our previous report describing the cyclization reaction of 24-noroxidosqualene **8**;[17] Me-30 of **8** is delivered to Me-29 (*α*-arrangement) of the *β*-amyrin skeleton, whereas H-24 is converted into the Me-30 position (*β*-orientation). Unambiguous NOEs, such as Me-28/H-18, Me-27/H-9, H-5/H-9, and H-3/H-5, made the overall stereochemistry of product **42** clear, as depicted in Figure 3.

The EIMS fission pattern of product **43** (Figure S6.1) is almost identical to that of product **42**, suggesting that product **43** also has the *β*-amyrin core. However, the retention time in the GC trace (Figure S3) was slightly different, suggesting that **43** and **42** may be diastereomers. Substrate **11** (8 mg) was incubated in the same manner as **10**, and product **43** was purified using SiO_2 column chromatography, yielding 2.3 mg in

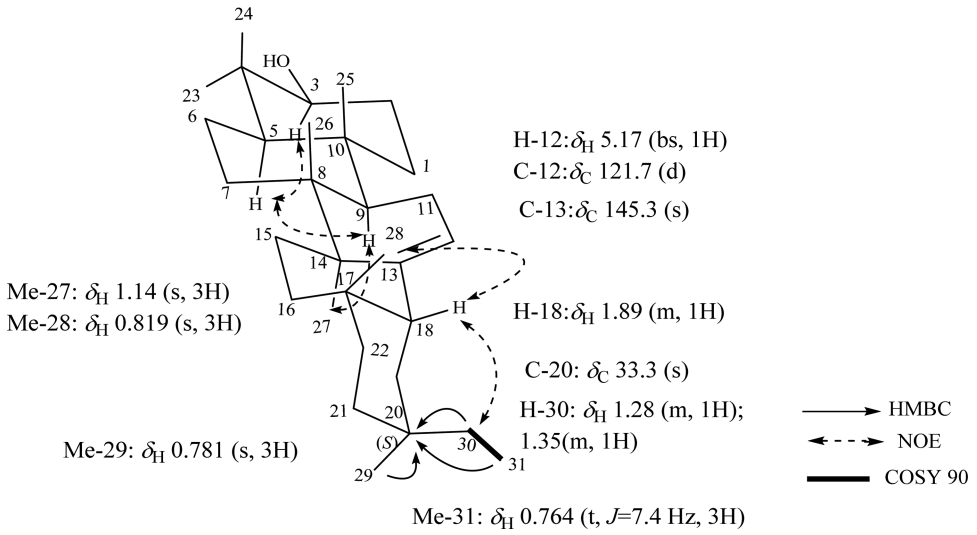

Figure 3. Two-dimensional NMR analyses for proposing the structure of product **42** dissolved in $CDCl_3$.

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657–6671

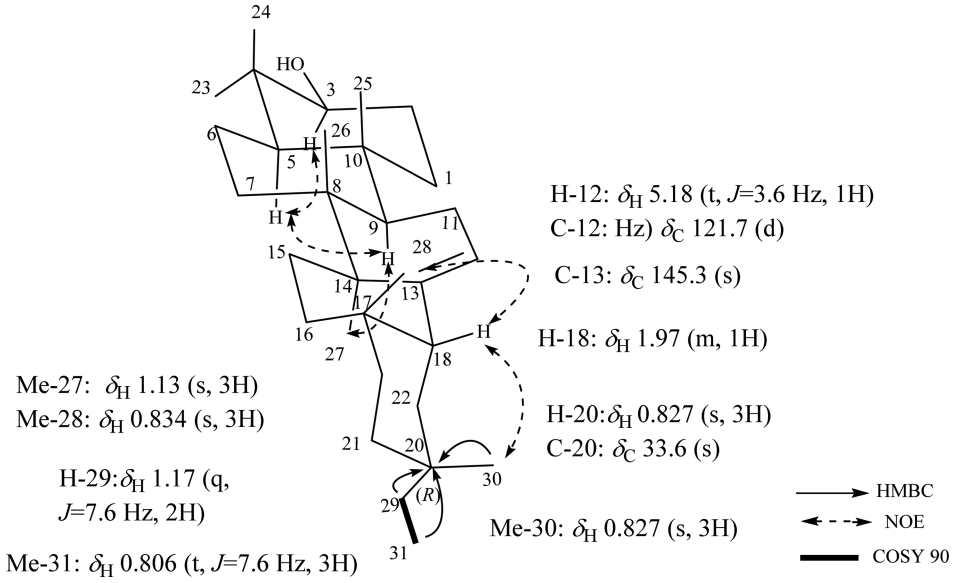

Figure 4. Important 2D NMR data for proposing the structure of product **43** dissolved in $CDCl_3$.

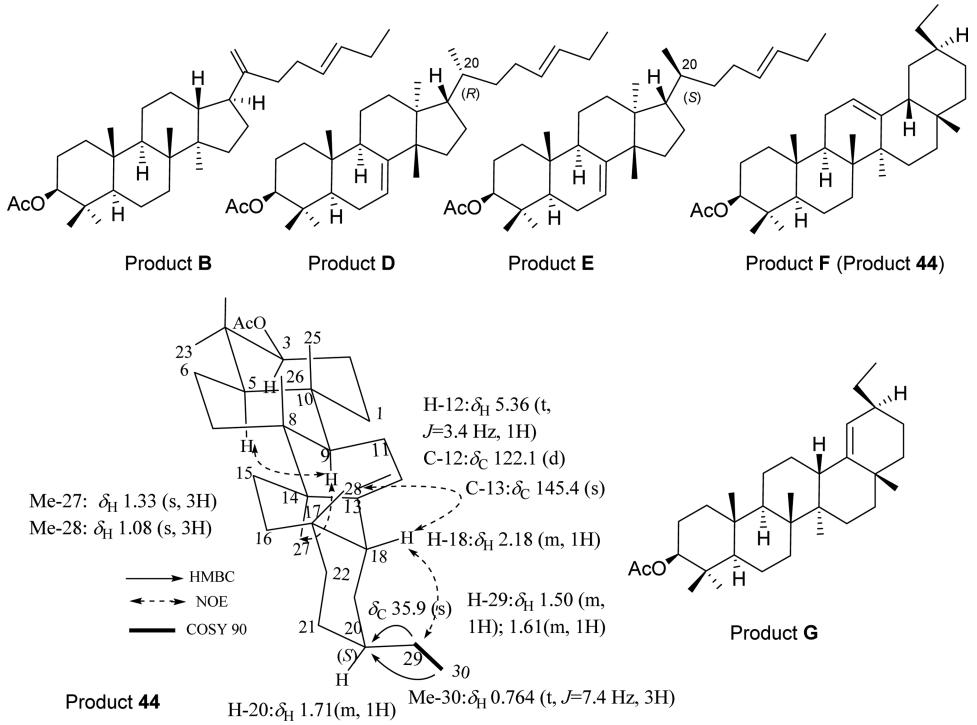

Figure 5. Proposed structures of the products, which were generated from **12** in a negligible amount, and the 2D NMR analyses for product **44** acetate dissolved in C_6D_6.

a pure state. The NMR spectra of product **43** dissolved in $CDCl_3$ are shown in Figures S6.2–S6.8. One olefinic proton was observed at δ_H 5.18 ppm (t, J = 3.6 Hz, 1H) in the ^1H NMR spectrum (400 MHz, $CDCl_3$), and two sp^2 carbons assigned for one double bond were observed at δ_C 121.7 ppm (d) and 145.3 ppm (s) in the ^{13}C NMR spectrum (100 MHz, $CDCl_3$). All the NMR data, including the 2D NMR spectra of **43**, further supported the assignment of the β-amyrin structure for **43**. However, a strong NOE was observed between H-18 (δ_H 1.97, m, 1H) and β-oriented Me-30 (δ_H 0.827, s, 3H) but not between H-18 and H-29 (δ_H 1.17, q, J = 7.6 Hz, 2H). These results definitively demonstrate that Me-30 is β-oriented and that the Et group (CH_2-29 and Me-31) is α-oriented (Figure 4). Thus, the stereochemistry at C-20 was determined to be *R*-configuration, which is opposite that of **42**, demonstrating that substrate **11** was also subjected to a cyclization pathway identical to that of **10** (see Scheme 1). That is, *E*-Me and *Z*-Et of **11** were converted in a stereospecific fashion into the Me-30 position (β-orientation) and into the Me-29 position (α-orientation) of **2**, respectively. The results of the incubation of **10** and **11** with the enzyme indicate that the larger steric bulk of the Et group could also be accepted at the recognition sites, irrespective of being in the *E*- or *Z*-configuration. The cyclization yields decreased compared to

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657–6671

that of **1** (Figure 2), but substantial amounts of β-amyrin homologues were produced.

Substrate **12** underwent almost no conversion (see Figure 2 and Figures S3D and S7.1.1). Removing unreacted substrate **12** using SiO_2 column chromatography (hexane/EtOAc = 100:5) led to detection of the enzymatic product by GC/MS. This product-enriched fraction was acetylated with Ac_2O/Py. Negligible amounts of the acetylated form of compounds **A**–**G** were observed, as shown in Figure S7.1.1. The EIMS spectra of **A**–**G** acetates were compared to those of products obtained from the incubation of 30-noroxidosqualene **9**,[17] which are shown in the Supporting Information of ref 17. Identification of peaks A and C was unsuccessful because EIMS fission patterns similar to those of the products from **9** could not be found. The retention times of the products were shorter than that of peak F (pentacyclic **44**), which suggests that the peaks are likely products of premature termination of the cyclization reaction. The EIMS of peak B was very similar to that of 27-nordammara-20(21),24-diene-3β-ol.[17] Peak D shows the characteristic ions m/z 393 (100%) and 453 (100%). This fragment pattern is very similar to that of 27-norbutyrospermol acetate [m/z 379 (100%) and 439 (98%)];[17] however, the mass unit increment observed for peak D represents CH_2 (m/z 14), suggesting that the structure of peak D would be a 27-norbutyrospermol homologue, as shown in Figure 5 (see also Figure S7.1.1). The difference of the EIMS spectra between peaks D and E was slight, indicating that compounds **D** and **E** share the same carbocyclic skeleton; the retention time of the 20*R*-form (butyrospermol) is known to be shorter than that of the 20*S*-form (tirucalladiene).[17] Thus, compound **E** would be the homologue of 27-nortirucalla-7(8),24-diene-3β-ol. Peak F exhibited the fragment ions (m/z 203 and 218) characteristic of the β-amyrin core. The fragment ion at m/z 189 likely corresponds to the structural unit shown in Figure S7.1.2.[18,19] Purification by HPLC (normal-phase, hexane/THF = 100:0.5) led to the successful isolation of the acetate of peak F but in a very small yield (0.4 mg from 60 mg of **12**). Detailed NMR analyses (Figures S7.2–S7.9) further revealed that peak F was a β-amyrin analogue (Figure 5). Complete assignments of the NMR data are shown in Figure S7.9. A clear NOE for H-29/H-18 indicates that the β-Et and α-H were substituted at the C-20 position (product **44**). Peak G shows the characteristic ions, such as m/z 177, 189, and 204, that correspond to increments of a CH_2 unit (m/z 14) to m/z 163, 175, and 190 observed in the EIMS of 29-norgermanicol.[17] Thus, careful inspection of the EIMS spectra of products from **9** and **12** indicate that products **B**, **D**, **E**, **F**, and **G** are homoderivatives (C_1 appendages, Et substituent) of 27-nordammara-20(21),24-diene-3β-ol, 27-norbutyrospermol (uph-7(8)-24-diene-3β-ol), 27-nortirucalla-7(8)-24-diene-3β-ol, 29-nor-β-amyrin, and 29-norgermanicol, respectively. These predicted structures and the 2D NMR analyses for **44** (peak F) are depicted in Figure 5.

By contrast, substrate **13** (the *Z*-isomer of **12**) was well-converted into product (yield: ca. 38%; see Figure 2). The EIMS spectrum of the acetate of product **45** (Figure S8.1.2) was almost identical to that of the acetate of product **44**; thus, the carbocyclic core of **45** is likely the same as that of **44**. Interpretation of the major fragment ions are shown in Figures S7.1.2 and S8.1.3.[18,19] Product **45** was roughly purified using SiO_2 column chromatography (hexane/EtOAc = 100:10), and the fraction including **45** was subsequently acetylated with Ac_2O/Py. Pure **45** acetate was obtained using normal-phase HPLC (hexane/THF = 100:0.3). The NMR spectra (400 MHz for ^1H, 100 MHz for ^{13}C NMR, C_6D_6) are shown in Figures S8.2–S8.8. The analyses of the NMR data are shown in Figure 6 (see also Figure S8.9); these data further indicate that **45** has

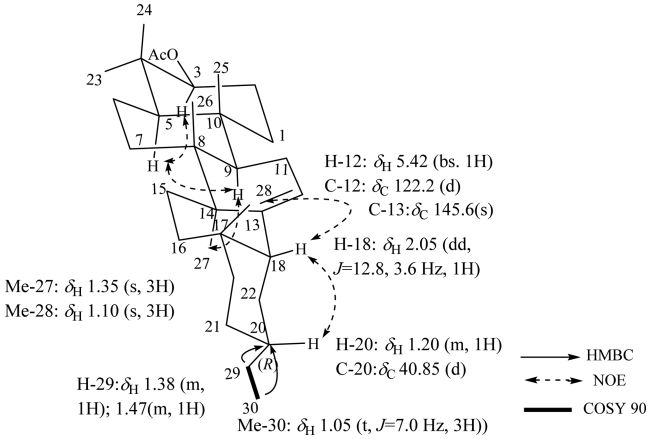

Figure 6. Two-dimensional NMR analyses for proposing the structure of product **45** dissolved in C_6D_6.

a β-amyrin scaffold similar to that of **44**. However, the NOESY spectrum demonstrated definitively the following NOEs: H-18/Me-28 and H-18/H-20 (H-18: δ_H 2.05, dd, J = 12.8, 3.6 Hz, 1H; H-20: δ_H 1.20, m, 1H; Me-28: δ_H 1.10, s, 3H). These findings indicated a 20*R*-configuration with regard to the arrangement of the β-H and the α-Et at C-20. No other products were found at detectable concentrations. Thus, the E-ring closure occurs in a stereospecific fashion, despite lacking Me-24 and despite Me-30 being replaced by a larger Et (C_1 appendage) at the C-23 position of **1**. The cyclization yield of **13** was approximately the same as that of **11** (Figure 2), further indicating that the substitution of the larger steric bulk (Et group) or the smaller size (H atom) at the 23*E* position of **1** had little effect on the cyclization pathway and yield. By contrast, substitution with a hydrogen atom at the 23*Z* position (comparison of **12** with **13**) notably affected the cyclization pathway and yield, strongly indicating that the presence of an alkyl group at the *Z* position is essential for the normal polycyclization cascade, as we demonstrated in a previous paper.[17]

To examine how the alkyl chain length at the *Z* position affects acceptance of a molecule as a suitable substrate, we incubated substrate **14** (*Z*-Pr group and *E*-H atom) with EtAS. Figure 2 shows that a small amount of product **46** was produced in ca. 4% yield (see also Figure S3F). The EIMS spectrum of this peak suggests that product **46** also has the β-amyrin scaffold; the fragment structures corresponding to the characteristic ions m/z 232 and 217 are shown in Figure S9.2. Repeated incubation experiments (3×) with **14** (10 mg) and purification by SiO_2 column chromatography afforded 0.3 mg of **46**. Pure **46** was obtained by normal-phase HPLC (hexane/THF = 100:3). The corresponding NMR spectra (600 MHz, $CDCl_3$) are shown in Figures S9.3–S9.9. A propyl group was observed at δ_H 0.863 (t, J = 7.3 Hz, 3H, Me-31), 1.30 (m, 2H, H-30) and 1.37 (m, 2H, H-29). A clear NOE was observed between H-18 (δ_H 1.79, m, 1H) and H-20, indicating the orientation was 20β-H and 20α-Pr (Figure S9.10). Thus, this enzymatic product **46** was produced in a stereospecific manner, as shown in Scheme 1. Notably, the cyclization yield of **14** (ca. 4%) was substantially decreased compared to that of **13** (ca.

DOI: 10.1021/acs.joc.6b01313
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38%), indicating that the EtAS enzyme did not accept the propyl group at the 23 position as well as it accepted the normal substrate (see Figure 2 and Figure S3F).

Interestingly, all the enzymatic products of substrates **10**−**14**, except that of substrate **12**, possessed a β-amyrin skeleton. A current model that can plausibly explain these results is proposed as follows: The alkyl group at the 23*Z* position plays a critical role in folding the substrate into an ordered chair−chair−chair−boat−boat conformation. Increasing the steric bulk at the 23*Z* position would place the epoxide ring of the substrates in a location somewhat distant from the DCTA motif, which is essential for initiating the polycyclization reaction by providing an acidic proton to activate the epoxide ring. A substituent with a larger steric size (Me → Et → Pr) at the 23*Z* position could move the epoxide ring farther from the DCTA motif inside the enzyme cavity. This change can explain the decrease in cyclization yields with increasing steric volume (100% → 40% → 4%; see Figure 2); however, the organized conformation may have still been retained, thereby enabling formation of the β-amyrin carbocyclic skeleton. Substrate **12**, which is substituted with a hydrogen atom at the 23*Z* position and an Et group at the 23*E* position, exhibited substantially decreased activity (almost no activity), whereas substrate **13** possessing an Et group at the 23*Z* position underwent the normal cyclization in a considerably high yield (ca. 38%). This difference in reactivity suggests that the hydrophobic interaction occurs between the *Z*-alkyl group (Me or Et group) and its recognition site of the cyclase and further indicates that the hydrophobic interaction with an alkyl group in the *Z* and not the *E* position is critical to creating the normal folding architecture. This model is consistent with the results of the comparative study between the reactions of **8** and **9** reported in our previous paper.[17] This finding is further supported by the fact that the reaction yields for substrates **11** and **13** were similar (40% for **11**; 38% for **13**). Thus, the contributions of hydrophobic interactions with the 23*E*-alkyl group are less important than the interaction with the 23*Z*-alkyl group. The importance of this hydrophobic interaction between the 23*Z*-alkyl group and its binding site was further established by the experiments described below involving substrates **15** and **16**, which have a hydrophilic CH_2OH group.

Structures of Products 47−50 from 15 and 16 and the Cyclization Mechanism. The hexane extract obtained from the incubation mixture of **15** (10 mg) with EtAS (1.12 mg) was subjected to normal HPLC (hexane/THF = 100:2), yielding ca. 3.5 mg of pure **47**. Incubation of **16** (10 mg) under the same conditions as **15** gave crude product mixtures (2.3 mg), which were acetylated with Ac_2O/Py. The resulting acetate mixtures were subjected to normal-phase HPLC (hexane/THF = 100:0.8), where the acetylated products were eluted in the order **50** → **48** → **49**. The isolation yields of **48**−**50** were 0.7, 0.7, and 0.4 mg, respectively.

No olefinic proton and no sp^2 carbon were observed in the ^1H and ^{13}C NMR spectra of **47** dissolved in $CDCl_3$ (Figures S10.2 and S10.3), suggesting that substrate **15** underwent the complete cyclization reaction by EtAS. In the HMBC spectrum (Figure S10.8), Me-27 (δ_H 0.914, s, 3H) showed correlations with C-15 (δ_C 27.2, t) and C-13 (δ_C 38.1, d), and Me-28 (δ_H 0.941, s, 3H) showed cross-peaks for C-16 (δ_C 36.2, t) and C-18 (δ_C 88.2, s). The chemical shift of C-18 indicates that an oxygen atom is connected to C-18. Furthermore, both H-13 (δ_H 2.12, dd, J = 12.4 and 2.8 Hz, 1H) and one of H_2-30 (δ_H 3.61, d, J = 7.6 Hz, 1H) showed a definitive HMBC cross-peak for C-18, suggesting that a THF ring is involved in **47**. One H_2-30 had a clear HMBC correlation with C-18, but the other exhibited no HMBC correlation (J = 8 Hz was used for detecting the long-range coupling constant). A three-bond C−H coupling constant follows a Karplus-type relationship. The dihedral angles for H_A−C(30)−O−C(18) and H_B−C(30)−O−C(18) are estimated to be 125 and 115°, respectively, from the corresponding Chem3D models (Figure S10.10), indicating that H_A has a clear HMBC correlation with C-18. The definitive NOEs between Me-27 and H_2-19 (δ_H 1.46, m, 1H; 1.62, m, 1H) indicate that the oxygen atom of the THF ring is β-oriented, and a clear NOE was observed for Me-29/H_B-30 (δ_H 3.28, d, J = 7.6 Hz, 1H); thus, Me-29 is determined to be in an α-arrangement. Additional detailed 2D NMR analyses allowed us to propose the whole structure of **47**, as shown in Figure 7 (see also Figure S10.9).

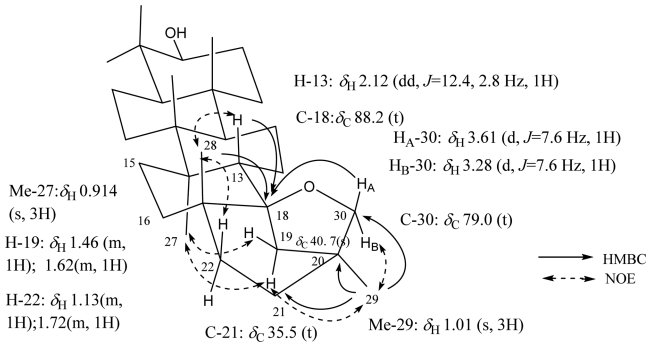

Figure 7. Two-dimensional NMR analyses for proposing the structure of product **47** dissolved in $CDCl_3$.

The ^1H (400 MHz) and ^{13}C NMR spectra (100 MHz) of product **48** acetate dissolved in C_6D_6 showed one olefinic proton (δ_H 5.57, br s) and one double bond (δ_C 125.3, d; 136.8, s). These results suggest that the polycyclization reaction of **16** terminated at the dammarenyl tetracyclic skeleton. Three carbons connected to an oxygen atom were observed (δ_C 80.6, d, C-3; 80.2, s, C-20; 64.7, t, C-27). In the HSQC spectrum, H-3 (δ_H 4.83, dd, J = 11.6, 2.4 Hz) was correlated with C-3. Me-21 (δ_H 1.29, s, 3H) had a clear HMBC cross-peak for C-20 and C-17 (δ_C 45.7, d). Me-26 (δ_H 1.61, br s, 3H) exhibited strong HMBC correlations with C-27, C-25 (δ_C 136.8, s), and C-24 (δ_C 125.3, d). In the HOHAHA spectrum, the olefinic proton H-24 (δ_H 1.61, br s, 1H) definitively showed the following ^1H−^1H network: H-24/H-27 (δ_H 3.91, br d, J = 16.4 Hz, 1H; 4.24, br d, J = 16.4 Hz, 1H)/Me-26 (δ_H 1.61, br s, 3H)/H-23 (δ_H 2.43, m, 1H; 2.17, m, 1H)/H-22 (δ_H 1.84, m, 1H; 1.72, m, 1H), indicating that a seven-membered ether ring (oxepane core) is involved in **48**. The characteristic fragment ion m/z 125 in the EIMS (Figure S11.1) further supported the presence of an oxepane moiety (Figure 8). A definitive NOE was observed between Me-30 and H-17 (δ_H 2.25, m), indicating that H-17 is α-oriented. Taking into consideration the analyses of the other NMR data, we can propose the whole structure of **48**, as depicted in Figure 8 (see also Figure S11.9). The EIMS spectrum of product **49** was almost identical to that of **48** (see Figures S11.1 and S12.1), indicating that a skeleton similar to that of **48** is assignable for **49**. Observation of the fragment ion m/z 125 further supported the seven-membered oxepane ring. This model was again supported by the detailed 2D NMR analyses of **49** (Figure S12.2−S12.9). Me-21 (δ_H 1.27, s, 3H)

DOI: 10.1021/acs.joc.6b01313
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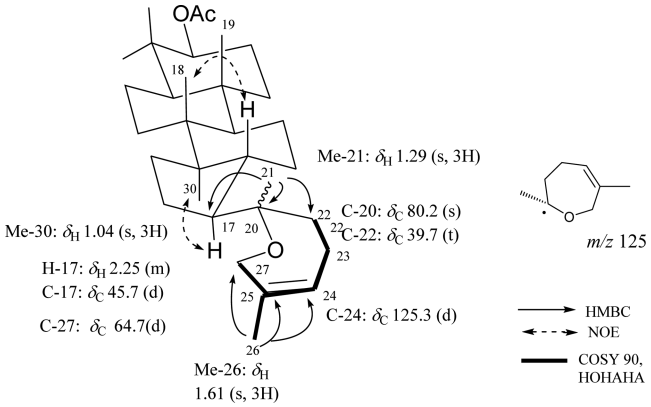


Figure 8. Two-dimensional NMR analyses for proposing the structure of product **48** or **49** dissolved in C_6D_6. Products **48** and **49** are diastereomers, with their configurations at C-20 being opposite. The chemical shifts of product **48** are shown.

had clear HMBC correlations with C-20, C-22, and C-17. The HOHAHA spectrum revealed the following $^1H-^1H$ spin-coupling network: H-24/Me-26/H-23/H-22/H-27, as observed in the HOHAHA spectrum of **48**. The α-orientation of H-17 was confirmed by the definitive NOE between H-17 and Me-30. The detailed NMR analyses (Figure S12.9) lead to a proposed structure identical to that of **48**. Product **48** was separable from **49** by normal HPLC and GC, indicating that **48** and **49** are diastereomers. Careful comparison of the NOESY spectra of **48** and **49** revealed that the configurations of the tetracyclic dammarenyl core including H-17 stereochemistry were identical between **48** and **49**, thus demonstrating that the C-20 stereochemistry is the only difference between the two isomers. The C-20 stereochemistry for **48** and **49** remained to be elucidated.

One olefinic proton (400 MHz, C_6D_6; δ_H 5.76, t, 1H) and one double bond (100 MHz, C_6D_6; δ_C 126.7, d; 132.9, s) were found in **50** acetate, implying that the polycyclization reaction of **16** terminated at the abortive tetracyclic stage to give **50**. The partial structure of $CH_2-C(Me)=CH-CH_2-CH_2$ was confirmed by the following HOHAHA correlations: H-21 (δ_H 5.76, t, J = 8.0 Hz, 1H)/H-20 (δ_H 1.98, m, 1H; 2.04, m, 1H)/H-19 (δ_H 1.65, m, 1H; 1.50, m, 1H)/Me-29 (δ_H 1.56, br s, 3H)/H-30 (δ_H 3.85, br d, J = 16.8 Hz, 1H; 4.47, br d, J = 16.8 Hz, 1H), with this partial structure being also observed in the structure of **48** and **49**. Two carbons connected to an oxygen atom were observed at δ_C 74.1 (t) and δ_C 84.5 (d) for **50**, suggesting an ether linkage. This result is in contrast to the ethereal carbons of **49**, which were at δ_C 64.7 (t) and 79.6 (s). Furthermore, the multiplicity for one of the two carbons was different, and the chemical shifts of the ethereal carbons were distinct between **50** and **49**. These data suggest that the structure of **50** is quite different from that of **48** or **49**, which possess an oxepane ring. Me-28 (δ_H 1.25, s, 3H) had clear HMBC correlations with C-16 (δ_C 36.5, t), C-17 (37.2, s), C-18 (84.5, d), and C-19 (45.9, t), indicating that the D-ring of **50** is six-membered and not five-membered as in **48** and **49**. These findings indicate that the E-ring of **50** is an eight-membered ring (oxocane ring), as shown in Figure 9. The strong NOE between H-18 (δ_H 3.20, d, J = 10.4 Hz, 1H) and Me-27 (δ_H 0.921, s, 3H) verified that H-18 was arranged in the α-configuration. Further detailed analyses of 2D NMR made clear the complete structure of **50**, as shown in Figure 9 (see also Figure S13.9).

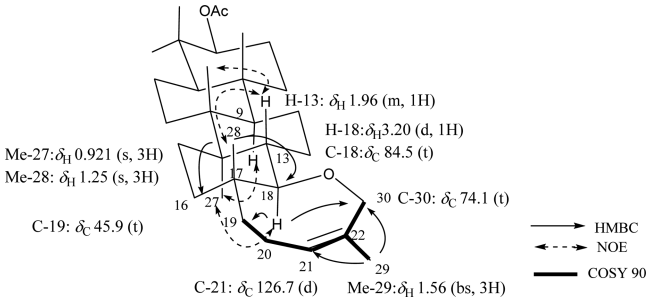


Figure 9. Two-dimensional NMR analyses for proposing the structure of product **50** acetate dissolved in $CDCl_3$.

Figure 10 summarizes the structures of all the products generated by incubating substrates **10**–**16** with EtAS enzyme. Substrates **10**–**14** afforded only β-amyrin skeleton, except for **12**, indicating that the *Z*-alkyl group at the 23 position was more strongly bound to the cyclase, as shown in Scheme 3A. Scheme 3B,C illustrates the product formation mechanism from substrates **15** and **16**. Substrate **15** was folded in the chair–chair–chair–boat–boat conformation and underwent the normal cyclization reaction according to Scheme 1, resulting in the formation of oleanyl cation **6′** with CH_2OH (C-19 cation, ca. 88% yield). A hydride shift of H-18 to C-19 cation afforded the oleanyl cation **6″** with a C-18 cation. The hydroxyl group subsequently attacked this cation as a nucleophile, yielding the 6–6–6–6–6–5-fused A–B–C–D–E–F hexacyclic ring system **47**. A nucleophilic attack of CH_2OH at the C-19 cation of **6′** would provide a 6–6–6–6–6–4-fused hexacyclic product. The four-membered ring has a larger steric strain, which triggered the 1,2-shift of hydride of H-18 to form a less-strained five-membered F-ring. Formation of **6″** likely leads to β-amyrin homologue **2′** according to Scheme 1 and Scheme 3A, but CH_2OH quickly attacked the generated cation **6″** because of its highly nucleophilic nature, yielding **47**. The 23*Z*-Me of **15** is anchored by the hydrophobic interaction, as shown in Scheme 3B, in a manner similar to that of **1** (Scheme 3A). This robust interaction could lead to the formation of the final cation **6′**. However, the reaction of substrate **16** terminated at the tetracyclic dammarenyl cation **3′** in a yield of ca. 19%. The *Z*-CH_2OH group then acted as a nucleophile to attack the C-20 cation and produce **48** and **49** [ca. 15% (7 or 8% each)]. The configuration at C-20 of **48** was opposite that of **49**, indicating that *Z*-CH_2OH of **16** was not anchored by the 23*Z* recognition site; thus, the side chain of **3′** underwent a free rotation to afford both the 20*R*- and 20*S*-configurations, as shown in Scheme 3C. This finding gives additional support to the essential nature of the alkyl group at 23*Z* position for formation of the final oleanyl cation intermediate **6′**. Further cyclization of **3′** led to a baccharenyl cation **4′** (ca. 4%) in a small amount, which was then converted into product **50** as a result of the nucleophilic attack of *Z*-CH_2OH at cation **4′**. Cation **4′** is the intermediary cation but not the final cation **6′**. Production of **50** also indicates that the chair–chair–chair–boat–boat structure was distorted because of the lack of a 23*Z*-alkyl group, resulting in the termination at the intermediary stage. As shown in Figure 2, the cyclization yield of **15** was outstandingly high (ca. 88%), whereas that of **16** was at most ca. 19%. This fact provides additional evidence that the alkyl group at the 23*Z* position is strongly bound to the binding site through a hydrophobic interaction and is essential for the normal polycyclization cascade, but this interaction of

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657–6671

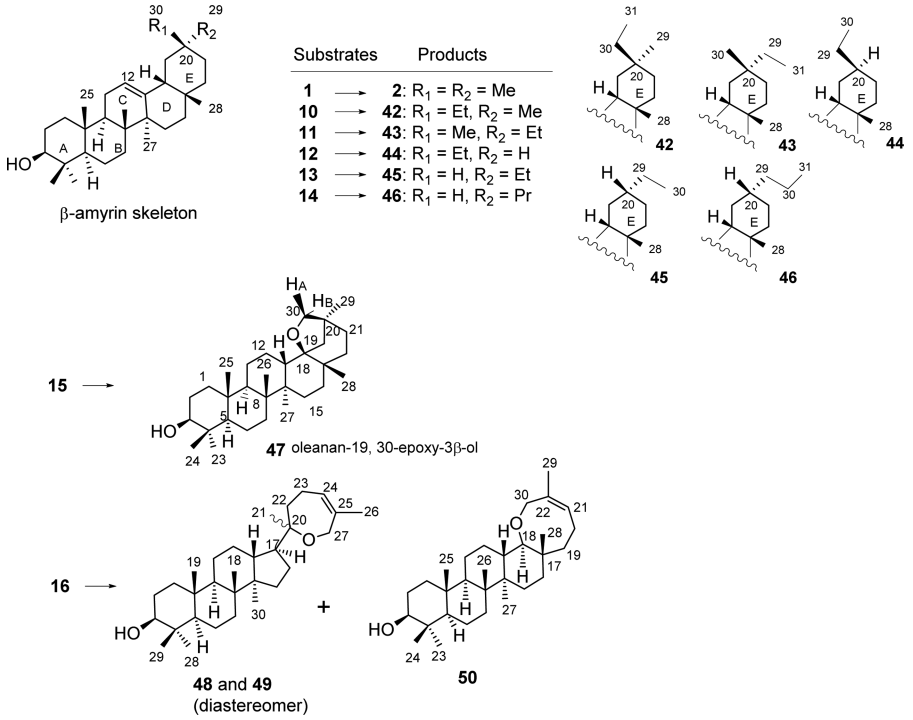


Figure 10. Structures of products **42**−**50** obtained from the incubation experiments with substrates **10**−**16**. The carbon numbering systems are also shown here to aid in interpretation of the NMR data.

23*E*-alky moiety is not robust. We propose that the robust interaction would be caused by CH−π interaction. Our experimental results clearly demonstrated that this robust interaction could sufficiently direct the enzymatic reaction toward pentacyclization, although a binding energy for CH/π affinity may be considered to be weak. We constructed site-directed mutants targeting the amino acids surrounding the terminus of substrate **1** (see Figure S14). The following residues were mutated into Ala residue: F124, F125, W219, C260, R261, V263, S412, F413, F552, I555, and C732. None of the variants produced tetracyclic products but instead produced only β-amyrin (Figure S15). Therefore, these residues are not responsible for the interaction with Me-30 (23*Z*-Me) on **1**. We have reported that the site-directed mutants of F728 afforded tetracyclic compounds along with pentacyclic products.[20,21] Kushiro et al. revealed that the residues of W259 and Y261 from *Panax ginseng* β-amyrin synthase stabilize the tetracyclic and the pentacyclic cation intermediates via cation/π interaction.[22] The W259 and Y216 from *P. ginseng* correspond to W257 and Y259 residues from EtAS, respectively, in the amino acid alignment. The functions of W257 and Y259 residues from EtAS identified by our experiments were consistent with those of W259 and Y261 reported by Kushiro et al.[22] These three aromatic residues—F728, W258, and Y259— (independently or together) may interact with Me-30 of **1** via CH/π interaction (perpendicular to the CH_3), as depicted in Scheme 3A, in addition to their function in stabilizing the intermediary cation(s) through cation/π interactions (horizontal to the planar sp^2 carbocation). Further studies may be necessary to validate this assumption.

CONCLUSION

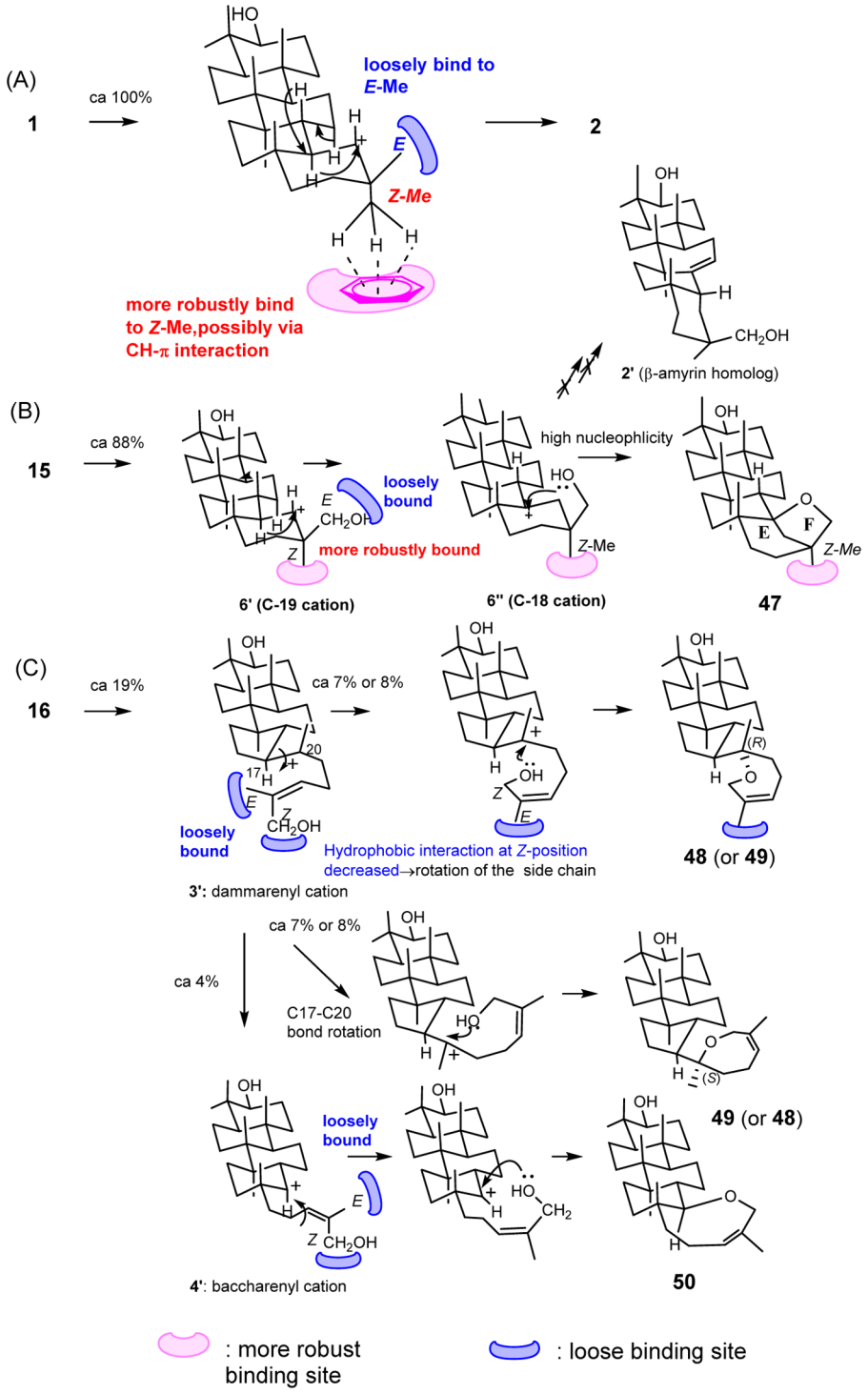
Through this investigation, we clarified that β-amyrin synthase tolerates ethyl substituents at position 23 in the substrate, as evidenced by the high yields of the corresponding products. The hydrophobic interaction of the *Z*-alkyl group with the enzyme is more critical to forming the normal folding architecture than that of the *E*-alkyl group. The contribution of the hydrophobic interaction with the 23*E*-alkyl group is much less than that of the 23*Z*-alkyl group. The importance of the hydrophobic interaction with the 23*Z*-alkyl group was further established by comparing the cyclization reactions of **15** and **16**, which possess a hydrophilic CH_2OH group. Compounds structurally similar **47** have been reported, such as 13,28-epoxyoleanan-3β-ol[23] and 19,28-epoxyoleanan-3β-ol;[24] however, the 19,30-epoxy derivative **47** had not been found before the present study. Products **48**−**50** are also new compounds; no similar structures are found in SciFinder (American Chemical Sociey, https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf). This study on the substrate analogues bearing a hydroxyl group has provided deep insight into the cyclization mechanism in addition to the creation of new compounds **47**−**50**.

EXPERIMENTAL SECTION

General Analytical Methods. NMR spectra were recorded at 600 or 400 MHz in the solution of $CDCl_3$ or C_6D_6. The chemical shifts (δ) are given in parts per million relative to the residual solvent peak as the internal reference (δ_H = 7.26 and δ_C = 77.0 ppm for $CDCl_3$; δ_H = 7.28 and δ_C = 128.0 ppm for C_6D_6; δ_H = 2.04 and δ_C = 29.80 ppm for acetone-d_6). GC analyses were performed by fitting a flame ionization detector and a nonpolar DB-1 capillary column (30 m × 0.32 mm); injection temp = 300 °C; column temp = 235−250 °C (3 °C/min) and 250−270 °C (0.5 °C/min); flow rate (He gas) = 1.50 mL/min. Several peaks of substrates **1** and **10**−**16** indicate that the thermal degradation of these substrates occurred during the GC analyses. GC/MS spectra were obtained under electronic impact at 70 eV with a Zebron ZB-5 ms capillary column (0.25 mm × 30 m), with the oven temperature being increased from 220 to 270 °C (3 °C/min). High-resolution mass spectra were obtained on a HRMS-TOF spectrometer.

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657−6671

Scheme 3. Cyclization Pathway of Substrates 1, 15, and 16 by the Cyclase Enzyme.[a]



[a]Robust binding of 23*Z*-Me of oxidosqualene **1** to the cyclase enzyme but with loose binding of 23*E*-Me (A). Reaction pathway of substrates **15** and **16** to produce **47**–**50** (B,C).

Synthetic Experiments of Substrate Analogues 10–16. As the general synthetic method, C_{27}-aldehyde **17** was subjected to Wittig reactions using reagents **18**–**21**. The starting material **17** was prepared as follows. Squalene was converted into the bromohydrin derivative by NBS treatment, the reaction of which was conducted in H_2O/THF solution. Subsequently, the epoxide ring was formed in the basic condition using K_2CO_3, followed by H_5IO_6 treatment, affording **17**.[17]

Preparations of 22 and 23. Esters **22** and **23** were produced by a Wittig–Horner reaction of **17** with NaH base. In the three-necked flask, triethyl 2-phosphonobutyrate **18** (1026.7 mg, 6.15 mmol) was dissolved in Et_2O (130 mL) and cooled to 0 °C under N_2 atmosphere. NaH (40% oil, 273.7 mg) was added bit-by-bit into the ethereal solution. Then, the solution was chilled to −40 °C and stirred for 15 min. Aldehyde **17** (472.9 mg, 1.23 mmol) dissolved in a small amount of Et_2O was added step-by-step and stirred for 30 min. The reaction was monitored by SiO_2 TLC. To the reaction mixture was added saturated aq NaCl, and the lipophilic materials were extracted with hexane (3×), which was dried over Na_2SO_4. The dried hexane was

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657–6671

evaporated under reduced pressure, yielding a mixture of **22** and **23** (571.0 mg, 96.4% yield). The *E*/*Z* mixture was separated by SiO_2 column chromatography (hexane/EtOAc = 100:5). The repeated chromatography afforded 85.3 mg of **22** and 100.2 mg of **23** in a pure state. Yet, an unseparated fraction was available in a yield of 380 mg.

Compound ***22:*** EIMS; *m*/*z* 482 (M^+, 0.8%); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.02 (t, *J* = 7.2 Hz, 3H), 1.30 (t, *J* = 7.2 Hz), 1.60 (s, 15H, 5 × Me), 1.68 (s, 3H, Me), 2.06−1.80 (m, 9 × CH_2), 2.25 (q, *J* = 7.2 Hz, 2H), 2.50 (q, *J* = 7.2 Hz, 2H), 4.20 (q, *J* = 7.2 Hz, 2H), 5.13−5.05 (5H, m), 5.82 (1H, t, *J* = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 13.7 (q), 14.3 (q),15.8 (q), 15.98(q), 16.00 (q, 3C), 17.7 (q), 25.7 (q), 26.6 (t, 2C), 27.5 (t), 27.9 (t), 28.2 (t, 2C), 39.2 (t), 39.7 (t, 3C), 59.9(t), 124.22(d), 124.24 (d), 124.26 (d), 124.36 (d), 124.9 (d), 131.2 (s), 133.6 (s), 134.1 (s), 134.9 (s), 135.0 (s), 135.1 (s), 140.0 (d), 168.3 (s). In the NOESY spectrum, a clear NOE was observed between δ_H 1.02 and 5.82, indicating that Me of Et at C-23 is oriented in a *E*-configuration and that CO_2Et group was in *Z*-configuration.

Compound ***23:*** EIMS; *m*/*z* 482 (M^+, 6%); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.00 (t, *J* = 7.2 Hz, 3H), 1.29 (t, *J* = 7.2 Hz, 3H), 1.60 (s, 12H, 3 × Me), 1.61 (s, 3H, Me), 1.68 (s, 3H), 2.10−1.90 (m, 10 × CH_2), 2.02 (m, 4H), 4.17 (q, *J* = 7.2 Hz, 2H), 5.13−5.05 (m, 5H), 6.70 (t, *J* = 7.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 13.9 (q),14.3 (q), 16.0 (q, 4Me),17.7 (q), 20.1 (t), 25.7 (q), 26.7 (t, 2C), 26.8 (t), 27.1 (t), 28.3 (t, 2C), 38.6 (t), 39.6 (t), 39.70 (t), 39.74 (t), 60.2 (t), 124.2 (d, 2 × C), 124.4 (d, 2 × C), 125.1 (d), 131.2 (s), 133.8 (s), 133.95 (s), 134.89 (s), 134.96 (s), 135.1 (s), 141.5 (d), 167.9 (s).

Preparations of 24 and 25. A solution of triphenylphosphine (17.06 g, 65.04 mmol) and 1-bromopropane dissolved in toluene (50 mL) was refluxed for 12 h. The precipitated phosphonium salts were collected by filtration and washed with toluene (3×) to remove unreacted materials. Salt **19** thus collected was dried over P_2O_5 under reduced pressure, giving 7.47 g (47.7% yield). Aldehyde **17** was subjected to a Wittig reaction with **19** in *n*-BuLi basic condition under N_2 atmosphere condition. Salt **19** (972.9 mg) was suspended in Et_2O (50 mL) at 0 °C in a three-necked flask, to which *n*-BuLi (1.58 mol/L, 3.20 mL) was added in a dropwise, and the solution was gradually changed into orange. The temperature was cooled to −78 °C and stirred for 15 min. Subsequently, an ethereal solution of **17** (193.9 mg, 0.505 mmol) was bit-by-bit added into the orange-colored solution. The reaction was monitored by TLC. After 30 min, the reaction mixture was poured into saturated aq NaCl, and the lipophilic materials were extracted with hexane, which were then dried over Na_2SO_4, followed by purification with a SiO_2 column (hexane/AcOEt = 100:10). The purified fraction contained a mixture of *E*- and *Z*-isomers. Separation of the *E*- and *Z*-isomers (**24** and **25**), which were produced in the presence of *n*-BuLi, was difficult even with the $AgNO_3$-impregnated SiO_2 column. Thus, the NMR data of compound **24** are not given. However, the separation of the epoxides **12** and **13** (*E*- and *Z*-isomers) succeeded by 5% $AgNO_3$−SiO_2 column chromatography (hexane/EtOAc = 100:5−100:50, stepwise elution). Bromohydrins **31** and **32** were produced by the reaction of NBS, followed by treatment of K_2CO_3, affording a mixture of **12** and **13** (NMR data of each product are described below). Argentation chromatography allowed the separation of **12** and **13**. The Wittig reaction of **21** with **17** was conducted using $NaN(SiMe_3)_2$ as base. Under the basic condition, *Z*-isomer **25** only was selectively produced. Phosphonium bromide **19** (649.5 mg, 1.68 mmol) was suspended in Et_2O (20 mL) at 0 °C under nitrogen gas in a three-necked flask. A solution of $NaN(SiMe_3)_2$ (1.01 mol/L, 2.3 mL) was slowly added, giving a clear orange solution. The reaction flask was cooled to −78 °C and stirred for 15 min. To the solution was slowly added **17** (64.7 mg, 0.169 mmol), and the reaction was continued for an additional 30 min. To the flask was added saturated aq NaCl, and the product was extracted with hexane, which was dried over Na_2SO_4. Pure **25** was obtained by SiO_2 column chromatography using a solvent of hexane/EtOAc = 100/1 (47 mg, yield 68%).

Compound ***25:*** EIMS *m*/*z* 410 (M^+, 1%); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 0.958 (t, *J* = 7.2 Hz, 3H), 1.60 (s, 15H, 5Me), 1.68 (s, 3H), 2.15−1.96 (m, 11CH_2), 5.15−5.00 (m, 5H), 5.37−5.26 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 14.4 (q), 15.95 (q), 15.99 (q), 16.02 (q, 2C), 17.7 (q), 20.5 (t), 25.7 (q), 25.8 (t), 26.63 (q) 26.66 (t), 26.8 (t), 28.3 (t, 2C), 39.67 (t, 2C), 39.74 (t, 2C), 124.27 (d), 124.30 (d), 124.34 (d), 124.41 (d), 124.48 (d), 128.8 (d), 131.2(s), 131.6 (d), 134.9 (s), 135.05 (s), 135.09 (s).

Preparation of 26. The Wittig reaction of **17** with $[Ph_3P$-$CH_2C_3H_7]^+Br^-$ **20** was conducted in the presence of $NaN(SiMe_3)_2$, selectively affording **31** with *Z*-configuration, and no contamination of the *E*-configuration was found. To the suspension of **20** (1.049 g, 2.63 mmol) in Et_2O was added 3.57 mL of $NaN(SiMe_3)_2$ (1.10 mol/L) drop-by-drop, whereby the suspension was changed into the solution colored with orange. These treatments were done at 0 °C under the atmosphere of N_2 gas. Next, the temperature was cooled at −78 °C. To the solution was added **17** (101.0 mg, 0.263 mmol), dissolved in a small amount of Et_2O, slowly during stirring. After 30 min, the reaction mixture was poured into saturated aq NaCl solution to quench this reaction, followed by extraction of product with hexane (3×). The hexane extract was dried over Na_2SO_4. Product **26** was purified by a SiO_2 column chromatography with hexane/AcOEt (100:1) as eluent, yielding 68.2 mg (61.1%).

Compound ***26:*** EIMS *m*/*z* 496 (M^+, 4%); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 0.904 (t, *J* = 7.2 Hz, 3H), 1.35 (q, *J* = 7.2 Hz, 2H), 1.60 (s, 15H, 5Me), 1.68 (s, 3H), 2.2−1.9 (m, 22H), 5.12 (m, 5H), 5.36 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 13.3 (q), 15.96 (q), 15.99 (q), 16.02 (q), 17.7 (q), 22.9 (t), 25.7 (q), 25.9 (t), 26.64 (t), 26.66 (t), 26.77 (t), 28.3 (t, 2C), 29.3 (t), 39.65 (t), 39.74 (t, 3C), 124.27 (d), 124.30 (d), 124.33 (d), 124.41 (d), 124.46 (d), 129.59 (d), 129.69 (d), 131.2 (s), 134.6 (s), 134.89 (s), 135.05 (s), 135.09 (s).

Preparations of 27 and 28. The synthetic protocols were the same as those of **22** and **23**. Ethyl 2-(diethoxyphosphoryl)propanoate **21** (257.0 mg, 1.02 mmol) was suspended in Et_2O (130 mL) at 0 °C under N_2 gas atmosphere. NaH (40% oil, 61.2 mg, 1.53 mmol) was slowly added to the suspension. The reaction flask was cooled to −40 °C and stirred for 15 min. To the solution was added aldehyde **17**, dissolved in a small amount of Et_2O, bit-by-bit. After 30 min, the reaction mixture was poured into saturated aq NaCl, and then the product was extracted with hexane, which was dried over Na_2SO_4. The hexane extract was dried under reduced pressure. The residue (302.0 mg, yield 94.9%) was applied to a SiO_2 column chromatography (hexane/EtOAc = 100:5) to separate *E*- and *Z*-isomers **27** and **28**. Isolation yields of **27** and **28** were 230.1 mg (72.3%) and 52.2 mg (25.8%), respectively.

Compound ***27:*** EIMS *m*/*z* 468 (M^+, 2%); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.29 (t, *J* = 7.2 Hz, 3H), 1.60 (s, 12H), 1.61 (s, 3H), 1.68 (s, 3H), 1.75 (s, 3H), 2.15−1.96 (m, 18H), 2.26 (q, *J* = 7.2 Hz, 2H), 4.18 (q, *J* = 7.2 Hz, 2H), 5.15−5.00 (m, 5H), 6.75 (t, *J* = 7.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 12.3 (q), 14.3 (q), 15.95 (q), 15.98 (q), 16.02 (q, 2C), 17.7 (q), 25.67 (q), 26.7 (t, 2C), 26.8 (t), 27.4 (t), 28.3 (t, 2C), 38.3 (t), 39.6 (t), 39.72 (t), 39.74 (t), 60.3 (t), 124.3 (d, 2C), 124.4 (d, 2C), 125.1 (d), 127.7 (s), 131.2 (s), 133.8 (s), 134.9 (s), 134.95 (s), 135.1 (s), 141.9 (d), 168.2 (s).

Compound ***28:*** EIMS *m*/*z* 468 (M^+, 2%); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.28 (t, *J* = 7.2 Hz, 3H), 1.60 (s, 15H, 5Me), 1.68 (s, 3H), 1.88 (s, 3H, s), 2.2−1.9 (m, 18H), 2.55 (q, *J* = 7.2 Hz, 2H), 5.15−5.00 (m, 5H), 5.90 (t, *J* = 7.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 14.5 (q),16.0 (q), 16.22 (q), 16.26 (q, 2C), 17.9 (q), 20.9 (q), 25.9 (q), 26.9 (t, 2C), 27.0 (t), 28.2 (t), 28.5 (t, 2C), 39.4 (t), 39.95 (t), 40.0 (t, 2C), 60.2 (t), 124.47(d), 124.49 (d), 124.53 (d), 124.61 (d), 124.75 (d), 127.3 (s), 131.5 (s), 134.4 (s), 135.1 (s), 135.27 (s), 135.33 (s), 142.8 (d), 168.3 (s).

Preparations of 29−35. The protocols for preparing the bromohydrin derivatives **29**−**35** were essentially the same between each of them. As a typical example, the experiments for the synthesis of **34** from **27** are described below. A solution of **27** (90 mg, 0.19 mmol), dissolved in THF (12 mL), was cooled at 0 °C. To the solution was added water until the solution became cloudy. Next, a small amount of THF was added until the transparent solution was formed. To the solution was added 51.3 mg (0.29 mmol) of NBS bit-by-bit. The reaction was monitored by SiO_2 TLC. After 30 min, saturated aq NaCl was added, and the crude product and unreacted material **27** were

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657−6671

extracted with hexane (3×), which was dried over Na_2SO_4. A SiO_2 column chromatography (hexane/EtOAc = 100:30) gave pure **34** (51.6 mg, yield 48.0%).

NMR data of **29**−**35** are given below.

Compound ***29:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.01 (t, J = 7.2 Hz, 3H), 1.30 (t, J = 7.2 Hz, 3H), 1.32 (s, 3H), 1.34 (s, 3H), 1.60 (s, 12H), 1.80 (m, 1H), 2.16−1.90 (m, 18H), 2.33−2.20 (m, 3H), 2.50 (q, J = 7.2 Hz, 2H), 3.98 (br d, J = 11.2 Hz, 1H), 4.20 (q, J = 7.2 Hz, 2H), 5.23−5.10 (m, 4H), 5.82 (t, J = 7.2 Hz, 1H).

Compound ***30:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 0.996 (t, J = 7.2 Hz, 3H), 1.29 (t, J = 7.2 Hz, 3H), 1.32 (s, 3H), 1.34 (s, 3H), 1.597 (s, 9H), 1.61 (s, 3H), 1.80 (m, 1H), 2.15−1.89 (m, 16H), 3.97 (br d, J = 11.2 Hz, 1H), 4.19 (q, J = 7.2 Hz, 2H), 5.23−5.10 (m, 4H), 6.94 (t, J = 7.2 Hz, 1H).

Compound ***31:*** The NMR data are not given because a mixture of **27** and **28** was produced by the reaction of **19** and **21** in the presence of *n*-BuLi. A mixture of **27** and **28** was used for the next reaction without separation. The NBS treatment afforded a mixture of **36** and **37**. The epoxides **12** and **13** only, which were produced from **36** and **37**, were separated by $AgNO_3$−SiO_2 column chromatography, as described above.

Compound ***32:*** This compound was synthesized from *Z*-isomer **25**, which was selectively produced from the reaction of **17** and **19** in the presence of $NaN(SiMe_3)_2$ as described above; ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 0.955 (t, J = 7.2 Hz, 3H), 1.33 (s, 3H), 1.34 (s, 3H), 1.60 (s, 12H), 1.78 (m, 1H), 2.13−2.18 (m, 20H), 2.30 (m, 1H), 3.98 (br d, J = 11.2 Hz, 1H), 5.22−5.01 (m, 4H), 5.33 (m, 2H).

Compound ***33:*** The ^{1}H NMR of **33** was not measured, but the epoxide structure of **14** synthesized from **33** was confirmed by ^{1}H and ^{13}C NMR data, which are described below.

Compound ***34:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.29 (t, J = 7.2 Hz, 3H), 1.33 (s, 3H), 1.34 (s, 3H), 1.60 (s, 9H), 1.61 (s, 3H), 1.81 (m, 1H), 1.82 (s, 3H), 2.21−1.94 (m, 18H), 2.35−2.21 (m, 3H), 3.97 (br d, J = 11.2 Hz), 4.17 (q, J = 7.2 Hz, 2H), 5.23−5.12 (m, 4H), 6.74 (t, J = 7.2 Hz, 1H).

Compound ***35:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.30 (t, J = 7.2 Hz, 3H), 1.33 (s, 3H), 1.34 (s, 3H), 1.60 (s, 12H), 1.79 (m, 1H), 1.88 (s, 3H), 2.15−1.93 (m, 18H), 2.54 (q, J = 7.2 Hz, 2H), 3.98 (br d, J = 11.2 Hz), 4.19 (q, J = 7.2 Hz, 2H), 5.20−5.12 (m, 4H), 5.90 (t, J = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 14.3 (q), 15.8 (q, 3C), 16.0 (q), 16.1 (q), 20.6 (q), 25.8 (q), 26.6 (t), 26.7 (t), 28.0 (t), 28.3 (t, 2C), 32.2 (t), 38.2 (t), 39.2 (t), 39.6 (t), 39.7 (t), 60.1 (t), 70.9 (s), 72.4 (d), 124.3 (d), 124.4 (d), 124.9 (d), 126.0 (d), 127.1 (s), 132.9 (s),134.2 (s), 134.9 (s), 135.1 (s), 142.5 (d), 168.2 (s).

Preparations of 36−39. The synthetic methods were essentially the same between the syntheses of **36**−**39**. As the typical experiment, the synthesis of **36** from **29** is described. Bromohydrin **29** (45.9 mg, 0.079 mmol) was dissolved in Et_2O (20 mL). The three-necked flask was cooled to −40 °C and was filled with N_2 gas. To the solution was added dropwise DIBAL-H reagent (1.03 M in hexane, 550 μL). The reaction was monitored with SiO_2 TLC. After we confirmed that the starting material **29** disappeared, the reaction was terminated by adding 10 mL of a mixture of EtOAc and H_2O and further stirred for 2 h, and then 10 mL of saturated aq NH_4Cl was added and left overnight. The product was extracted with hexane, and the organic layer was dried over Na_2SO_4. Pure **36** was isolated by a SiO_2 column chromatography with hexane/EtOAc (100:30) in a yield of 32.2 mg (75.6%).

Compound ***36:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.03 (t, J = 7.2 Hz, 3H), 1.32 (s, 3H), 1.34 (s, 3H), 1.60 (s, 12H), 1.78 (m, 1H), 2.17−1.93 (m, 20H), 2.31 (m, 1H), 3.98 (br d, J = 11.2 Hz, 1H), 4.12 (s, 2H), 5.21−5.07 (m, 4H), 5.28 (t, J = 7.2 Hz, 1H).

Compound ***37:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 0.997 (t, J = 7.2 Hz, 3H), 1.32 (s, 3H), 1.34 (s, 3H), 1.60 (s, 12H), 1.78 (m, 1H), 2.17−1.93 (m, 20H), 2.31 (m, 1H), 3.97 (br d, J = 11.2 Hz, 1H), 4.03 (s, 2H), 5.22−5.08 (m, 4H), 5.36 (t, J = 7.2 Hz, 1H).

Compound ***38:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.32 (s, 3H), 1.34 (s, 3H), 1.60 (s, 12H), 1.66 (s, 3H), 1.78 (m, 1H), 2.15−1.94 (m, 18H), 2.30 (m, 1H), 3.97 (br d, J = 11.0 Hz, 1H), 3.98 (s, 2H), 5.22−5.08 (m, 4H), 5.38 (t, J = 7.0 Hz, 1H).

Compound ***39:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.33 (s, 3H), 1.34 (s, 3H), 1.79 (m, 1H), 1.81 (3H, s), 2.17−1.94 (m, 18H), 2.30 (m, 1H), 3.97 (br d, J = 11.2 Hz, 1H), 4.10 (s, 2H), 5.22−5.08 (m, 4H), 5.28 (t, J = 7.0 Hz, 1H).

Preparations of 40 and 41. Protocols for the syntheses of **40** and **41** were essentially the same between them. Therefore, the synthetic experiment of **40** from **36** only is described here. Compound **36** (25.0 mg, 0.047 mmol) was dissolved in 5 mL of CH_2Cl_2. The three-necked flask was cooled to 0 °C and filled with N_2 gas. To the flask was added Et_3N (70.7 mg, 0.70 mmol), dissolved in a small amount of CH_2Cl_2. Next, MsCl (80.5 mg, 0.70 mmol), dissolved in a small quantity of CH_2Cl_2, was added slowly and then stirred overnight. The reaction mixture was washed successively with 5% aq HCl, saturated aq $NaHCO_3$, and saturated aq NaCl, all of which were cooled at 0 °C in advance. The organic layer containing mesylate **40** was dried over Na_2SO_4. Mesylates are generally labile, thus the reaction product was used for the next reaction without purification. Therefore, we did not measure the ^{1}H NMR spectrum of the reaction products **40** and **41**.

Preparations of 10 and 11. The protocols were identical between them. The MeOH solution (10 mL) of **49** (ca. 10 mg) was added bit-by-bit into dry MeOH solution of K_2CO_3 (10 mg/10 mL). After 30 min, the reaction mixture was poured into saturated aq NaCl, and the epoxide product was extracted with hexane (3×). The hexane extract was subjected to a short SiO_2 column (hexane/EtOAc = 100:10) to remove just the high-polar impurities found at the origin of TLC and then employed for the next reaction without further purification. The epoxide (ca. 10 mg) thus obtained was subjected to reduction with $LiBEt_3H$ to convert the $-CH_2OMs$ into $-CH_3$. The epoxide was dissolved in Et_2O (10 mg/10 mL). Under the atmosphere of N_2 gas, the THF solution of $LiBEt_3H$ (50 μL, 1.0 M) was added in a small portion. The reaction was monitored with TLC. After 2 h, the mesylate having the epoxide ring disappeared. To the reaction mixture was added saturated aq NaCl, followed by extraction with hexane (3×). The hexane extract was dried over Na_2SO_4. Complete purification was done by normal-phase HPLC (hexane/THF = 100:0.8, Inertsil, GL Science), giving ca. 7.0 mg of **10**.

Compound ***10:*** EIMS m/z 440 (M^+, 0.6%); HRMS (EI) m/z calcd for $C_{31}H_{52}O$ 440.40181; found 440.40187; ^{1}H NMR (400 MHz, C_6D_6) δ_H 1.07 (t, J = 7.6 Hz, 3H), 1.23 (s, 3H), 1.28 (s, 3H), 1.67 (s, 3H), 1.73 (s, 9H), 1.81 (s, 3H), 2.33−2.10 (m, 22H), 2.69 (t, J = 6.0 Hz, 1H), 5.44−5.35 (m, 5H); ^{13}C NMR (100 MHz, C_6D_6) δ_C 13.1 (q), 16.05 (q), 16.13 (q), 16.15 (q, 2C), 18.9 (q), 25.0 (q, 2C), 27.10 (t), 27.14 (t), 28.1 (t), 28.8 (t, 3C), 32.8 (t), 36.9 (t), 40.1 (t), 40.2 (t, 2C), 57.4 (s), 63.5 (d), 123.4 (d), 124.8 (d), 124.9 (d), 125.2 (d), 134.4 (s), 135.0 (s), 135.1 (s), 135.2 (s), 136.7 (s).

Compound ***11:*** EIMS m/z 440 (M^+, 0.3%); HRMS (EI) m/z calcd for $C_{31}H_{52}O$ 440.40181; found 440.40206; ^{1}H NMR (400 MHz, C_6D_6) δ_H 1.07 (t, J = 7.2 Hz, 3H), 1.23 (s, 3H), 1.28 (s, 3H), 1.67 (s, 3H), 1.73 (s, 3H), 1.81 (s, 3H), 2.34−2.10 (m, 22H), 2.70 (t, J = 6.0 Hz, 1H), 5.45−5.28 (m, 5H); ^{13}C NMR (150 MHz, C_6D_6) δ_C 13.0 (q), 16.0 (q), 16.12 (q), 16.14 (q, 2C), 18.9 (q), 23.0 (q), 24.9 (q), 25.1 (t), 26.8 (t), 27.07 (t), 27.10 (t), 28.0 (t), 28.7 (t, 2C), 36.8 (t), 40.1 (t), 40.2 (t), 40.5 (t), 57.3 (s), 63.5 (d), 124.6 (d), 124.8 (d, 2C), 124.9 (d), 125.1 (d), 134.4 (s), 134.9 (s), 135.0 (s), 135.2 (s), 136.9 (s).

Preparations of 12−16. The synthetic methods of these epoxide compounds from the bromohydrin precursors were identical. The experiment for the synthesis of only **15** is described here. The starting material **38** (12.0 mg, 0.023 mmol) was dissolved in a small amount of MeOH. This solution was added into the methanolic suspension (15 mg/10 mL) of K_2CO_3 and stirred for 30 min. The reaction mixture was added into saturated aq NaCl solution, and the lipophilic materials were extracted with hexane (3×). Product **15** was isolated by a SiO_2 column chromatography (hexane/EtOAc = 100:5) in a yield of 8.9 mg (87.4% yield).

Compound ***12:*** EIMS m/z 426 (M^+, 0.6%); HRMS (EI) m/z calcd for $C_{30}H_{50}O$ 426.38617; found 426.38723; ^{1}H NMR (400 MHz, C_6D_6) δ_H 1.08 (t, J = 7.2 Hz, 3H), 1.23 (s, 3H), 1.28 (s, 3H), 1.67 (s, 3H), 1.71 (s, 3H), 1.74 (s, 6H), 2.35−2.08 (m, 22H), 2.69 (t, J = 6.0 Hz, 1H), 5.45−5.35 (m, 4H), 5.58 (m, 2H); ^{13}C NMR (100 MHz,

DOI: 10.1021/acs.joc.6b01313
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C_6D_6) δ_C 14.2 (q), 16.0 (q), 16.1 (q), 16.2 (q, 2C), 18.9 (q), 25.0 (q), 26.0 (t), 27.1 (t, 2C), 28.0 (t), 28.7 (t, 2C), 31.7 (t), 36.9 (t),40.1 (t), 40.2 (t, 2C), 57.3 (s), 63.5 (d), 124.82 (d), 124.84 (d), 124.91 (d), 125.12 (d), 129.3 (d), 132.2 (d), 134.3 (s), 134.8 (s), 135.1 (s), 135.2 (s).

Compound ***13:*** EIMS m/z 426 (M^+, 0.4%); HRMS (EI) m/z calcd for $C_{30}H_{50}O$ 426.38617; found 426.38625; ^{1}H NMR (400 MHz, C_6D_6) δ_H 1.05 (t, J = 7.6 Hz, 3H), 1.23 (s, 3H), 1.28 (s, 3H), 1.67 (s, 3H), 1.71 (s, 3H), 1.73 (s, 6H), 2.34−2.10 (m, 22H), 2.69 (t, J = 6.0 Hz, 1H), 5.45−5.34 (m, 4H), 5.56 (t-like, J = 6.0 Hz, 2H); ^{13}C NMR (100 MHz, C_6D_6) δ_C 14.6 (q), 16.0 (q, 2C), 16.1 (q, 2C), 18.9 (q), 20.9 (t), 25.0 (q), 27.1 (t, 2C), 28.0 (t), 28.7 (t, 2C), 36.9 (t), 40.09 (t), 40.13 (t), 40.2 (t), 57.3 (s), 63.5 (d), 124.8 (d), 124.9 (d), 125.0 (d), 125.1 (d), 129.1 (d), 131.8 (d), 134.3 (s), 134.7 (s), 135.1 (s), 135.15 (s).

Compound ***14:*** EIMS m/z 440 (M^+, 0.4%); HRMS (EI) m/z calcd for $C_{31}H_{52}O$ 440.40182; found 440.40313; ^{1}H NMR (400 MHz, C_6D_6) δ_H 0.989 (t, J = 7.2 Hz, 3H), 1.21 (s, 3H), 1.26 (s, 3H), 1.45 (q, J = 7.2 Hz, 2H), 1.65 (s, 3H), 1.70 (s, 3H), 1.72 (s, 6H), 2.35−2.08 (m, 22H), 2.67 (t, J = 6.0 Hz, 1H), 5.44−5.32 (m, 4H), 5.60−5.50 (m, 2H); ^{13}C NMR (100 MHz, C_6D_6) δ_C 13.9 (q), 16.1 (q), 16.2 (q, 3C), 18.9 (q), 23.2 (t), 25.0 (q), 26.3 (t), 27.1 (t, 2C), 28.0 (t), 28.7 (q, 2C), 29.7 (t), 36.9 (t), 40.1 (t), 40.13 (t), 40.19 (t), 57.3 (s), 63.5 (d), 124.85 (d), 124.92 (d), 124.99 (d), 125.14 (d), 129.9 (d, 2C), 134.4 (s), 134.7 (s), 135.1 (s), 135.2 (s).

Compound ***15:*** EIMS m/z 424 ($M^+ - H_2O$, 0.8%), 442 (M^+, 0.2%); HRMS (EI) m/z calcd for $C_{30}H_{50}O_2$ 442.38018; found 440.38216; ^{1}H NMR (400 MHz, C_6D_6) δ_H 1.23 (s, 3H), 1.28 (s, 3H), 1.67 (s, 3H), 1.69 (s, 3H), 1.72 (s, 3H), 1.737 (s, 3H), 1.743 (s, 3H), 2.35−2.13 (m, 20H), 2.70 (t, J = 6.0 Hz), 3.93 (s, 2H), 5.53−5.35 (m, 5H); ^{13}C NMR (100 MHz, C_6D_6) δ_C 13.6 (q), 16.05 (q), 16.09 (q), 16.16 (q, 2C), 18.9 (q), 24.9 (q), 26.7 (t), 27.1 (t, 2C), 28.0 (t) 28.7 (t, 2C), 36.8 (t), 39.9 (t), 40.1 (t), 40.2 (t), 57.4 (s), 63.6 (d), 68.7 (t), 124.88 (d), 124.92, 124.94 (d), 125.2 (d), 125.3 (d), 134.8 (s), 135.1 (s), 135.2 (s), 135.4 (s), 135.5 (s).

Compound ***16:*** EIMS m/z 424 ($M^+ - H_2O$, 1%), 442 (M^+, 0.1%); HRMS (EI) m/z calcd for $C_{30}H_{50}O_2$ 442.38018; found 440.38205; ^{1}H NMR (400 MHz, C_6D_6) δ_H 1.23 (s, 3H), 1.27 (s, 3H), 1.67 (s, 3H), 1.69 (s, 3H), 1.74 (s, 6H), 1.90 (s, 3H), 2.33−2.10 (m, 20H), 2.69 (t, J = 6.0 Hz, 1H), 4.09 (s, 2H), 5.40−5.33 (m, 5H); ^{13}C NMR (100 MHz, C_6D_6) δ_C 16.05 (q), 16.08 (q), 16.14 (q, 2C), 18.9 (q), 21.4 (q), 24.94 (q), 26.5 (t), 27.1 (t, 2C), 28.0 (t), 28.7 (t, 2C), 36.8 (t), 40.1 (t), 40.2 (t), 40.3 (t), 57.4 (s), 61.4 (t), 63.6 (d), 124.89 (d), 124.92 (d), 125.15 (d, 2C), 127.3 (d), 134.4 (s), 134.7 (s), 135.1 (s), 135.14 (s), 135.36 (s).

Incubation Experiments of Analogues 10−16. The purification of EtAS and the incubation conditions were carried out according to the published protocol.[9] The detailed incubation conditions and GC conditions for the product profiles are described in Figure S3.

Spectroscopic Data for Products 42−50. *Product* ***42:*** ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.730 (br d, J = 12.5 Hz, 1H, H-5), 0.764 (t, J = 7.4 Hz, 3H, Me-31), 0.781 (s, 3H, Me-29), 0.789 (s, 3H, Me-24), 0.819 (s, 3H, Me-28), 0.820 (m, 1H, H-16), 0.934 (s, 3H, Me-25), 0.960 (m, 1H, H-1), 0.961 (s, 3H, Me-26), 0.970 (m, 1H, H-15), 0.995 (s, 3H, Me-23), 1.11 (m, 1H, H-19), 1.14 (s, 3H, Me-27), 1.16 (m, 1H, H-22), 1.22 (m, 2H, H-21), 1.28 (m, 1H, H-30), 1.33 (m, 1H, H-7), 1.35 (m, 1H, H-30), 1.38 (m, 1H, H-22), 1.39 (m, 1H, H-6), 1.50 (m, 1H, H-7), 1.54 (m, 1H, H-9), 1.55 (m, 1H, H-6), 1.58 (m, 1H, H-19), 1.59 (m, 2H, H-2), 1.62 (m, 1H, H-1),1.76 (dd, J = 14.0, 4.5 Hz, 1H, H-15), 1.85 (m, 2H, H-11), 1.89 (m, 1H, H-18), 2.01 (ddd, J = 13.0, 13.0, 4.5 Hz, 1H, H-16), 3.22 (dd, J = 11.4, 4.8 Hz, 1H, H-3), 5.17 (br s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 7.75 (q, C-31), 15.4 (q, C-24), 15.5 (q, C-25), 16.8 (q, C-26), 18.4 (t, C-6), 23.5 (t, C-11), 25.9 (q, C-27), 26.2 (t, C-15), 27.18 (t, C-30), 27.26 (t, C-2), 27.29 (t, C-16), 28.1 (q, C-23), 28.4 (q, C-28), 29.0 (q, C-29), 32.3 (s, C-17), 32.7 (t, C-7), 32.8 (t, C-21), 33.3 (s, C-20), 36.6 (t, C-1), 37.0 (s, C-10), 38.6 (t, C-22), 38.8 (s, C-4), 39.8 (s, C-8), 41.8 (s, C-14), 44.6 (t, C-19), 46.7 (d, C-18), 47.7 (d, C-9), 55.2 (d, C-5), 79.0 (d, C-3), 121.7 (d, C-12), 145.3 (s, C-13). Assignment of C-2 and C-16, that of C-7 and C-21, that of C-8 and C-14, and that of C-24 and C-25 may be exchangeable because the chemical shifts are very similar: MS (EI) m/z 203 (75%), 217 (22%), 232 (100%), 411 (5%), 440 (5%); [M^+]; HRMS (EI) m/z calcd for $C_{31}H_{52}O$ 440.40181; found 440.40267; $[\alpha]_D^{25}$ = +97.0 (c = 0.115, $CHCl_3$); solid, mp 191−193 °C.

Product ***43:*** ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.740 (br d, J = 12.0 Hz, H-5), 0.792 (s, 3H, Me-24), 0.806 (t, J = 7.6 Hz, 3H, Me-31), 0.82 (m, 1H, H-16), 0.827 (s, 3H, Me-30), 0.834 (s, 3H, Me-28), 0.939 (s, 3H, Me-25), 0.95 (m, 1H, H-15), 0.97 (m, 1H, H-1), 0.970 (s, 3H, Me-26), 0.998 (s, 3H, Me-23), 1.00 (m, 1H, H-19), 1.10 (m, 1H, H-21), 1.13 (s, 3H, Me-27), 1.17 (q, J = 7.6 Hz, 2H, H-29), 1.24 (m, 1H, H-22), 1.28 (m, 1H, H-21), 1.34 (m, 1H, H-7), 1.41 (m, 1H, H-6), 1.42 (m, 1H, H-22), 1.50 (m, 1H, H-15), 1.51 (m, 1H, H-7), 1.54 (m, 1H, H-6), 1.56 (m, 1H, H-9), 1.60 (m, 2H, H-2), 1.62 (m, 2H, H-1, H-19), 1.62 (m, 1H, H-19), 1.87 (m, 2H, H-11), 1.97 (m, 1H, H-18), 1.98 (ddd, J = 12.8, 12.8, 4.4 Hz, 1H, H-16), 3.22 (dd, J = 11.2, 4.4 Hz, 1H, H-3), 5.18 (t, J = 3.6 Hz, 1H, H-12); ^{13}C NMR (150 MHz, $CDCl_3$) δ 7.67 (q, C-31), 15.5 (q, C-25), 15.6 (q, C-24), 16.8 (q, C-26), 18.4 (t, C-6), 20.6 (q, C-30), 23.5 (t, C-11), 26.0 (q, C-27), 26.2 (t, C-15), 27.0 (t, C-16), 27.2 (t, C-2), 28.1 (q, C-23), 28.4 (q, C-28), 32.4 (t, C-21), 32.7 (t, C-7), 32.9 (s, C-17), 33.6 (s, C-20), 36.8 (t, C-22), 36.9 (s, C10), 38.5 (t, C-29), 38.6 (t, C-1), 38.8 (s, C-4), 39.8 (s, C-8), 41.7 (s, C-14), 44.7 (t, C-19), 46.9 (d, C-18), 47.6 (d, C-9), 55.2 (d, C-5), 79.0 (d, C-3), 121.7 (d, C-12), 145.3 (d, C-13). Assignment of C-8 and C-14 and that of C-24 and C-25 may be exchangeable because their chemical shifts are very similar: MS (EI) m/z 203 (48%), 217 (26%), 232 (100%), 411 (5%), 440 (M^+, 2%); [M^+]; HRMS (EI) m/z calcd for $C_{31}H_{52}O$ 440.40181; found 440.40218; $[\alpha]_D^{25}$ = +96.25 (c = 0.2312, $CHCl_3$); solid, mp: 190−195 °C.

Product ***44*** *Acetate:* ^{1}H NMR (600 MHz, C_6D_6) δ 0.881 (br d, J = 10.8 Hz, 1H, H-5), 0.94 (m, 1H, H-1), 0.969 (s, 3H, Me-25), 1.00 (m, H-16), 1.05 (s, 6H, Me-23, Me-24), 1.05 (t, J = 7.2 Hz, 3H, Me-30), 1.08 (s, 3H, Me-28), 1.12 (m, H-15), 1.13 (s, 3H, Me-26), 1.33 (s, 3H, Me-27), 1.33 (m, 1H, H-22), 1.42 (m, 2H, H-6, H-7), 1.50 (m, 2H, H-1, H-29), 1.55 (m, 1H, H-21), 1.57 (m, 2H, H-6, H-19), 1.58 (m, 2H, H-7, H-22), 1.61 (m, 1H, H-29), 1.64 (m, 1H, H-9), 1.71 (m, 2H, H-2, H-20), 1.82 (m, 2H, H-2, H-21), 1.88 (s, 3H, CH_3CO), 1.89 (m, 2H, H-11), 1.93 (m, 1H, H-15), 2.17 (m, 1H, H-19), 2.18 (m, 1H, H-18), 2.24 (m, 1H, H-16), 4.84 (dd, J = 12.0, 4.8 Hz, 1H, H-3), 5.36 (t, J = 3.4 Hz, 1H, H-12); ^{13}C NMR (150 MHz, C_6D_6) δ 12.6 (q, C-30), 15.7 (q, C-25), 17.0 (q, 2C, C-24, C-26), 18.6 (t, C-6), 20.8 (q, CH_3CO), 23.6 (t, C-29), 23.8 (t, C-2), 23.9 (t, C-11), 25.5 (t, C-21), 26.2 (q, C-27), 26.6 (t, C-15), 27.6 (t, C-16), 28.2 (q, C-23), 28.8 (q, C-28), 32.9 (t, C-7), 33.2 (s, C-17), 35.8 (t, C-22), 35.9 (d, C-20), 37.0 (s, C-10), 37.3 (t, C-19), 37.9 (s, C-4), 38.4 (t, C-1), 40.1 (s, C-8), 42.0 (s, C-14), 45.8 (d, C-18), 47.8 (d, C-9), 55.6 (d, C-5), 80.6 (d, C-3), 122.1 (d, C-12), 145.4 (s, C-13), 169.9 (s, CH_3CO); MS (EI) m/z 189 (100%), 203 (22%), 218 (84%), 468 (M^+, 5%); [M^+]; HRMS (EI) m/z calcd for $C_{32}H_{52}O_2$ 468.39673; found 468.39496; $[\alpha]_D^{25}$ = +88.3 (c = 0.0115, $CHCl_3$); solid.

Product ***45*** *Acetate:* ^{1}H NMR (400 MHz, C_6D_6) δ 0.881 (br d, J = 11.6 Hz, 1H, H-5), 0.960 (m, H-1), 0.975 (s, 3H, Me-25), 1.02 (m, 1H, H-16), 1.045 (t, J = 7.0 Hz, 3H, Me30), 1.049 (s, 3H, Me-24), 1.10 (s, 6H, Me-23, Me-28), 1.12 (m, 1H, H-15), 1.14 (s, 3H, Me-26), 1.20 (m, 1H, H-20), 1.22 (m, 1H, H-21), 1.35 (s, 3H, Me-27), 1.38 (m, 1H, H-29), 1.41 (m, 1H, H-22), 1.42 (m, 2H, H-6, H-7), 1.47 (m, 1H, H-29), 1.52 (m, 1H, H-1), 1.57 (m, 1H, H-6), 1.58 (m, 1H, H-7), 1.64 (m, 2H, H-21, H-22), 1.67 (m, 1H, H-9), 1.69 (m, 1H, H-2), 1.73 (m, 2H, H-19), 1.83 (m, 1H, H-2), 1.88 (s, 3H, CH_3CO), 1.92 (m, 2H, H-11), 1.95 (m, 1H, H-15), 2.05 (dd, J = 12.8, 3.6 Hz, 1H, H-18), 2.17 (ddd, J = 12.8, 12.8, 4.4 Hz, 1H, H-16), 4.83 (dd, J = 12.0, 4.4 Hz, 1H, H-3), 5.42 (br s, 1H, H-12); ^{13}C NMR (100 MHz, C_6D_6) δ 11.8 (q, C-30), 15.7 (q, C-25), 17.1 (q, 2C, C-24, C-26), 18.5 (t, C-6), 20.8 (q, CH_3CO), 23.8 (t, C-2), 23.9 (t, C-11), 26.2 (q, C-27), 26.7 (t, C-15), 27.6 (t, C-16), 28.2 (q, C-23), 28.8 (q, C-28), 28.9 (t, C-21), 30.3 (t, C-29), 32.9 (t, C-7), 33.3 (s, C-17), 37.0 (s, C-10), 37.9 (s, C-4), 38.4 (t, C-1), 40.1 (s, C-8), 40.8 (d, C-20), 41.3 (t, C-19), 41.9 (t, C-22), 42.0 (s, C-14), 47.8 (d, C-9), 52.1 (d, C-18), 55.5 (d, C-5), 80.5 (d, C-3), 122.2 (d, C-12), 145.6 (s, C-13), 169.9 (s, CH_3CO). The assignment of C-2 and C-11 and that of C-8 and C-14 may be exchangeable between the two carbons: MS (EI) m/z 189 (100%),

DOI: 10.1021/acs.joc.6b01313
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203 (25%), 218 (82%), 468 (M^+, 4%); [M^+]; HRMS (EI) m/z calcd for $C_{32}H_{52}O_2$ 468.39673; found 468.39405; $[\alpha]_D^{25}$ = +97.92 (c = 0.0534, $CHCl_3$); solid.

Product ***46****:* ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.742 (br d, J = 11.1 Hz, 1H, H-5), 0.791 (s, 3H, Me-24), 0.806 (s, 3H, Me-28), 0.85 (m, 1H, H-16), 0.863 (t, J = 7.3 Hz, 3H, Me-31), 0.938 (s, 3H, Me-25), 0.971 (s, 3H, Me-26), 0.98 (m, 2H, H-1, H-15), 0.997 (s, 3H, Me-23), 1.02 (m, 1H, H-21), 1.13 (m, 1H, H-20), 1.134 (s, 3H, Me-27), 1.21 (m, 1H, H-22), 1.30 (m, 2H, Me-30), 1.35 (m, 1H, H-7), 1.37 (m, 4H, H-19, H-29), 1.40 (m, 1H, H-6), 1.43 (m, 1H, H-22), 1.44 (m, 1H, H-21), 1.51 (m, 1H, H-8), 1.54 (m, 1H, H-6), 1.56 (m, 1H, H-9), 1.60 (m, 2H, H-2), 1.63 (m, 1H, H-1), 1.77 (m, 1H, H-15), 1.79 (m, 1H, H-18), 1.88 (m, 2H, H-11), 1.96 (m, 1H, H-16), 3.22 (dd, J = 11.1, 4.1 Hz, 1H, H-3), 5.21 (br t, J = 3.3 Hz, 1H, H-12); ^{13}C NMR (150 MHz, $CDCl_3$) δ 14.4 (q, C-31), 15.5 (q, C-25), 15.6 (q, C-24), 16.9 (q, C-26), 18.4 (t, C-6), 20.0 (t, C-30), 23.6 (t, C-11), 25.9 (q, C-27), 26.4 (t, C-15), 27.29 (t, C-16), 27.33 (t, C-2), 28.1 (q, C-23), 28.5 (q, C-28), 29.0 (t, C-21), 32.8 (t, C-7), 33.0 (s, C-17), 37.0 (s, C-10), 38.5 (d, C-20), 38.7 (d, 2C, C-1, C-3), 38.8 (s, C-4), 39.7 (t, C-29), 39.9 (s, C-8), 41.3 (t, C-19), 41.8 (s, C-14), 41.8 (t, C-22), 47.7 (d, C-9), 51.8 (d, C-18), 55.3 (d, C-5), 121.7 (d, C-12), 145.6 (s, C-13). The assignment of C-2 and C-16 and that of C-24 and C-25 may be exchangeable: MS (EI) m/z 189 (20%), 203 (25%), 217 (21%), 232 (100%), 440 (M^+, 4%); [M^+]; HRMS (EI) m/z calcd for $C_{31}H_{52}O$ 440.40181; found 440.40177; $[\alpha]_D^{25}$ = +97.92 (c = 0.038, $CHCl_3$); solid.

Product ***47****:* ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.678 (br d, J = 11.0 Hz, 1H, H-5), 0.762 (s, 3H, Me-24), 0.824 (s, 3H, Me-25), 0.91 (m, 1H, H-15), 0.914 (s, 3H, Me-27), 0.92 (m, 1H, H-1), 0.941 (s, 3H, Me-28), 0.970 (s, 3H, Me-23), 1.01 (s, 3H, Me-28), 1.05 (s, 3H, Me-26), 1.13 (m, 1H, H-22), 1.21 (m, 1H, H-9), 1.28 (m, 1H, H-12), 1.30 (m, 2H, H-7, H-16), 1.33 (m, 1H, H-11), 1.40 (m, 1H, H-6), 1.41 (m, 2H, H-7, H-21), 1.45 (m, 1H, H-2), 1.46 (m, 2H, H-19, H-21), 1.54 (m, 1H, H-6), 1.55 (m, 2H, H-11, H-16), 1.57 (m, 1H, H-2), 1.62 (m, 1H, H-19), 1.68 (m, 1H, H-12), 1.71 (m, 1H, H-1), 1.72 (m, 1H, H-22), 1.73 (m, 1H, H-15), 2.12 (dd, J = 12.4, 2.8 Hz, 1H, H-13), 3.20 (dd, J = 11.2, 5.2 Hz, H-3), 3.28 (d, J = 7.6 Hz, 1H, H-30), 3.61 (d, J = 7.6 Hz, 1H, H-30); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.4 (q, C-24), 16.0 (q, C-25), 16.2 (q, 2C, C-26, C-27), 18.4 (t, C-6), 20.8 (t, C-11), 20.9 (t, C-12), 22.5 (q, C-28), 23.7 (q, C-29), 27.2 (t, C-15), 27.4 (t, C-2), 28.0 (q, C-23), 32.6 (t, C-22), 33.3 (t, C-7), 35.5 (t, C-21), 36.2 (t, C-16), 37.1 (s, C-10), 38.1 (d, C-13), 38.8 (t, C-1), 38.9 (s, C-4), 39.2 (s, C-17), 40.6 (s, C-20), 41.9 (t, C-19), 42.1 (s, C-8), 42.5 (s, C-14), 50.6 (d, C-9), 55.3 (d, C-5), 76.0 (d, C-3), 79.0 (t, C-30), 88.2 (s, C-18). The assignment of C-8 and C-14 may be exchangeable: MS (EI) m/z 189 (90%), 203 (68%), 220 (90%), 234 (38%), 411 (100%), 442 (M^+, 18%); [M^+]; HRMS (EI) m/z calcd for $C_{30}H_{50}O_2$ 442.38108; found 442.38148; $[\alpha]_D^{25}$ = +45.0 (c = 0.1428, $CHCl_3$); solid, mp 211−214 °C.

Product ***48*** *Acetate:* ^{1}H NMR (400 MHz, C_6D_6) δ 0.853 (dd, J = 11.6, 2.4 Hz, 1H, H-5), 0.90 (m, 1H, H-1), 0.904 (s, 3H, Me-19), 1.040 (s, 3H, Me-28), 1.044 (br s, 3H, Me-30), 1.05 (br s, 3H, Me-29), 1.09 (s, 3H, Me-18), 1.22 (m, 1H, H-16), 1.29 (s, 3H, Me-21), 1.35 (m, 1H, H-11), 1.37 (m, 2H, H-7, H-9), 1.38 (m, 2H, H-15), 1.39 (m, 1H, H-12), 1.46 (m, 1H, H-6), 1.55 (m, 1H, H-6), 1.60 (m, 1H, H-1), 1.61 (br s, 3H, Me-26), 1.62 (m, 1H, H-7), 1.72 (m, 1H, H-22), 1.73(m, 1H, H-2), 1.74 (m, 1H, H-11), 1.80 (m, 1H, H-16), 1.84 (m, 1H, H-22), 1.85 (m, 1H, H-2), 1.88 (s, 3H, CH_3CO), 1.94 (m, 1H, H-13), 2.17 (m, 1H, H-23), 2.25 (m, 1H, H-17), 2.34 (m, 1H, H-12), 2.43 (m, 1H, H-23), 3.91 (d, J = 16.4 Hz, 1H, H-27), 4.24 (d, J = 16.4 Hz, 1H, H-27), 4.83 (dd, J = 11.2, 4.8 Hz, 1H, H-3), 5.57 (br s, 1H, H-24); ^{13}C NMR (100 MHz, C_6D_6) δ 15.8 (q, C-18), 16.4 (q, C-19), 16.6 (q, C-30), 16.8 (q, C-29), 18.5 (t, C-6), 19.1 (q, C-21), 20.8 (q, CH_3CO), 21.2 (q, C-26), 21.8 (t, C-11), 23.3 (t, C-23), 24.1 (t, C-2), 26.1 (t, C-16), 27.1 (t, C-12), 28.1 (q, C-28), 31.5 (t, C-15), 35.6 (t, C-7), 37.2 (s, C-10), 38.1 (s, C-4), 38.8 (t, C-1), 39.7 (t, C-22), 40.7 (s, C-8), 43.0 (d, C-13), 45.7 (d, C-17), 49.9 (s, C-14), 51.0 (d, C-9), 56.2 (d, C-5), 64.7 (t, C-27), 80.2 (s, C-20), 80.6 (d, C-3), 125.3 (d, C-24), 136.8 (s, C-25), 169.9 (s, CH_3CO). The assignments of C-29 and C-30 may be exchangeable: MS (EI) m/z 125 (100%), 343 (10%), 484 (M^+, 0.4%); [M^+]; HRMS (EI) m/z calcd for $C_{32}H_{52}O_3$ 484.39165; found 484.39158; $[\alpha]_D^{25}$ = +23.6 (c = 0.043, C_6D_6); solid, mp 209−213 °C.

Product ***49*** *Acetate:* ^{1}H NMR (400 MHz, C_6D_6) δ 0.839 (dd, J = 11.6, 2.0 Hz, 1H, H-5), 0.89 (m, 1H, H-1), 0.910 (s, 3H, Me-19), 1.032 (s, 3H, Me-30), 1.036 (s, 6H, Me-18, Me-28), 1.05 (s, 3H, Me-29), 1.20 (m, 1H, H-11), 1.22 (m, 1H, H-15), 1.27 (s, 3H, Me-21), 1.30 (m, H-12), 1.34 (m, 2H, H-7, H-9), 1.47 (m, 1H, H-6), 1.50 (m, 1H, H-11), 1.56 (m, 1H, H-6), 1.58 (m, 1H, H-7), 1.59 (m, 1H, H-1), 1.61 (br s, 3H, Me-26), 1.65 (m, 1H, H-13), 1.76 (m, 1H, H-2), 1.82 (m, 3H, H-12, H-15, H-22), 1.85 (m, 1H, H-2), 1.89 (s, 3H, CH_3CO), 1.92 (m, 1H, H-22), 1.99 (m, 2H, H-16), 2.22 (m, 1H, H-23), 2.27 (m, 1H, H-17), 2.43 (m, 1H, H-23), 4.02 (d, J = 16.4, 1H, H-27), 4.24 (d, J = 16.4 Hz, 1H, H-27), 4.83 (dd, J = 11.6, 4.8 Hz, 1H, H-3), 5.59 (br s, 1H, H-24); ^{13}C NMR (100 MHz, C_6D_6) δ 15.6 (q, C-18), 16.4 (q, C-19), 16.7 (q, C-30), 16.8 (q, C-29), 18.5 (t, C-6), 20.5 (q, C-21), 20.8 (q, CH_3CO), 21.8 (q, C-26), 21.8 (t, C-11), 23.9 (t, C-23), 24.1 (t, C-2), 25.0 (t, C-16), 27.5 (t, C-12), 28.1 (q, C-28), 31.6 (t, C-15), 35.5 (t, C-7), 37.2 (s, C-10), 38.1 (s, C-4), 38.8 (t, C-1), 40.3 (t, C-22), 40.6 (s, C-8), 43.3 (d, C-13), 47.9 (d, C-17), 50.8 (d, 2C, C-9), 50.8 (s, C-14), 56.2 (d, C-5), 64.7 (t, C-27), 79.6 (s, C-20), 80.5 (d, C-3), 125.3 (d, C-24), 136.9 (s, C-25), 169.9 (s, CH_3CO). The assignments of C-29 and C-30 may be exchangeable: MS (EI) m/z 125 (100%), 343 (8%), 484 (M^+, 0.4%); [M^+]; HRMS (EI) m/z calcd for $C_{32}H_{52}O_3$ 484.39165; found 484.39160; $[\alpha]_D^{25}$ = +16.7 (c = 0.017, C_6D_6); solid.

Product ***50*** *Acetate:* ^{1}H NMR (400 MHz, C_6D_6) δ 0.776 (br d, J = 10.8 Hz, H-5), 0.882 (s, 3H, Me-25), 0.92 (m, 1H, H-1), 0.921 (s, 3H, 1H, Me-27), 1.01 (s, 3H, Me-23), 1.03 (s, 3H, Me-24), 1.05 (s, 3H, Me-26), 1.17 (m, 1H, H-12), 1.25 (s, 3H, Me-28), 1.32 (m, 1H, H-9), 1.35 (m, 1H, H-11), 1.38 (m, 4H, H-7, H-15, 2H for each, and m, 1H for H-6, total 5H), 1.39 (m, 2H, H-16), 1.50 (m, 1H, H-19), 1.51 (m, 1H, H-6), 1.52 (m, 1H, H-11), 1.56 (s, 3H, Me-28), 1.65 (m, 2H, H-1, H-19), 1.73 (m, 1H, H-2), 1.86 (m, 1H, H-2), 1.88 (s, CH_3CO), 1.96 (m, 1H, H-13), 1.98 (m, 1H, H-20), 2.04 (m, 1H, H-20), 2.09 (m, 1H, H-12), 3.20 (d, J = 10.4 Hz, 1H, H-18), 3.85 (d, J = 16.8 Hz, 1H, H-30), 4.47 (d, J = 16.8 Hz, 1H, H-30), 4.82 (dd, J = 11.6, 4.4 Hz, 1H, H-3), 5.76 (t, J = 8.0 Hz, 1H, H-21); ^{13}C NMR (100 MHz, C_6D_6) δ 14.9 (q, C-27), 15.9 (q, C-26), 16.5 (q, C-25), 16.8 (q, C-24), 18.0 (q, C-28), 18.4 (t, C-6), 20.8 (q, CH_3CO), 21.2 (t, C-20), 21.4 (q, C-29), 22.1 (t, C-11), 24.1 (t, C-2), 25.2 (t, C-12), 28.1 (q, C-23), 33.9 (t, C-7), 36.5 (t, 2C, C-15, C-16), 37.1 (s, C-10), 37.2 (s, C-17), 38.0 (s, C-4), 38.6 (t, C-1), 40.9 (s, C-8), 41.0 (d, C-13), 42.6 (s, C-14), 45.9 (t, C-19), 50.9 (d, C-9), 55.8 (d, C-5), 74.1 (t, C-30), 80.5(d, C-3), 84.5 (d, C-18), 126.7 (d, C-21), 132.9 (s, C-22), 169.9 (s, CH_3CO); MS (EI) m/z 189 (20%), 402 (100%), 424 (0.9%), 469 (0.03%), 484 (M^+, 0.02%); [M^+]; HRMS (EI) m/z calcd for $C_{32}H_{52}O_3$ 484.39165; found 484.39167; $[\alpha]_D^{25}$ = +64.2 (c = 0.02, C_6D_6); solid, mp 192−195 °C.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01313.

EIMS and NMR data of products **10**−**16**, **22**−**39**, and **42**−**50**; product profiles (GC) for the enzymatic reactions of **10**−**16** and for those for the mutants targeting amino acid residues surrounding the E-ring (PDF)

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Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657−6671

■ ACKNOWLEDGMENTS

This work was supported in part by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science, Nos. 25450150 (C) and 18380001(B).

■ REFERENCES

(1) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890−1904.
(2) Wendt, K. U.; Schulz, G. E.; Corey, E. J.; Liu, D. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 2812−2833.
(3) Hoshino, T.; Sato, T. *Chem. Commun.* **2002**, *2002*, 291−301.
(4) Yoder, R. A.; Johnston, J. N. *Chem. Rev.* **2005**, *105*, 4730−4756.
(5) Abe, I. *Nat. Prod. Rep.* **2007**, *24*, 1311−1331.
(6) Wu, T.-K.; Chang, C.-H.; Liu, Y.-T.; Wang, T.-T. *Chem. Rec.* **2008**, *8*, 302−325.
(7) Nes, W. D. *Chem. Rev.* **2011**, *111*, 6423−6451.
(8) Xu, R.; Fazio, G. C.; Matsuda, S. P. T. *Phytochemistry* **2004**, *65*, 261−291.
(9) Ito, R.; Masukawa, Y.; Hoshino, T. *FEBS J.* **2013**, *280*, 1267−1280.
(10) Rees, H. H.; Mercer, E. I.; Goodwin, T. W. *Biochem. J.* **1966**, *99*, 726−734.
(11) Rees, H. H.; Britton, G.; Goodwin, T. W. *Biochem. J.* **1968**, *106*, 659−665.
(12) Barton, D. H. R.; Mellows, G.; Widdowson, D. A.; Wright, J. J. *J. Chem. Soc. C* **1971**, *1971*, 1142−1148.
(13) Suga, T.; Shishibori, T. *Phytochemistry* **1975**, *14*, 2411−2417.
(14) Seo, S.; Tomita, Y.; Tori, K. *J. Am. Chem. Soc.* **1981**, *103*, 2075−2080.
(15) Seo, S.; Yoshimura, Y.; Uomori, A.; Takeda, K.; Seto, H.; Ebizuka, Y.; Sankawa, U. *J. Am. Chem. Soc.* **1988**, *110*, 1740−1745.
(16) Hoshino, T.; Yamaguchi, Y.; Takahashi, K.; Ito, R. *Org. Lett.* **2014**, *16*, 3548−3551.
(17) Hoshino, T.; Miyahara, Y.; Hanaoka, M.; Takahashi, K.; Kaneko, I. *Chem. - Eur. J.* **2015**, *21*, 15769−15784.
(18) Budzikiewicz, H.; Wilson, J. M.; Djerassi, C. *J. Am. Chem. Soc.* **1963**, *85*, 3688−3699.
(19) Karliner, J.; Djerassi, C. *J. Org. Chem.* **1966**, *31*, 1945−1956.
(20) Ito, R.; Hashimoto, I.; Masukawa, Y.; Hoshino, T. *Chem. - Eur. J.* **2013**, *19*, 17150−17158.
(21) Ito, R.; Masukawa, Y.; Nakada, C.; Amari, K.; Nakano, C.; Hoshino, T. *Org. Biomol. Chem.* **2014**, *12*, 3836−3846.
(22) Kushiro, T.; Shibuya, M.; Masuda, K.; Ebizuka, Y. *J. Am. Chem. Soc.* **2000**, *122*, 6816−6824.
(23) Narayanan, C. R.; Natu, A. A. *J. Org. Chem.* **1974**, *39*, 2639−2641.
(24) Dehaen, W.; Mashentseva, A. A.; Seitembetov, T. S. *Molecules* **2011**, *16*, 2443−2466.

DOI: 10.1021/acs.joc.6b01313
J. Org. Chem. 2016, 81, 6657−6671