



Substrate specificity of the CYC2 enzyme from *Kitasatospora griseola*: production of sclarene, biformene and novel bicyclic diterpenes by the enzymatic reactions of labdane- and halimane-type diterpene diphosphates

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ABSTRACT

The enzymatic reaction of copalyl diphosphate (CDP), *ent*-CDP, *syn*-CDP, and tuberculosinyl diphosphate with the CYC2 enzyme from *Kitasatospora griseola* afforded sclarene, biformene, and novel diterpenes having a buta-1,3-diene moiety in the side chain. The substrate specificity of the CYC2 is discussed.

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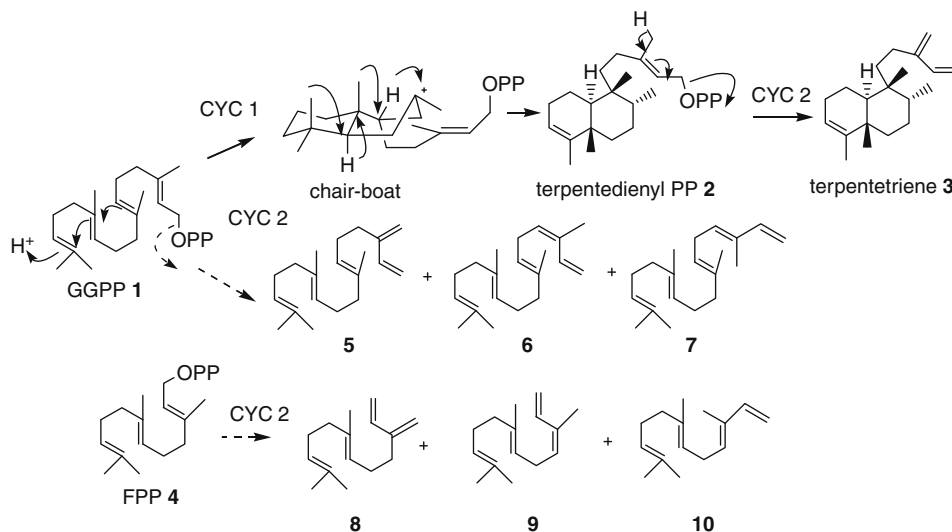
Isoprenoids are abundant in nature and their structural diversity is remarkable. Terpene synthases catalyze the committed step in natural products biosynthetic pathways and mediate complex reactions.¹ Dairi et al. discovered the *cyc1* and *cyc2* genes from the eubacterium *Kitasatospora griseola*.^{2,3} The *cyc1* gene product is a diterpene cyclase that mediates the reaction of converting a linear molecule of geranylgeranyl diphosphate (GGPP, **1**) to bicyclic terpenedienyl diphosphate **2** possessing a clerodane scaffold.^{3,4} The CYC2 enzyme catalyzes the generation of terpentetriene **3** from **2** through a diphosphate (PP)-removal reaction (Scheme 1).² Eukaryotic diterpene synthases such as abietadiene synthase,^{5,6} copalyl diphosphate synthases,^{7–9} and *ent*-kaurene synthases¹⁰ have been well characterized. In addition to the physiological reactions, the CYC2 enzyme also catalyses the reactions of the linear isoprenoids of **1** and farnesyl diphosphate (FPP, **4**) to afford acyclic diterpenes **5–7** and sesquiterpenes **8–10** through a PP-removal reaction, as shown in Scheme 1.³ The unexpected reactions motivated us to further investigate the substrate specificity of the CYC2. We examined whether labdane-type diterpene diphosphates such as (+)-copalyl diphosphate ((+)-CDP, **11**), (–)-*ent*-CDP **12** and *syn*-CDP **13**, and halimane-type diterpene diphosphate such as tuberculosinyl diphosphate (TBPP, **14**) are accepted as substrates

of the CYC2. The structures of these diphosphates are shown in Figure 1. We demonstrate here that all of these four bicyclic diphosphates are allowed to react with the CYC2, and found that the CYC2 has considerable potential for recognizing the various bicyclic diterpene diphosphates.

Three bicyclic CDPs, that is, (+)-CDP, (–)-*ent*-CDP (enantiomer of CDP), and *syn*-CDP (diastereomer of CDP and *ent*-CDP), usually undergo further cyclization reaction by Type-A cyclase to form tri- or tetracyclic skeletons,¹ as found in the biosyntheses of the carbocycles of pimarane, abietane, cassane, kaurane, beyerane, stemarane, and so on. The abovementioned CDPs are the common precursors of the tri- and tetracyclic diterpenes. The *syn-ent*-copalyl skeleton has not been isolated from any organism so far. We examined whether the CYC2 recognizes the three CDPs as substrates to afford further cyclized skeletons during the PP-removal reaction. Substrate **11** was prepared by employing the recombinant CDP synthase from *Phomopsis amygdali*;¹¹ **12** and **13** were obtained by employing the recombinant enzymes from *Oryza sativa* L.;⁷ and **14** was prepared with the recombinant Rv3377c enzyme from *Mycobacterium tuberculosis* H37 genome.^{12,13}

Optimal temperatures for *ent*-CDP,⁹ *syn*-CDP,⁹ and TBPP synthases¹³ were reported to be around 30 °C and that for CDP synthase was reported to be 25 °C.¹¹ At an approximately neutral pH range of 6–7.5, high activities were observed for the above four cyclases. On the other hand, the optimal temperature and pH for

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Scheme 1. The reaction mechanisms of GGPP **1** into terpenedienyl diphosphate **2** and terpenetriene **3**, which are mediated by the CYC1 and CYC2 enzymes, and the acyclic products **5–10** obtained by the enzymatic reaction of **1** and FPP **4** with the CYC2. Products **6** and **9** have (Z)-butadiene moiety, while **7** and **10** have (E)-butadiene residues.

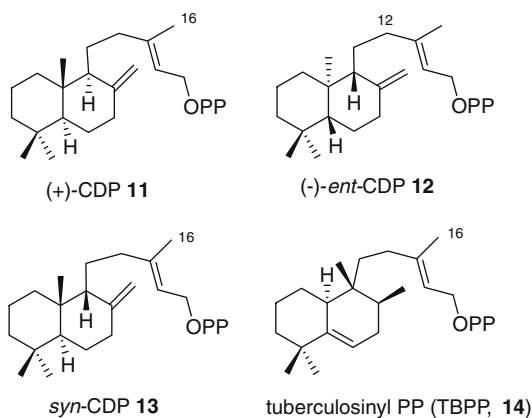


Figure 1. The substrate structures for the CYC2 employed in this study.

the CYC2 were 50 °C and 6.8 (active at a pH range of 6.8–7.5), respectively.³ The divalent metal ion of Mg²⁺ is essential for the enzyme activities of all diterpene cyclases including the dephosphorylating CYC2 enzyme. To examine whether CDP, ent-CDP, syn-CDP, and TBPP are accepted as the substrates of the CYC2, each of these diterpene cyclases was mixed separately with the CYC2, and the mixtures were incubated with **1** for 15 h at a pH of 7.5 and a temperature of 30 °C in the presence of 1 mM MgCl₂. When the incubation temperature was 50 °C (optimal temperature for the CYC2), no bicyclic product was obtained; this was possibly due to the deactivation of all the cyclase enzymes (CDPs and TBPP diterpene synthases) at the high temperature. Previously, we found that cell-free extracts obtained from *Escherichia coli* cells exhibit phosphatase activity.^{12,13} In order to remove the endogenous phosphatase, all of the enzymes were purified by affinity column chromatography using a Ni²⁺-NTA column or glutathione-Sepharose 4B. The CYC2 and TBPP synthases were expressed as the His-tag fusion proteins in *E. coli*. The expression vectors used for the CYC1 and CYC2 were pQE-30 and that for TBPP synthase was pET-22b (+), whereas the pGEX-4T-3 vector was used to express the GST-fusion proteins for the three CDP synthases. The enzyme reactions¹⁴ were terminated by adding 15% KOH/MeOH and heating at 75 °C for 30 min. The hexane extract obtained from the reaction mixture was subjected to GC analyses (data not shown), which

showed that each of the four reactions (CDP + CYC2, ent-CDP + CYC2, syn-CDP + CYC2, and TBPP + CYC2) afforded a single product. The enzymatic products were purified by SiO₂ column chromatography eluting with hexane.

Reaction of a mixture of CDP synthase and the CYC2 with GGPP: Thus far, (+)-CDP synthase has been isolated only from *P. amygdali* as PaDC2.¹¹ This CDP synthase was used for this enzymatic reaction with the CYC2, affording **15** as the single product with a yield of 0.9 mg from 3 mg of **1** (yield: 49.6%). The structure was determined by the detailed NMR analyses including DEPT, ¹H–¹H COSY, HOHAHA, NOESY, HMQC, and HMBC, which were measured in C₆D₆. GGPP **1** involves five-allylic Me, all of which disappeared in the ¹H NMR spectrum of **15**. In turn, three methyl groups appeared at a higher field (δ_{H} 0.829, 3H, s; 0.906, 3H, s; and 0.957, 3H, s) accompanying three methylene moieties [δ_{C} 106.7 (t), 113.3 (t), and 115.8 (t)], all of which were assigned to CH₂ by the DEPT 135 analysis. The HMQC spectrum revealed the following correlations: δ_{C} 106.7 (t)/ δ_{H} 5.06 (s) and 4.79 (s); δ_{C} 113.3 (t)/ δ_{H} 5.40 (d, J = 17.8 Hz) and 5.14 (d, J = 10.8 Hz); δ_{C} 115.8 (t)/ δ_{H} 5.17 (s) and 5.15 (s). The methylene protons at δ_{H} 4.79 (s) and 5.06 (s) exhibited a clear HMBC correlation with C-9 (δ_{C} 56.75, d), indicating that these protons are assignable to H-17. Thus, **15** has a bicyclic core of copalyl skeleton. The methylene protons at δ_{H} 5.40 (d, J = 17.8 Hz) and 5.14 (d, J = 10.8 Hz) exhibited a strong HMBC correlation with C-13 (δ_{C} 147.5, s) and C-14 (δ_{C} 139.6, d), indicating that these protons are assignable to H-15. H-14 (δ_{H} 6.54) exhibited a dd splitting pattern (J = 17.8, 10.8 Hz) due to the spin–spin couplings with H-15a (*trans*) and H-15b (*cis*). The methylene protons at δ_{H} 5.17 (s) and 5.15 (s) were assigned to H-16 because it had clear cross-peaks with C-13, C-14, and C-12 (δ_{C} 30.69, t) in the HMBC spectrum. Thus, **15** has a buta-1,3-diene moiety in the side chain. The complete assignments of the ¹H and ¹³C NMR data unambiguously supported the structure of **15** (Fig. 2).¹⁵ This compound, also called sclarene, was previously isolated from *Dacrydium cupressinum*.¹⁶ The structure of sclarene indicates that the CYC2 does not catalyze the further cyclization reaction of **11**.

Reaction of a mixture of ent-CDP synthase and the CYC2 with GGPP: Product **16** was obtained with a yield of 1.1 mg from 3 mg of **1** (yield: 60.6%). Two methylene moieties were found in the ¹H and ¹³C NMR spectra; H-17 [δ_{H} 5.06 (d, J = 1.4 Hz) and 4.78 (d, J = 1.4 Hz)]; and H-15 [δ_{H} 5.34 (1H, d, J = 16.9 Hz) and 5.24 (1H, d, J = 10.8 Hz)]; δ_{C} 108.1 (t) and 113.3 (t). H-15 was spin-cou-

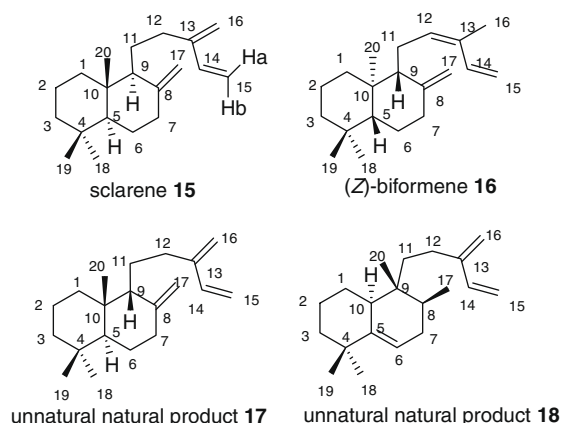


Figure 2. Structures of the enzymic products.

pled with H-14 [δ_{H} 7.08 (dd, $J = 16.9, 10.8$ Hz)], indicating the involvement of one vinyl group in **16**. H-17 exhibited a strong HMBC correlation with C-9 (δ_{C} 57.58, d); on this basis, we suggested that **16** contains a copalyl skeleton. In addition, one allylic Me (Me-16) was found at δ_{H} 1.94 (3H, d, $J = 1.2$ Hz), which exhibited a clear cross-peak with C-12 (δ_{C} 132.2, d) in the HMBC spectrum. A strong NOE between Me-16 and H-12 (δ_{H} 5.59, t, $J = 6.4$ Hz) demonstrated that the double bond at C12–C13 has a *Z*-configuration. The detailed NMR analyses¹⁷ indicated that **16** was (*Z*)-biformene that was previously isolated from *Helianthus decapetalus*.¹⁸ (*E*)-Biformene with *E*-configured double bond (like **7** and **10**) was not detected.

Reaction of a mixture of syn-CDP synthase and the CYC2 with GGPP: One mg of **17** was obtained from 3 mg of **1** (yield: 55.1%). As found in **15**, one vinyl group and two sets of methylidene protons were found: H-15 (δ_{H} 5.40, d, $J = 17.6$ Hz; 5.14, d, $J = 10.8$ Hz) and H-14 (δ_{H} 6.53, dd, $J = 17.6, 10.8$ Hz) for the vinyl group, and H-16 (δ_{H} 5.16, s; 5.14, s) and H-17 (δ_{H} 4.93, t, $J = 2.4$ Hz; 4.76, dd, $J = 2.4, 1.6$ Hz) for the methylidene protons. These functional groups were further confirmed by DEPT experiments: δ_{C} 113.2 (t) for C-15, 139.5 δ_{C} (d) for C-14, δ_{C} 115.9 (t) for C-16, and 110.1 (t) for C-17. Definitive HMBC cross-peaks were found for H-17/C-9 (δ_{C} 58.53, d) and for Me-20 (δ_{H} 1.10, 3H, s)/C-9. A strong NOE between Me-20 and H-9 (δ_{H} 1.60, m) indicated a *syn*-copalyl skeleton. The detailed analyses of the HMBC and COSY spectra led to the complete assignments of the copalyl skeleton of **17** (Fig. 2).¹⁹ Product **17** is a novel compound and has never been found in nature.

Reaction of a mixture of TBPP synthase and the CYC2 with GGPP: 0.9 mg of **18** was obtained from 3 mg of **1** (yield: 49.6%). NMR analyses in a manner similar to those for **15** and **17**, showed the presence of one vinyl group and one methylidene protons in **18**. The detailed analyses of the 2D NMR data indicated that this product is a novel compound containing a halimane bicyclic skeleton. The complete structure²⁰ is shown in Figure 2.

The present study revealed that the substrate specificity of the CYC2 is remarkably broad. Three scaffolds of the bicyclic diterpenes—labdane, halimane, and clerodane—occur in nature, but other bicyclic diterpene skeletons have not been found until now.²¹ All of the three scaffolds were accepted as substrates of the CYC2. It is of particular interest that the three stereoisomers of the labdane-type diterpenes such as CDP, *ent*-CDP, and *syn*-CDP were equally recognized as substrates of the CYC2, resulting in nearly the same yields for each product (50–60%). The deprotonation of **11**, **13**, and **14** from Me-16 in conjunction with the PP-removal reaction afforded **15**, **17**, and **18**, respectively. This reaction mechanism is identical to that for the formation of **3** from **2**

(Scheme 1). In the case of **16**, the deprotonation reaction occurred from H-12. As all the enzymatic products did not consist of tri- or tetracyclic skeletons, no further cyclization was induced by the CYC2. In other words, the CYC2 is not a cyclase. The products **3** and **15–18** have a similar partial structure—buta-1,3-diene moiety—in the side chain. Thus, it appears that the bicyclic skeletons of labdane, halimane, and clerodane are not captured by the CYC2. However, (*−*)-*ent*-sclarene, which was isolated from *Dacrydium intermedium foliage*,²² was not detected in the enzyme mixture of *ent*-CDP and the CYC2. Instead, (*Z*)-biformene **16** was specifically produced without the formation of (*E*)-biformene. These findings suggest that the CYC2 may recognize the bicyclic scaffold to some extent. Further studies will be required to account for the substrate recognition. No protein homologous to the CYC2 has been found thus far, but we succeeded in synthesizing naturally occurring sclarene **15** and biformene **16** by the CYC2, thus the genes homologous to *cyc2* are likely to be present in living organisms other than the eubacterium species *K. griseola*. In general, cyclic diterpene cores found in nature are produced by the actions of the cyclases of Type A, Type B, or a combination thereof.²³ The CYC2 enzyme involves a DDXXD motif in the sequence that is characteristic to Type-A cyclase and responsible for the PP-removal reaction through Mg^{2+} chelation.¹ Therefore, the CYC2 did catalyze Type A-like reaction, that is, the PP-removal reaction followed by the formation of allylic carbocation. However, the CYC2 is not responsible for a cyclization reaction after the carbocation formation, but is for a deprotonation reaction, as clearly demonstrated by the present study and Hamano et al.³ Isolation of the genes homologous to *cyc2* from various biological sources might lead to the proposal of a new class of terpene synthases, that is, deprotonation-type through a PP-removal reaction. Furthermore, the CYC2 would be a promising tool for introducing a buta-1,3-diene moiety into molecules by the enzymatic reactions. The enzymatic syntheses of unnatural natural products with a high level of plasticity to tolerate a variety of substrates are attractive areas of research.²⁴

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- The purified CYC2 was obtained from 1.5 L culture in a LB medium (26.5 mg). The purified CDPs, and TBPP synthases were prepared from 6 L culture in $2 \times \text{YT}$ medium and LB medium, respectively. Incubation conditions: **1** (3 mg, 44.4 μM), MgCl_2 (1 mM), DTT (2 mM), each of the purified proteins, Tris–HCl

- buffer (pH 7.5, 50 mM) containing 0.1% Triton X-100 (total volume, 150 mL), 30 °C for 15 h. When the purified CDPs and TBPP synthases thus prepared were used under this incubation condition, followed by the treatment with acid phosphatase, no geranylgeraniol was detected by GC analyses of the hexane extract, indicating that the cyclization reactions had completed.
15. **Product 15:** oil; $[\alpha]_D^{24} +37.8$ (c 0.09, CHCl₃), cf. lit. +45.0 (c 1.0, CHCl₃);²⁵ EIMS: m/z (%): 69 (97), 81 (100), 93 (73), 109 (37), 137 (37), 257 (84), 272 (9, M⁺); HR-EIMS: m/z : calcd for C₂₀H₃₂: 272.2504; found: 272.2504. ¹H NMR (400 MHz, C₆D₆): $\delta_H = 6.54$ (H-14, dd, $J = 17.8, 10.8$ Hz), 5.40 (Ha-15, d, $J = 17.8$ Hz), 5.17 (H-16, s), 5.15 (H-16, s), 5.14 (Hb-15, d, $J = 10.8$ Hz), 5.06 (H-17, s), 4.79 (H-17, s), 2.65 (H-12, m), 2.49 (H-7, m), 2.25 (H-12, m), 2.07 (H-7, ddd, $J = 12.8, 12.8, 5.2$ Hz), 1.85 (H-11, m), 1.75 (H-1, m), 1.73 (H-9, m), 1.72 (H-6, m), 1.70 (H-11, m), 1.60 (H-2, m), 1.50 (H-2, m), 1.46 (H-3, m), 1.39 (H-6, ddd, $J = 12.8, 12.8, 4.1$ Hz), 1.25 (H-3, ddd, $J = 12.0, 12.0, 4.0$ Hz), 1.10 (H-5, dd, $J = 12.6, 2.8$ Hz), 1.03 (H-1, ddd, $J = 12.8, 12.8, 4.0$ Hz), 0.957 (Me-18, 3H, s), 0.829 (Me-20, 3H, s). ¹³C NMR (100 MHz, C₆D₆): $\delta_C = 148.7$ (C-8, s), 147.5 (C-13, s), 139.6 (C-14, d), 115.8 (C-16, t), 113.3 (C-15, t), 106.7 (C-17, t), 56.75 (C-9, d), 55.56 (C-5, d), 42.38 (C-3, t), 39.82 (C-10, s), 39.10 (C-1, t), 38.67 (C-7, t), 33.71 (C-18, q), 33.63 (C-4, s), 30.69 (C-12, t), 24.73 (C-6, t), 22.69 (C-11, t), 21.86 (C-19, q), 19.71 (C-2, t), 14.72 (C-20, q).
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 17. **Product 16:** Oil; $[\alpha]_D^{24} -23.7$ (c 0.11, CHCl₃), cf. lit. -16.0 (c 0.25, CHCl₃);¹⁸ EIMS: m/z (%): 69 (70), 81 (100), 95 (69), 107 (44), 135 (56), 201 (27), 216 (37), 257 (35), 272 (42, M⁺); HR-EIMS: m/z : calcd for C₂₀H₃₂: 272.2504; found: 272.2504. ¹H NMR (400 MHz, C₆D₆): $\delta_H = 7.08$ (H-14, dd, $J = 16.9, 10.8$ Hz), 5.59 (H-12, t, $J = 6.4$ Hz), 5.34 (H-15, d, $J = 16.9$ Hz), 5.24 (H-15, d, $J = 10.8$ Hz), 5.06 (H-17, d, $J = 1.4$ Hz), 4.78 (H-17, d, $J = 1.4$ Hz), 2.56 (H-11, m), 2.45 (H-7, m), 2.35 (H-11, m), 2.06 (H-7, ddd, $J = 12.8, 12.8, 4.8$ Hz), 1.93 (H-16, 3H, d, $J = 1.2$ Hz), 1.81 (H-9, br d, $J = 11.2$ Hz), 1.75 (H-m), 1.38 (H-6, dddd, $J = 13.2, 13.2, 13.2, 4.4$ Hz), 1.22 (H-3, ddd, $J = 13.2, 13.2, 3.6$ Hz), 1.07 (dd, $J = 12.6, 2.8$ Hz), 1.05 (H-1, m), 0.946 (Me-18, 3H, s), 0.909 (Me-19, 3H, s), 0.852 (Me-20, 3H, s). ¹³C NMR (100 MHz, C₆D₆): $\delta_C = 148.5$ (C-8, s), 134.3 (C-14, d), 132.2 (C-12, d), 131.9 (C-13, s), 113.3 (C-15, t), 108.1 (C-17, t), 57.58 (C-9, d), 55.37 (C-5, d), 42.31 (C-3, t), 39.78 (C-10, s), 39.21 (C-1, t), 38.39 (C-7, t), 33.70 (C-18, q), 33.59 (C-4, s), 24.49 (C-6, t), 22.47 (C-11, t), 21.90 (C-19, q), 19.99 (C-16, q), 19.72 (C-2, t), 14.63 (C-20, q).
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 19. **Product 17:** Oil; $[\alpha]_D^{24} +22.7$ (c 0.1, CHCl₃), EIMS: m/z (%): 69 (100), 81 (98), 93 (73), 109 (42), 123 (28), 149 (21), 163 (21), 177 (23), 189 (22), 203 (15), 257 (64), 272 (13, M⁺); HR-EIMS: m/z : calcd for C₂₀H₃₂: 272.2504; found: 272.2508. ¹H NMR (400 MHz, C₆D₆): $\delta_H = 0.918$ (Me-19, 3H, s), 0.948 (Me-18, 3H, s), 1.105 (Me-20, 3H, s), 1.14 (H-1, m), 1.28 (H-3, ddd, $J = 13.2, 13.2, 4.1$ Hz), 1.36 (H-5, m), 1.37 (H-6, m), 1.48 (H-3, m), 1.50 (H-2, m), 1.60 (H-9, m), 1.61 (2H, H-6 & H-1, m), 1.70 (H-11, m), 1.73 (H-2, m), 1.91 (H-11, m), 2.13 (H-12, m), 2.43 (H-12, m), 4.76 (H-17, dd, $J = 2.4, 1.6$ Hz), 4.93 (H-17, t, $J = 2.4$ Hz), 5.14 (H-15, d, $J = 10.8$ Hz), 5.14 (H-16, s), 5.16 (H-16, s), 5.40 (H-15, d, $J = 17.6$ Hz), 6.53 (H-14, dd, $J = 17.6, 10.8$ Hz). ¹³C NMR (100 MHz, C₆D₆): $\delta_C = 149.8$ (C-8, s), 147.4 (C-13, s), 139.5 (C-14, d), 115.9 (C-16, t), 113.2 (C-15, t), 110.1 (C-17, t), 58.53 (C-9, d), 46.07 (C-5, d), 42.83 (C-3, t), 38.22 (C-10, s), 36.99 (C-1, t), 35.59 (C-18, q), 33.27 (C-4, s), 31.86 (C-7, t), 30.68 (C-12, t), 25.51 (C-11, t), 23.94 (C-6, t), 22.60 (C-20, q), 22.33 (C-19, q), 19.48 (C-2, t).
 20. **Product 18:** oil; $[\alpha]_D^{24} +29.9$ (c 0.09, CHCl₃), EIMS: m/z (%): 69 (27), 80 (58), 93 (33), 107 (29), 119 (100), 175 (32), 189 (100), 190 (88), 191 (92), 257 (5), 272 (2, M⁺); HR-EIMS: m/z : calcd for C₂₀H₃₂: 272.2504; found: 272.2508. ¹H NMR (400 MHz, C₆D₆): $\delta_H = 6.53$ (H-14, dd, $J = 17.6, 10.8$ Hz), 5.65 (H-6, ddd, $J = 4.8, 2.4, 2.4$ Hz), 5.42 (H-15, d, $J = 17.6$ Hz), 5.15 (H-16, s), 5.14 (H-15, d, $J = 10.8$ Hz), 5.12 (H-16, s), 2.44 (H-10, m), 2.38 (H-12, m), 2.27 (H-12, m), 1.95 (H-7, 2H, m), 1.86 (H-1, m), 1.82 (H-11, m), 1.68 (H-8, m), 1.68 (H-2, 2H, m), 1.62 (H-11, m), 1.51 (H-3, m), 1.37 (H-3, m), 1.260 (Me-19, 3H, s), 1.207 (Me-18, 3H, s), 1.18 (H-1, m), 0.922 (Me-17, d, $J = 6.8$ Hz), 0.790 (Me-20, 3H, s). ¹³C NMR (100 MHz, C₆D₆): $\delta_C = 147.8$ (C-13, s), 146.1 (C-5, s), 139.5 (C-14, d), 116.7 (C-6, d), 115.8 (C-16, t), 113.1 (C-15, t), 41.23 (C-3, t), 40.24 (C-10, d), 37.44 (C-9, s), 36.29 (C-4, s), 36.00 (C-11, t), 33.62 (C-8, d), 31.91 (C-7, t), 30.02 (C-19, q), 29.13 (C-18, q), 27.98 (C-1, t), 25.06 (C-12, t), 22.58 (C-2, t), 16.39 (C-20, q), 15.34 (C-17, q).
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