Microbial Production of Plant Gibberellins and Related Compounds from *ent*-Kaurene Derivatives in *Gibberella fujikuroi*

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ent-15 α -Hydroxykaurenoic acid (**8**) was synthesized and fed to a mycelium suspension of *Gibberella fujikuroi* in the presence of 1-*n*-decylimidazole, a gibberellin biosynthesis inhibitor. The metabolites included 15 β -hydroxy GA₂₄, GA₄₅ (GA of *Pyrus communis*), 15 β -hydroxy GA₁₅ and 15 β -hydroxy GA₂₅. Microbial production of 12 α -hydroxy GAs from *ent*-12 β -hydroxykaurene is also described.

Many gibberellins have been isolated from higher plants. Each of these plant gibberellins may have a specific physiological activity or activities, for example, shoot elongation, stimulation of amylase biosynthesis, setting and development of fruits, and initiation of flowering *etc.*¹⁾ Their specific activities remain to be examined, and practical supply of plant gibberellins is required for the detailed study of gibberellin physiology. However, syntheses of many plant gibberellins still are not accomplished. Recently, we demonstrated that microbial transformation of synthesized *ent*-kaurene derivatives by *G. fujikuroi* in the presence of a gibberellin biosynthesis inhibitor is useful for preparing gibberellins and related compounds in large scale.²⁾ Bearder,³⁾ Cross⁴⁾ and Murofushi⁵⁾ also reported similar microbial transformation of *ent*-kaurene derivatives in *G. fujikuroi*.

We wish report here bioorganic productions of plant gibberellins and related compounds through combination of synthesis of *ent*-kaurene derivatives and their microbial transformations.

Synthesis and microbial transformation of *ent*-15 α -hydroxykaurenoic acid (**8**)

ent-15 α -Hydroxykaurenoic acid (**8**) was synthesized from *ent*-kaurenoic acid (**1**) with six steps as an artificial substrate of the enzyme for gibberellin biosynthesis in *G. fujikuroi*.

Methyl ester (**2**) of *ent*-kaurenoic acid was rearranged to a mixture of the starting material and *ent*-kaur-15-en-19-oic acid methyl ester (**3**) by refluxing in acetic acid. The mixture was converted to *ent*-15 β -hydroxy compound (**4**) and *ent*-kaur-15-en-17-ol (**5**) by photooxygenation and reduction following the procedure reported in the literature.⁶⁾ The *ent*-15 β -hydroxy compound (**4**) was oxidized to α,β -unsaturated ketone (**6**) with manganese dioxide. NaBH₄ reduction and alkaline hydrolysis of the compound (**6**) gave the *ent*-15 α -hydroxy compound (**8**). The physicochemical data except melting point of the product was identical with those of an authentic sample.⁷⁾

FIG. 1. GC of the Methyl Esters of Acidic Metabolites Produced from *ent*-15 α -Hydroxykaurenoic Acid in *G. fujikuroi*.

Peak 1, A-III; 2, A-I; 3, *ent*-7 α ,15 α -dihydroxykaurenoic acid; 4, A-IV.

Lowering of melting point of our sample may result from admixture of the dihydroderivative of (8), which is a byproduct formed in the NaBH₄ 1,4-reduction of 6. GC-MS of the sample (7) showed the presence of 4% of the dihydroderivative.

In the preliminary time coarse culture; *ent*-15 α -hydroxykaurenoic acid (8) was incubated with mycelium of *G. fujikuroi* suspended in N 0%-ICI medium, in which endogenous GA biosynthesis was blocked by the addition of 1-*n*-decylimidazole. Small portion of the culture were removed in the 1st, 3rd, 5th and 7th days, and the crude acidic extracts of these culture filtrate were analyzed by GC-MS. Even in the 24 hr culture, the artificial substrate (8) was completely metabolized to *ent*-7 α ,15 α -dihydroxykaurenoic acid (18).

The other metabolites were then formed at the expense of 18 in longer incubation, and almost all of 18 disappeared in 7th days (see Fig. 1).

The acidic metabolites (39 mg) obtained from the preliminary microbial transformation of 60 mg of the substrate (8) were separated into 7 fractions by silica gel TLC, using a solvent mixture of CH₂Cl₂-EtOAc-AcOH (10:1:1).

The main GA metabolites (A-I, -III and

-IV) were isolated from the fraction 2 by a reverse phase HPLC (Merk, Lichrosorb RP-18, MeOH-CH₃CN-1% H₃PO₄, 10:40:50) (see Figs. 2 and 3).

A large-scale microbial transformation of *ent*-15 α -kaurenoic acid (8) (1.5 g) was performed under the conditions identical with those of the time coarse culture for 7 days. As shown in Fig. 4, 9 acidic metabolites (A-I to A-IX) were isolated from the extract of the culture filtrate by silica gel TLC and reverse phase HPLC.

The metabolite A-I (9) is a colorless powder, $[\alpha]_D^{32} -81.0^\circ$. The methyl ester of A-I, C₂₂H₃₀O₆, showed hydroxyl (3520 cm⁻¹), carbonyl (1720-1710) and olefinic (1660, 900) absorption in its IR spectrum. The ¹H NMR spectrum contained one quaternary Me, two-OMe, two olefinic protons, a secondary alcohol, an aldehyde, and the typical gibberellin AB system (δ 2.13 and 3.96, *J*=13.1 Hz) assigned to the *trans* 5-H and 6-H protons.⁸⁾ By addition of D₂O, signal of C-15 proton (δ 3.97, bd) collapsed to a sharp triplet and a hydroxyl proton signal (δ 3.42, d, *J*=3.7 Hz) disappeared, suggesting that an intra molecular hydrogen bond between the hydroxyl group at C-15 and C-7 carbonyl group is formed. The MS shows similarities to that of GA₁₉ methyl

FIG. 2. Silica Gel TLC of the Acidic Metabolites from 8 (60 mg).
Solvent, CH_2Cl_2 -EtOAc-AcOH (10:10:1).

FIG. 3. HPLC Separation of Fraction 2.

ester by possessing ions at M-28, M-32, M-46, M-78, M-88 and M-147/148 characteristic of the loss of two methoxycarbonyl groups and a carbon monoxide from a C_{20} gibberellin.⁹⁾ The compound A-I was thus 15 β -hydroxyl GA₂₄.

The second metabolite A-II (10) was isolated as its dimethyl ester $\text{C}_{22}\text{H}_{32}\text{O}_6$, which showed hydroxyl (3530 cm^{-1}) and carbonyl (1720 – 1715) absorption in its IR spectrum. The ^1H NMR contained one quaternary Me,

one secondary Me, an aldehyde, and a secondary alcohol. Spin decoupling experiments confirmed the presence of the typical gibberellin AB system (δ 2.08 and 3.86, $J = 12.9$ Hz) assigned to the C-5 and C-6 protons. The MS was similar to that of A-I dimethyl ester with M-28, M-32, M-46, M-88 and M-147/148 shifted to higher mass by 2 a.m.u. Hence A-II was assigned the structure (10). This metabolite is presumably derived

FIG. 4. Isolation of Acidic Metabolites from *ent*-15 α -Hydroxykaurenoic Acid (8). FIG. 5. Structures of the Acidic Metabolites from *ent*-15 α -Kaurenoic Acid.

from the dihydroderivative of **8** admixed in the artificial substrate.

The metabolite A-III has mp. 204~205°C and $[\alpha]_D^{33} -44.0^\circ$. The methyl ester of A-III, C₂₀H₂₆O₅, showed hydroxyl (3500 cm⁻¹), γ -lactone (1765), ester (1720) and olefinic (1600, 900) absorption in its IR spectrum. In the ¹H NMR spectrum, the chemical shifts of the signals (C₁₈-Me, *trans* 5-H and 6-H) were almost identical with those of GA₉ methyl ester. The MS of MeTMSi derivative of A-III showed fragmentation pattern and relative intensities of each peaks identical with those of GA₄₅MeTMSi.^{10,11)} A-III was thus GA₄₅.

The metabolite A-IV has mp 265~267°C

and $[\alpha]_D^{29} -66.7^\circ$. The methyl ester of IV, C₂₁H₂₈O₅, showed hydroxyl (3530 cm⁻¹), carbonyl (1720), olefinic (1600, 900) absorption in its IR spectrum. The ¹H NMR showed proton signals (quaternary Me, -OCH₂- at C-20 and *trans* 5-H and 6-H) analogues to GA₁₅ methyl ester. The MS showed ions at M-32, M-60 and M-78 characteristic of the loss of one methoxycarbonyl group. The presence of a significant ion at M-46 is associated with δ -lactone ring in C₂₀ gibberellins.⁹⁾ The NaBH₄ reduction of A-I gave quantitatively A-IV. Thus, A-IV was assigned the structure (12), 15 β -hydroxy GA₁₅.

The metabolite A-V, mp. 232~233°C, $[\alpha]_D^{33}$

FIG. 6. Synthesis of *ent*-12 β -Hydroxykaur-16-ene.

FIG. 7. Variation in the Chemical Shift for the Protons of **28** with Increasing Concentration of $\text{Eu}(\text{fod})_3$. Deviations from linearity at high shift reagent concentration originate from the precipitation of $\text{Eu}(\text{fod})_3$ from the solution.

-27.8° , was identified as the dihydroderivative of A-III from the spectral data. The configurations of $\text{C}_{15}\text{-OH}$ and $\text{C}_{16}\text{-Me}$ group were assigned *cis* on β -side because the coupling constant between 15-H and 16-H was

10.5 Hz.¹²⁾ Thus, A-V was assigned the structure (**13**). The ^1H NMR of the methyl ester of A-VI showed three OMe signals, and similar to that of GA_{25} methyl ester.¹³⁾ The MS showed strong peaks at M-120 and M-180

characteristic of the loss of three methoxycarbonyl groups from a C₂₀ gibberellin. A-VI was thus 15 β -hydroxy GA₂₅ (**14**). The metabolite VII was assigned *ent*-6 α ,7 α ,15 α -trihydroxykaurenoic acid (**15**) from its ¹H NMR and the MS of its MeTMSi derivative, which showed signals and fragmentation pattern similar to those of *ent*-6 α ,7 α ,13-trihydroxykaurenoic acid derivative.³⁾ The ¹H NMR spectra of the methyl esters of A-VIII and A-IX were similar to those of the methyl esters of A-III and A-VI, respectively, except the deshielded signals of 5-H ($\Delta\delta$ 0.63 and 0.64), which show the presence of a hydroxy group on 1 β or 3 β -position. In addition, the MS of MeTMSi derivatives of both metabolites showed a prominent ion at *m/z* 129, characteristic of the TMSi ether of a 1 or 3-hydroxy GA.⁹⁾ Thus, the metabolites, A-VIII and A-IX were tentatively assigned 1 β - or 3 β -hydroxy GA₄₅ (**16**) and 1 β ,15 β - or 3 β ,15 β -dihydroxy GA₂₅ (**17**), respectively.

Synthesis and microbial transformation of ent-12 β -hydroxykaur-16-ene (**28**)

ent-12 β -Hydroxykaur-16-ene (**28**) was synthesized from *ent*-kaurene (**19**) by eight steps as shown in Fig. 6. The structure of the synthesized material (**28**) was confirmed as follows: The triplet signal at δ 2.62 (13-H) in its ¹H NMR collapsed to a doublet on irradiation of the 12-H signal at 3.80 (t, *J* = 4.7 Hz). In the ¹H NMR of the ketone (**29**) which was obtained by CrO₃-pyridine oxidation of **28**, the 13-H signal shifted to δ 3.21 by the anisotropy of the carbonyl group and appeared as a doublet. The high downfield shifts of proton signals of C-11 α , -13, -14 α and -20 in the spectra recorded using a concentration ratio of Eu (fod)₃ to **28** of 0.2~0.6 indicate that the configuration of the hydroxyl group in **28** is 12 α (see Fig. 7).

Microbial transformation of *ent*-12 β -hydroxykaurene (**28**) in *G. fujikuroi* was performed under conditions used for that of *ent*-15 α -hydroxykaurenoic acid except chlorocholine (CCC) was used as GA biosynthesis inhibitor. After 5 days, the acidic metabolites

FIG. 8. Gas Chromatogram of the Methyl Esters of the Acidic Metabolites from *ent*-12 β -Hydroxykaur-16-ene.

3% OV-1 on 100~120 mesh Gaschrom Q; N₂ 40 ml/min, 210°C.

were extracted, and their methyl esters were analyzed by GC-MS. As shown in Fig. 8, the main metabolites (peaks, 1, 2, 3, 6, and 7) were tentatively assigned 12 α -hydroxy GA₁₂ (**30**), 12 α -hydroxy GA₂₅ (**31**), *ent*-7 α ,12 β -dihy-

droxykaurenoic acid, *ent*-6 α ,7 α ,12 β -trihydroxykaurenoic acid and GA₃₉ (**32**) which has been found in immature seeds of *Cucurbitae pepo*.¹⁴⁾

Since *ent*-kaurenoic acid and *ent*-kaurene are reasonably available from the heads of sunflower¹⁵⁾ and the leaves of *Cryptomeria japonica*, respectively, the microbial transformations of their derivatives provide a practical route to inaccessible plant gibberellins and their analogues. Studies on the specific biological activities of GA₄₅ and related 15 β -hydroxyl GAs are now in progress.

EXPERIMENTAL

Melting points were determined with a Kofler hot-stage apparatus. IR spectra were recorded with a JASCO A-III spectrometer, ^1H NMR spectra with a JEOL JNM-MH-100 (100 MHz) spectrometer (tetramethylsilane as internal standard). Mass spectra and GC-MS were measured with a JEOL JMS-D100 spectrometer equipped with a JEOL JGC-20K gas chromatography. Gas chromatographic conditions were as follows: column, $1\text{ m} \times 2\text{ mm}$ glass column packed with 3% OV-1 on Gas Chrom Q; oven temperature, 230°C ; Carrier gas, He, ca. 40 ml/min. Methylation was carried out by treatment with ethereal diazomethane and trimethylsilylation was with TMS-HT reagent (Tokyo Kasei).

Optical rotations were measured with JEOL DIP-4 polarimeter. Silica gel 60 PF₂₅₄ (Merk) was used for analytical and preparative TLC. Mallinckrodt silica gel was used for column chromatography. HPLC were performed with Hitachi 635.

Synthesis of ent-15 α -hydroxykaurenoic acid (8).

Isomerization of ent-kaur-16-en-19-oic acid methyl ester (2). ent-Kaurenoic acid methyl ester (3.5 g), dissolved in glacial acetic acid (200 ml), was refluxed for 5 hr. The reaction mixture was then poured into water and extracted with ethyl acetate. The extract was washed with saturated aqueous sodium hydrogen carbonate, water and brine, dried over Na_2SO_4 , and evaporated to afford an inseparable mixture of the starting material and ent-kaur-15-en-19-oic acid methyl ester (3) (1:2.6). The ratio of 2 and 3 was determined by NMR and GC analysis. NMR (CDCl_3) δ : 0.80 (3H, s), 0.84 (3H, s), 1.01 (3H, s), 1.71 (2.2H, s), 4.74 (0.6H, bs) and 5.08 (0.7H, bs).

Photo-sensitized oxygenation and reduction of the mixture of 2 and 3. A mixture of 2 and 3 (3.2 g) and 45 mg of hematoporphyrin was dissolved in 42 ml of dry pyridine, and oxygen was passed through the solution under irradiation with fluorescent tubes (20W $\times$ 4). After 69 hr, the reaction mixture was concentrated *in vacuo* below 40°C . To the residue was added a solution of KI (18 g) in 140 ml of ethanol and 3 ml of acetic acid, and the mixture was kept at room temperature overnight. After decomposition of iodine with $\text{Na}_2\text{S}_2\text{O}_3$ solution, the solvent was evaporated to leave an oily residue, which was suspended in 300 ml of water, and extracted with ethyl acetate. The extract was washed with water and brine, and dried over Na_2SO_4 .

Removal of solvent gave an oil (3.3 g), which was then chromatographed on a column of silica gel (150 g) in *n*-hexane containing increasing amounts of ethyl acetate. Elution with ethyl acetate-*n*-hexane (2:8) gave two products, ent-15 β -hydroxykaurenoic acid methyl ester (4) (1.36 g) and ent-17-hydroxykaur-15-en-19-oic acid methyl ester (5) (507 mg).

4, mp $102\sim 103^\circ\text{C}$. $[\alpha]_{\text{D}}^{34}$, -114° ($c=0.39$, EtOH). MS

(m/z): 332 (M^+), 317 ($\text{M}^+ - \text{CH}_3$), 314 ($\text{M}^+ - \text{H}_2\text{O}$), 299, 273, 255, 239 and 207. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3300 and 1720. NMR (CDCl_3) δ : 0.86 (3H, s), 1.21 (3H, s), 2.75 (1H, m), 3.66 (3H, s), 3.83 (1H, bs), 5.10 (1H, s), and 5.23 (1H, s).

5, mp $114\sim 116^\circ\text{C}$. $[\alpha]_{\text{D}}^{30}$, -59.2° ($c=0.90$, CHCl_3). MS (m/z): 332 (M^+), 317 ($\text{M}^+ - \text{CH}_3$), 314 ($\text{M}^+ - \text{H}_2\text{O}$), 299, 273, 255, 239, and 207. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3300 and 1720. NMR (CDCl_3) δ : 0.96 (3H, s), 1.97 (3H, s), 2.51 (1H, m), 3.61 (3H, s), 4.16 (2H, s) and 5.32 (1H, bs). Anal. Found: C, 74.71; H, 9.57. Calcd for $\text{C}_{21}\text{H}_{32}\text{O}_3$: C, 75.85; H, 9.70.

Manganese dioxide oxidation of 4. Methyl ester (4) (1.0 g) dissolved in dry CH_2Cl_2 (50 ml) was stirred with active manganese dioxide (10 g) for 3 hr. The reaction mixture was then filtered and washed with CH_2Cl_2 , and the filtrate was evaporated under reduced pressure to give ent-15-oxokaurenoic acid methyl ester (6) (850 mg). Recrystallization from methanol gave 6 as colorless prisms, mp $148\sim 149^\circ\text{C}$. $[\alpha]_{\text{D}}^{34}$, -189° ($c=0.38$, EtOH). MS (m/z): 330 (M^+), 315 ($\text{M}^+ - \text{CH}_3$), 271, 270, 255 and 237. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 1720, 1640, 1160 and 940. NMR (CDCl_3) δ : 0.93 (3H, s), 1.21 (3H, s), 3.03 (1H, m), 3.65 (3H, s), 5.24 (1H, s) and 5.93 (1H, s). Anal. Found: C, 76.09; H, 9.11. Calcd for $\text{C}_{21}\text{H}_{30}\text{O}_3$: C, 76.31; H, 9.15.

Sodium borohydride reduction of ent-15-oxokaurenoic acid methyl ester (6). A solution of 6 (640 mg) in 60 ml of methanol was treated with 500 mg of sodium borohydride at -20°C for 3 hr. The reaction mixture was then poured into water and extracted with ethyl acetate. The extract was washed with brine, and dried over Na_2SO_4 . The solution, on evaporation, gave 612 mg of ent-15 α -hydroxykaurenoic acid methyl ester (7), which was recrystallized from methanol.

7, mp $169\sim 171^\circ\text{C}$. $[\alpha]_{\text{D}}^{30}$, -91.0 ($c=0.93$, CHCl_3). MS (m/z): 332 (M^+), 317 ($\text{M}^+ - \text{CH}_3$), 314 ($\text{M}^+ - \text{H}_2\text{O}$), 299, 274, 257, 255 and 239. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3535, 3080, 1700, 1660. NMR (CDCl_3) δ : 0.87 (3H, s), 1.19 (3H, s), 2.66 (1H, m), 3.66 (3H, s), 3.76 (1H, bs), 4.96 (1H, bd, $J=2.3\text{ Hz}$) and 5.10 (1H, bs). Anal. Found: C, 75.39; H, 9.63. Calcd for $\text{C}_{21}\text{H}_{32}\text{O}_3$: C, 75.85; H, 9.70, identical in optical rotation, and IR and NMR spectra with authentic material.⁷⁾

GC-MS analysis of this material showed it is a mixture of 7 and its dihydroderivative (96:4).

Saponification of ent-15 α -hydroxykaurenoic acid methyl ester (7). A solution of 7 (500 mg) and potassium hydroxide (7.5 g) in 50 ml of ethylene glycol was refluxed for 4.5 hr. The solution was then diluted with water (150 ml), washed with ether to remove neutral materials, acidified with cold conc. HCl, and extracted with ether. The ether extract was washed with brine and dried over Na_2SO_4 . The solution, on evaporation and recrystallization from aqueous methanol, gave ent-15 α -hydroxykaurenoic acid (8) as colorless prisms, mp $115\sim 117^\circ\text{C}$. $[\alpha]_{\text{D}}^{34}$, -94.8° ($c=0.66$, EtOH). MS (m/z): 318 (M^+), 303 ($\text{M}^+ - \text{CH}_3$),

300 ($M^+ - H_2O$), 285, 260, 257, 245, 243, 239 and 189. $IR \nu_{\max}^{KBr} \text{ cm}^{-1}$: 3400, 3070, and 1690. NMR ($CDCl_3$) δ : 1.00 (3H, s), 1.27 (3H, s), 2.66 (1H, m), 3.76 (1H, bs), 4.96 (1H, bs) and 5.10 (1H, bs). *Anal.* Found: C, 74.69; H, 9.45. Calcd. for $C_{20}H_{30}O_3$: C, 74.44; H, 9.50.

Microbial transformation of ent-15 α -hydroxykaurenoic acid (8). In a time course incubation, the artificial substrate, **8** (60 mg) and washed mycelium obtained from 200 ml of the preculture of *G. fujikuroi* were added to a Sakaguchi flask containing 110 ml of N-0% ICI medium³⁾ and 10 ml of 8% Tween 20, and incubated at 30°C on a reciprocal shaker. Small portions of the suspension (ca. 10 ml) were removed in the 1st, 3rd, 5th and 7th days and filtered, and their acidic metabolites were extracted with ethyl acetate.

After evaporation these extracts were methylated with diazomethane and subjected to GC-MS, which showed four main peaks corresponding to the methyl esters of A-I, A-III, A-IV and ent-7 α ,15 α -dihydroxykaurenoic acid (see Fig. 1).

In the large scale culture, 1.5 g of **8** was distributed between Sakaguchi flasks (25 $\times$ 500 ml). Wet mycelium of *G. fujikuroi*, resuspended in 110 ml of N-0% ICI medium and 10 ml of 8% Tween 20 containing 9 mg of 1-*n*-decylimidazole was added to each of the flasks. After 7 days incubation the culture filtrate was extracted with ethyl acetate (3 liters) at pH 2.0. The extract concentrated to ca. 500 ml was extracted with 5% $NaHCO_3$ twice. The aqueous solution was acidified to pH 2.0, and extracted with ethyl acetate (1 liter) twice. The ethyl acetate extract, on evaporation, gave 947 mg of gummy acidic material. The acidic metabolites was fractionated into 7 fractions with preparative TLC on silica gel developed with $CHCl_3$ –EtOAc–AcOH (10:10:1). Each fraction was then subjected to HPLC on Lichrosorb RP-18 (8 $\times$ 250 mm) with CH_3CN –MeOH–1% H_3PO_4 (40:10:50), and the eluates were monitored by RI and UV detectors. The eluate collected was neutralized to pH 6.0, and the solvent were evaporated. The residue was acidified to pH 2.0, and extracted with ethyl acetate.

From HPLC of the fraction II, A-I (105 mg), A-II (28 mg), A-III (46 mg), A-IV (104 mg) and A-V (16 mg) were obtained. HPLC of the other fraction (III to VII) gave the metabolites, A-VI (35 mg), A-VII (47 mg), A-VIII (2.9 mg) and A-IX (14 mg), as described in Fig. 2.

A-I, 15 β -Hydroxy GA₂₄(9). A-I was isolated as a powder, $[\alpha]_D^{32} -81^\circ$ ($c=0.40$, MeOH). Methyl ester, (Found: M^+ , 390.2040. $C_{22}H_{30}O_6$ requires 390.2042), $IR \nu_{\max}^{CHCl_3} \text{ cm}^{-1}$: 3530, 1720, 1715, 1660 and 910. NMR ($CDCl_3$) δ : 1.10 (3H, s, 18- H_3), 2.13 (1H, d, $J=13.2$ Hz, 5-H), 3.42 (1H, d, $J=3.7$ Hz, -OH, exchanged with D_2O), 3.63 (3H, s, CO_2CH_3), 3.79 (3H, s, CO_2CH_3), 3.96 (1H, d, $J=13.2$ Hz, 6-H), 3.97 (1H, bd, $J=3.7$ Hz, reduced to a sharp triplet, $J=1$ Hz, in addition of D_2O , 15-H), 5.04 and 5.14 (both bs, 17- H_2) and 9.63 (1H, s, -CHO). MS (m/z):

390 (M^+ , 6%), 362 (4), 358 (17), 344 (4), 340 (82), 330 (61), 328 (16), 312 (51), 302 (60), 298 (28), 284 (97), 270 (100), 242 (51) and 225 (58). MeTMSi derivative: MS (m/z): 462 (M^+ , 16%), 447 (36), 434 (17), 430 (26), 402 (79), 374 (40), 341 (28), 312 (59), 284 (100), 225 (52) and 156 (100).

A-II (10) was isolated as its methyl ester. Methyl ester of A-II, (Found: $M^+ - 28$ 364.2261. $C_{21}H_{32}O_5$ requires 364.2249), $IR \nu_{\max}^{CHCl_3} \text{ cm}^{-1}$: 3530, 1720 and 1715. NMR ($CDCl_3$) δ : 0.86 (3H, d, $J=7.3$ Hz, 17- H_3), 1.08 (3H, s, 18- H_3), 2.08 (1H, d, $J=12.7$ Hz, 5-H), 3.63 and 3.77 (both 3H, s, $2 \times CO_2CH_3$), 3.89 (1H, d, $J=12.9$ Hz, 6-H), and 9.63 (1H, s, -CHO). MS (m/z): 392 (M^+ , 2%), 364 (2), 360 (5), 346 (5), 342 (5), 332 (20), 328 (5), 314 (30), 304 (22), 301 (11), 300 (8), 286 (49), 271 (25), 255 (16), 245 (53), 227 (46), 178 (100) and 150 (37). MeTMSi derivative, MS (m/z): 464 (M^+ , 4%), 449 (20), 432 (13), 404 (22), 374 (20), 342 (93), 314 (67), 310 (27), 286 (100), 285 (82), 255 (38), 227 (42) and 130 (22).

A-III (11) was recrystallized from ethyl acetate. Colorless prisms, mp 204~205°C. $[\alpha]_D^{33} \pm 40.0$ ($c=0.21$, MeOH). Methyl ester (Found: M^+ 346.1786. $C_{20}H_{26}O_5$ requires 346.1780). $IR \nu_{\max}^{CHCl_3} \text{ cm}^{-1}$: 3450, 1765 and 1718. NMR ($CDCl_3$) δ : 1.08 (3H, s, 18- H_3), 2.45 (1H, d, $J=10.7$ Hz, 5-H), 2.75 (1H, d, $J=10.7$ Hz, 6-H), 3.76 (3H, s, CO_2CH_3), 3.93 (1H, b, 15-H), and 5.08 (2H, m, 17- H_2). MS (m/z): 346 (M^+ , 45%), 328 (1), 314 (100), 300 (7), 286 (23), 284 (20), 268 (20), 242 (35), 241 (30), 225 (28), 214 (27), 213 (27), and 185 (27). MeTMSi derivative: MS (m/z): 418 (M^+ , 100%), 403 (23), 386 (5), 375 (6), 372 (7), 358 (43), 329 (9), 328 (9), 313 (8), 296 (6), 284 (18), 269 (22), 241 (9), 225 (18), 223 (13) and 156 (64).

A-IV (12). Colorless prisms (from ethyl acetate), mp 265~267°C. $[\alpha]_D^{29} -66.7^\circ$ ($c=0.12$, MeOH). Methyl ester (Found: M^+ 360.1931. $C_{21}H_{28}O_5$ requires 360.1937). $IR \nu_{\max}^{CHCl_3} \text{ cm}^{-1}$: 3530, 1720, 1660 and 900. NMR ($CDCl_3$) δ : 1.11 (3H, s, 18- H_3), 2.11 (1H, d, $J=12.9$ Hz, 5-H), 2.81 (1H, d, $J=12.9$ Hz, 6-H), 3.26 (1H, d, $J=3.0$, -OH, exchanged with D_2O), 3.78 (3H, s, CO_2CH_3), 3.88 (1H, bd, $J=3$, reduced to a sharp triplet, $J=1$ in addition of D_2O , 15-H), 4.14 (1H, d, $J=12.2$) and 4.41 (1H, d, $J=12.2$) (20- H_2), and 5.05 (1H, bs) and 5.12 (1H, bs) (17- H_2). MS (m/z): 360 (M^+ , 3%), 342 (2), 329 (27), 328 (100), 314 (3), 310 (12), 300 (27), 282 (24), 271 (10), 255 (20), 254 (19), 243 (13), 242 (13), 241 (12), 225 (14), 175 (12) and 159 (21). MeTMSi derivative: MS (m/z): 432 (M^+ , 29%), 417 (24), 414 (1), 400 (18), 386 (4), 385 (5), 372 (16), 349 (9), 342 (5), 317 (4), 310 (27), 284 (14), 283 (14), 282 (19), 242 (13), 237 (15), 225 (13), 181 (13) and 156 (100).

A-V (13). Colorless prisms, mp 232~233°C. $[\alpha]_D^{33} -27.8$ ($c=0.09$, MeOH). Methyl ester (Found: M^+ 348.1952. $C_{20}H_{28}O_5$ requires 348.1936). $IR \nu_{\max}^{CHCl_3} \text{ cm}^{-1}$: 3550, 1765 and 1720. NMR ($CDCl_3$) δ : 0.85 (3H, d, $J=7.3$ Hz, 17- H_3), 1.07 (3H, s, 18- H_3), 2.39 (1H, d,

$J=10.7$ Hz, 5-H), 2.72 (1H, d, $J=10.7$ Hz, 6-H), 3.63 (1H, d, $J=10.5$ Hz, 15-H), and 3.74 (3H, s, CO_2CH_3). MS (m/z): 348 (M^+ , 10%), 330 (9), 316 (73), 304 (42), 302 (26), 300 (20), 289 (42), 271 (42), 257 (95), 245 (100), 243 (97), 230 (63), 185 (100) and 183 (63). *MeTMSi derivative*: MS (m/z): 420 (M^+ , 18%), 405 (31), 388 (17), 373 (5), 360 (10), 343 (10), 330 (79), 312 (11), 298 (60), 284 (57), 270 (69), 256 (59) and 130 (100).

Methyl ester of A-VI (14). High MS (Found: M^+ 420.2151. $\text{C}_{23}\text{H}_{32}\text{O}_7$ requires 420.2148). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3530 and 1722. NMR (CDCl_3) δ : 1.10 (3H, s, 18- H_3), 2.05 (1H, d, $J=12.9$ Hz, 5-H), 3.60 (3H, s, CO_2CH_3), 3.63 (3H, s, CO_2CH_3), 3.78 (3H, s, CO_2CH_3), 3.89 (1H, b, 15-H), 3.95 (1H, d, $J=12.7$ Hz, 6-H), 5.00 (1H, bd, $J=2.7$ Hz) and 5.10 (1H, bs) (17- H_2). MS (m/z): 420 (M^+ , 3%), 388 (16), 370 (3), 360 (20), 356 (8), 328 (100), 324 (5), 311 (13), 310 (12), 300 (75), 296 (10), 283 (22), 268 (49), and 240 (42). *MeTMSi derivative*: MS (m/z): 492 (M^+ , 10%), 473 (20), 460 (15), 432 (100), 400 (82), 372 (85), 342 (41), 313 (28), 311 (36), 310 (54), 283 (54), 282 (54), 250 (18), 223 (46), and 156 (100).

A-VII (15). Colorless prisms, mp 234~235°C. *Methyl ester* (Found: M^+ 364.2270. $\text{C}_{21}\text{H}_{32}\text{O}_5$ requires 364.2249). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3530, 1700, 1665 and 900. NMR (CDCl_3) δ : 0.87 (3H, s, 20- H_3), 1.41 (3H, s, 18- H_3), 1.88 (1H, d, $J=10.7$ Hz, 5-H), 3.69 (1H, d, $J=2.2$ Hz, 7-H), 3.74 (3H, s, CO_2CH_3), 4.23 (1H, dd, $J=10.7$, 2.4 Hz, 6-H), 4.33 (1H, d, $J=2.4$ Hz, 15-H), 4.97 (1H, bd, $J=2.9$ Hz) and 5.14 (1H, bs) (17- H_2).

Methyl ester of A-VIII (16). High MS (m/z): (Found: M^+ 362.1710. $\text{C}_{20}\text{H}_{26}\text{O}_6$ requires 362.1729). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3520, 1765 and 1720. NMR (CDCl_3) δ : 1.15 (3H, s, 18- H_3), 2.73 (1H, d, $J=11.2$ Hz, 6-H), 3.09 (1H, d, $J=11.0$ Hz, 5-H), 3.76 (3H, s, CO_2CH_3), 3.83 (1H, bs, 1 α -H or 3 α -H), 3.96 (1H, b, 15-H), and 5.11 (2H, bs, 17- H_2). MS (m/z): 362 (M^+ , 6%), 344 (5), 331 (25), 330 (100), 316 (5), 312 (7), 302 (7), 284 (20), 255 (15), 239 (13), and 148 (20). *MeTMSi derivative*: MS (m/z): 506 (M^+ , 6%), 491 (32), 474 (4), 446 (23), 416 (15), 384 (6), 377 (8), 372 (7), 356 (6), 287 (25), 282 (15), 223 (10), 156 (38) and 129 (11).

Methyl ester of A-IX (17): High MS (m/z): (Found: M^+ 436.2121. $\text{C}_{23}\text{H}_{32}\text{O}_8$ requires 436.2097). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3520 and 1720. NMR (CDCl_3) δ : 1.20 (3H, s, 18- H_3), 2.51 (1H, d, $J=12.9$ Hz, 5-H), 3.60 (3H, s, CO_2CH_3), 3.65 (3H, s, CO_2CH_3), 3.78 (3H, s, CO_2CH_3), 3.90 (1H, bs, 15-H), 3.96 (1H, d, $J=12.7$ Hz, 6-H), 5.02 (1H, bs) and 5.10 (1H, bs) (17- H_2). MS (m/z): 436 (M^+ , 12%), 404 (42), 386 (9), 376 (49), 374 (52), 372 (39), 358 (19), 344 (100), 326 (36), 316 (57), 298 (46), 284 (36), 270 (31), 239 (32), 176 (29) and 149 (39). *MeTMSi derivative*: MS (m/z): 586 (M^+ , 54%), 565 (63), 550 (39), 548 (27), 524 (36), 520 (93), 479 (37), 478 (51), 460 (44), 430 (67), 419 (20), 399 (41), 398 (32), 371 (41), 370 (42), 156 (100) and 129 (98).

Synthesis of ent-12 β -hydroxykaur-16-ene (28).

Hydroboration-oxidation of the acetate (22). The acetate (22) (860 mg), which was prepared from *ent*-kaurene (19) according to McAlees and McCrindle,¹⁶ was dissolved in 64 ml of diglyme and treated with 608 mg of sodium borohydride and 2.6 ml of borontrifluoride etherate for 2 hr. To the ice-cooled reaction mixture was added 6.4 ml of 10% NaOH in 30% aqueous ethanol and 12.8 ml of 30% H_2O_2 , and stirred at room temperature overnight. The reaction mixture was then poured into 400 ml of water, and extracted with 500 ml of ethyl acetate. The extract was washed with 250 ml of brine and dried over Na_2SO_4 .

The solution, on evaporation and recrystallization from ethanol, gave 603 mg of diol (23) as colorless needles, mp 222~224°C. MS (m/z): 306 (M^+), 291 ($\text{M}^+ - \text{CH}_3$), 288 ($\text{M}^+ - \text{H}_2\text{O}$), 273, 254, 203. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3280.

Monotosylation of the diol (23). 520 mg of diol (23) was treated with 17 ml of pyridine and 391 mg of tosyl chloride at 4°C for 20 hr. The reaction mixture was poured into water (400 ml), and extracted with 300 ml of ethyl acetate twice. The combined extracts were washed with 5% HCl, 5% NaHCO_3 and brine, and dried over Na_2SO_4 . The solution, on evaporation, gave 694 mg of an oily material, which was purified by preparative TLC with 40% ethyl acetate in *n*-hexane. Recrystallization from methanol and ethyl acetate gave 491 mg of monotosylate (24) as colorless prisms, mp 145~148°C. MS (m/z): 460 (M^+), 445 ($\text{M}^+ - \text{CH}_3$), 442 ($\text{M}^+ - \text{H}_2\text{O}$), 305, 289, 270, 255. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3500 and 1600. NMR (CDCl_3) δ : 0.79 (3H, s), 0.83 (3H, s), 1.14 (3H, s), 2.42 (3H, s), 3.72 (1H, m, 12-H), 3.98 (2H, d, $J=8$ Hz, 17- H_2), 7.24 (2H, d, $J=8$ Hz) and 7.70 (2H, d, $J=8$ Hz).

16 β -ent-12 β -Acetoxy-17-tosyloxy-kaurane (25). Acetylation of 24 (486 mg) with acetic anhydride (6 ml) and pyridine (6 ml) and usual work-up gave 600 mg of the acetate (25) as an oily material, MS (m/z): 444 ($\text{M}^+ - 60$), 429, 369, 367, 289, 272 and 204. IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 1735 and 1580. NMR (CDCl_3) δ : 0.80 (3H, s), 0.85 (3H, s), 1.05 (3H, s), 2.00 (3H, s, $\text{OCO}-\text{CH}_3$), 2.42 (3H, s), 4.01 (2H, d, $J=8$ Hz), 4.67 (1H, m, 12-H), 7.26 (2H, d, $J=8$ Hz) and 7.72 (2H, d, $J=8$ Hz).

16 β -ent-12 β -Acetoxy-17-phenylseleno-kaurane (26). To a solution of phenylselenide anions generated from sodium borohydride (175 mg) and diphenyldiselenide (713 mg) in 9 ml of dimethylformamide under argon at 0°C was added dropwise 591 mg of the acetate (25) in 4.5 ml of dimethylformamide.

The reaction mixture warmed to 40°C was stirred for 4 hr, and poured into ice-water (400 ml) and extracted with ethyl acetate. The extract was washed with brine (300 ml) and dried over Na_2SO_4 . Removal of the solvent, followed by preparative TLC with 20% ethyl acetate in *n*-hexane afforded 415 mg of phenylseleno compound (26) as

a colorless solid, MS (m/z): 488 (M^+), 428 ($M^+ - 60$), 351, 289, 271, and 230. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 1730 and 1600. NMR (CDCl_3) δ : 0.81 (3H, s), 0.85 (3H, s), 1.07 (3H, s), 2.02 (3H, s, OCO-CH₃), 2.96 (2H, t, $J=8$, 17-H₂), 4.92 (1H, m, 12-H), 7.17 (3H, m) and 7.41 (2H, m).

ent-12 β -Acetoxy-kaur-16-ene (**27**). To a solution of **26** (408 mg) in THF (20 ml) cooled to 0°C was added 30% H₂O₂ (1.2 ml). The reaction mixture was stirred for 30 min at 0°C, and kept at room temperature overnight. The mixture was poured into water (300 ml), and extracted with ethyl acetate (250 ml) twice. The extract was washed with brine (300 ml) and dried over Na₂SO₄. Preparative TLC of the crude material afforded 247 mg of the acetate (**27**) as a colorless solid. MS (m/z): 330 (M^+), 315 ($M^+ - \text{CH}_3$), 271, 270, 256 and 227. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 1735. NMR (CDCl_3) δ : 0.82 (3H, s), 0.86 (3H, s), 1.10 (3H, s), 2.02 (3H, s, OCO-CH₃), 2.71 (1H, m, 13-H), 4.68 (1H, m, 12-H), 4.77 (1H, bs, 17-H), and 4.90 (1H, bs, 17-H).

ent-12 β -Hydroxykaur-16-ene (**28**). The acetate (**27**) (242 mg) dissolved in 1% sodium hydroxide in methanol (25 ml) was stirred at 50°C for 3 hr. Usual work-up gave **28** as a colorless solid, $[\alpha]_{\text{D}}^{20} -0.4$ ($c=1.0$, CHCl₃). High MS (m/z) (Found: M^+ 288.2442. C₂₀H₃₂O requires 288.2453). MS (m/z): 288 (M^+), 273, 270, 255, 244, 231, 215 and 199. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3450. NMR (CDCl_3) δ : 0.83 (3H, s), 0.86 (3H, s), 1.20 (3H, s), 2.62 (1H, t, $J=4.4$ Hz, 13-H), 3.80 (1H, t, $J=4.7$ Hz, 12-H), 4.76 (1H, bs, 17-H), and 4.80 (1H, bs, 17-H).

ent-Kaur-16-en-12-one (**29**). To a stirred chromic acid-pyridine complex (30 mg of CrO₃ in 1 ml of pyridine) was added 10 mg of **28** in 0.2 ml of pyridine. The reaction mixture was stirred at room temperature for 5 hr.

Usual work-up gave 9.5 mg of the ketone (**29**) as colorless solid. NMR (CDCl_3) δ : 0.80 (3H, s), 0.87 (6H, s), 3.21 (1H, d, $J=4$ Hz, 13-H), 4.86 (1H, s, 17-H) and 4.98 (1H, s, 17-H).

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REFERENCES

- 1) M. Katsumi, "Gibberellins," ed. by S. Tamura, University of Tokyo Press, 1969, Chapter 6.
- 2) K. Wada, T. Imai and K. Shibata, *Agric. Biol. Chem.*, **43**, 1157 (1979).
- 3) J. R. Bearder, J. MacMillan and C. M. Wels and B. O. Phinney, *Phytochemistry*, **14**, 1741 (1975).
- 4) B. E. Cross and A. Erasmuson, *J. Chem. Soc., Chem. Commun.*, 1013 (1978).
- 5) N. Murofushi, S. Nagura and N. Takahashi, *Agric. Biol. Chem.*, **43**, 1159 (1979).
- 6) E. Fujita, T. Fujita and H. Katayama, *Tetrahedron*, **26**, 1009 (1970).
- 7) J. R. Cannon, P. W. Chow, P. R. Jefferies and G. V. Meehan, *Aust. J. Chem.*, **19**, 861 (1966).
- 8) N. Takahashi, in "Gibberellins," ed. by S. Tamura, University of Tokyo Press, 1969, pp. 150~158.
- 9) R. Binks, J. MacMillan and R. J. Pryce, *Phytochemistry*, **8**, 271 (1969).
- 10) J. R. Bearder, F. G. Dennis, Jr, J. MacMillan, G. C. Martin and B. O. Phinney, *T. Letters*, 669 (1975).
- 11) G. C. Martin, F. G. Dennis, Jr, P. Gaskin and J. MacMillan, *Phytochemistry*, **16**, 605 (1977).
- 12) J. MacMillan and R. H. Walker, *J. Chem. Soc., Perkin Trans. I*, 1272 (1972).
- 13) D. M. Harrison and J. MacMillan, *J. Chem. Soc. (C)*, 631 (1971).
- 14) H. Fukui, R. Nemori, K. Koshimizu and Y. Yamazaki, *Agric. Biol. Chem.*, **41**, 175 (1977).
- 15) J. St. Pyrek, *Tetrahedron*, **26**, 5029 (1970).
- 16) A. J. McAlees and R. McCrindle, *Can. J. Chem.*, **51**, 4103 (1973).