Tetrahedron Vol 24. pp 231 to 237 Pergamon Press. 1968 Printed in Great Britain

THE BIOSYNTHESIS OF THE GIBBERELLINS—II[1]

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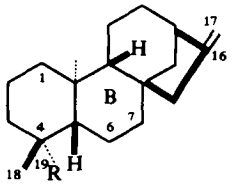
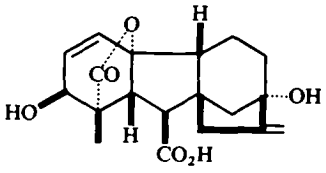
(*Received in the UK April* 1967; *accepted for publication* 17 *April* 1967)

Abstract—^{14}C-Labelled 7-hydroxykaurenolide (VI), the related triol (XI) and 7α-hydroxykaurenolide (X) were not incorporated into gibberellic acid by *Gibberella fujikuroi*, but 7-hydroxykaurenolide was transformed in high yield into 7,18-dihydroxykaurenolide (IX). In comparative studies the incorporation of kaur-16-en-19-ol (II) into gibberellic acid was much higher than that of kaur-6,16-dien-19-ol (XIV).

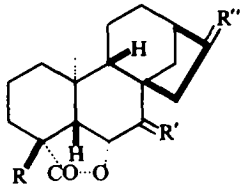
(−)-KAURENE (I) was shown by Cross *et al.*[1] to act as a precursor in the biosynthesis of gibberellic acid (V) by *Gibberella fujikuroi*. Subsequently it has been shown[2–5] that the related compounds kaur-16-en-19-ol (II), kaur-16-en-19-al (III) and kaur-16-en-19-oic acid (IV) are all incorporated into gibberellic acid by *G. fujikuroi*. The transformation of (−)-kaurene into gibberellic acid involves the contraction of ring B of (−)-kaurene with the extrusion of one carbon atom which ultimately forms the



I: R = Me
II: R = CH_2OH
III: R = CHO
IV: R = CO_2H



V



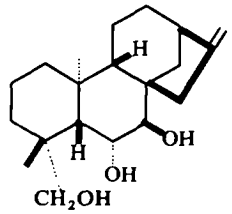
VI: R = Me, R′=  , R″ = CH_2

VII: R = Me, R′=  , R″ = O

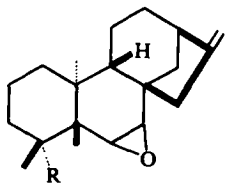
VIII: R = Me, R′=  , R″ = O

IX: R = CH_2OH, R′=  , R″ = CH_2

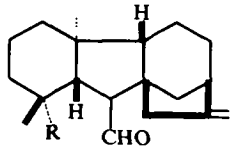
X: R = Me, R′=  , R″ = CH_2



XI



XII : R = CH_2OH, CHO or CO_2H



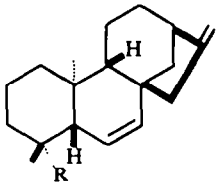
XIII: R = CH_2OH, CHO or CO_2H

free carboxyl group of gibberellic acid. However, there has been no investigation of the reaction sequence by which this ring contraction takes place. The first steps in this ring contraction must involve the oxygenation of ring B of (−)-kaurene at positions 6 and/or 7 and some speculations on the mechanism of the ring contraction have been advanced.[6]

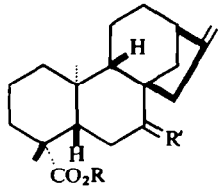
Inspection of the structure of 7-hydroxykaurenolide (VI),[7] which co-occurs[8] with (−)-kaurene, suggested[9] that it might be an intermediate in the biosynthesis of gibberellic acid. The α-oriented carbon atom attached to C-4 is in the form of a carboxyl group as required in the gibberellins, and ring B of 7-hydroxykaurenolide is at a suitable oxidation level for ring contraction, although both oxygen substituents have the axial orientation which is unfavourable mechanistically. [17-^{14}C]7-hydroxykaurenolide was prepared by reacting the acetate (VII) of 7-hydroxykaurenolide norketone with the Wittig reagent $^{14}CH_2{=}PPh_3$. The labelled compound was added to a fermentation of *G. fujikuroi* which was harvested 72 hr later and the products isolated in the usual manner.[8] The incorporation into gibberellic acid was only 0·1%, however the 7-hydroxykaurenolide had been hydroxylated in 43% yield to 7,18-dihydroxykaurenolide[10] (IX).* The failure of the fungus to utilize 7-hydroxykaurenolide as a precursor of gibberellic acid could be due to the inability of the substrate to reach the appropriate enzyme systems. Alternatively it might be due to factors such as the stereochemistry of the ring B substituents or to the lactone ring blocking further reaction at C-6. The latter was excluded when it was found that the labelled triol XI, prepared by LAH reduction of [17-^{14}C]7-hydroxykaurenolide, was not incorporated into gibberellic acid by *G. fujikuroi*. Ring contraction of a (−)-kaurene-6,7-diol would be expected to be more likely to occur if either or both of the OH groups were equatorial. Furthermore, it is known[6] that it is C-7 of (−)-kaurene which is extruded to form the free carboxyl group of gibberellic acid and this would be expected to take place if the 6-hydroxyl group was equatorial. However, no 6β-hydroxy derivatives have been prepared from 7-hydroxykaurenolide. On the other hand 7α-hydroxykaurenolide (X) is readily available[7] and it seemed worth testing as a precursor. [17-^{14}C]7α-Hydroxykaurenolide was prepared from [17-^{14}C]7-hydroxykaurenolide, but under the usual fermentation conditions no incorporation into gibberellic acid or gibberellin A_{13}[2] (XX) took place.

If, as seems likely, 7-hydroxykaurenolide is not a precursor of the gibberellins, then it is possible that the kaurenolides and the gibberellins are derived from a common intermediate. The epoxide XII might be such an intermediate, since it could either undergo ring contraction to the aldehyde XIII, or opening of the epoxide ring to give a diaxial 6,8-diol closely related to 7-hydroxykaurenolide. The epoxide XII is not readily accessible, but it is known that fungi which can introduce axial OH groups into saturated steroids are able to transform the unsaturated analogues into epoxides.[11] Consequently, the 6,16-dien-19-ol (XIV), which should serve as a precursor of the epoxide XII, was prepared in labelled form. [17-^{14}C]7-Hydroxykaurenolide was converted by the literature method[12] into the 7-keto-acid (XVI) which was reduced with sodium borohydride to the hydroxy-acid XVII. Attempts to form the toluene-*p*-sulphonate and methanesulphonate of this hydroxy-acid gave, in each case, a gum (ν_{max} 1780 cm^{-1}) as the major product. However, the methyl ester

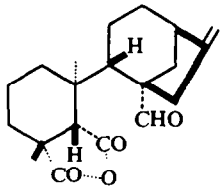
* For a preliminary account of this work see Ref. 9.



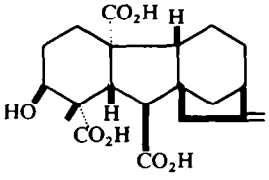
XIV: R = CH_2OH
XV: R = CO_2Me



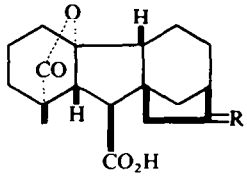
XVI: R = H, R′ = O
XVII: R = H, R′ = H, OH
XVIII: R = Me, R′ = H, OH



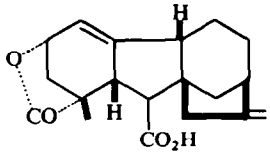
XIX



XX



XXI: R = CH_2
XXII: R = OH, Me



XXIII

XVIII readily gave a toluene-*p*-sulphonate which on refluxing in collidine gave the diene-ester XV. LAH reduction of the diene-ester yielded [17-^{14}C](−)-kaur-6,16-dien-19-ol (XIV). Comparison of the incorporation of the dienol with that of [17-^{14}C](−)-kaur-16-en-19-ol (II) under identical fermentation conditions showed that whereas the former was only incorporated into gibberellic acid to the extent of 0·48%, the incorporation of the latter was 4·9% (cf. Refs 2 and 3). In each case ozonolysis of the gibberellic acid in the form of its methyl ester showed that at least 97% of the radioactivity resided in the terminal methylene group. Further investigation of the metabolites from the dienol led to the isolation of [^{14}C]fujenal (XIX)[13] (1·2% incorporation) and [^{14}C]gibberellin A_{13} (XX)[2]. It is concluded that (−)-kaur-6,16-dien-19-ol, unlike (−)-kaur-16-en-19-ol, is not on the main biosynthetic pathway to gibberellic acid.

In Part I[1] it was reported that [^{14}C]gibberellin A_9 (XXI) was converted by *G. fujikuroi* into radioactive gums. These gums have now been shown, by TLC and autoradiography, to contain [^{14}C]gibberellin A_{10}[14] (XXII) (cf. Ref. 9) and the labelled 1 → 3-lactone[15] XXIII.

EXPERIMENTAL

M.ps were determined on a Kofler hot-stage apparatus. IR spectra were determined as Nujol mulls and NMR spectra were measured in $CDCl_3$ soln with a Varian Associates A-60 spectrometer and had TMS as internal standard. The following chromatographic materials were used: silica gel (M.F.C., Hopkin and Williams) and (Whatman Chromedia SG31), Celite 545 (Johns-Mandeville) and alumina (Woelm neutral alumina, grade II). Light petroleum refers to the fraction b.p. 60–80°.

TLC and autoradiography were carried out as previously described.[1]

Mould metabolites and their derivatives were identified by their IR spectra.

Radioactive assay. Unless otherwise stated, activities were measured in NE 220 liquid scintillator [Nuclear Enterprises (G.B.) Ltd.] on an I.D.L. tritium scintillation counter, Type 6012A. Incorporations of radioactivity were determined by counting a standard sample of [1-^{14}C]hexadecane with each batch of specimens. Determinations of activity were made in duplicate.

7β-Acetoxy-6α-hydroxy-16-oxo-17-norkauren-19-oic acid 19→6-lactone (VII)

Treatment of VIII with Ac_2O in pyridine at room temp gave the *acetate* which crystallized from EtOH–light petroleum in needles m.p. 241–242° (Found: C, 70·1; H, 8·0. $C_{21}H_{28}O_5$ requires C, 70·0; H, 7·8%).

Preparation of [17-^{14}C]*7-hydroxykaurenolide*

A suspension of [^{14}C]triphenylmethylphosphonium iodide (1·08 g; 0·5 Mc) in THF (20 ml) was stirred in an atm of N_2 and treated with n-Bu-Li (0·6N; 3·9 ml; 1 mole). The resultant orange soln was stirred for 30 min, then the acetate VII of 7- hydroxykaurenolide norketone (300 mg) in THF (15 ml) was added and a white ppt was immediately formed. Unlabelled triphenylmethylphosphonium iodide (30 mg) was added to remove any unreacted n-Bu-Li and the soln stirred for a further 15 min. The mixture was heated overnight at 70°, the solvent was removed *in vacuo*, and water was added. Recovery of the product in AcOEt gave a gum (540 mg) which was diluted with unlabelled 7-hydroxykaurenolide (245 mg) and chromatographed on alumina. Elution with 20% AcOEt in light petroleum gave a mixture of triphenylphosphine oxide and 7-hydroxykaurenolide, which, after further chromatography on alumina and crystallization from benzene–light petroleum, afforded [17-^{14}C]7-hydroxykaurenolide as needles, m.p. 185–188°, (290 mg; 45 μc, $7·08 \times 10^7$ counts/100 sec/mmol).

[17-^{14}C]*6,7,19-Trihydroxykaur-16-ene* (XI)

[17-^{14}C]7-Hydroxykaurenolide (130 mg; 4 μc) in ether (80 ml) was refluxed with LAH (300 mg) for 10 hr. AcOEt and NH_4Cl soln were added and the soln was concentrated *in vacuo*. Recovery with AcOEt gave a solid (130 mg) which crystallized from AcOEt–light petroleum in prisms (119 mg; 3·69 μc) of the triol (XI)[10], m.p. 205–208° ($1·83 \times 10^7$ counts/100 sec/mmol).

[17-^{14}C]*7α-Hydroxykaurenolide* (X)

[17-^{14}C]7α-Hydroxykaurenolide (340 mg; 1·9 μc), prepared from [17-^{14}C]7-hydroxykaurenolide by the literature method,[7] had m.p. 175–178°.

[17-^{14}C]*7α-Hydroxykaur-16-en-19-oic acid* (XVII)

[17-^{14}C]7-Hydroxykaurenolide (1·63 g; 20 μc) was converted[7] into [17-^{14}C]7-oxokaurenolide (1·61 g). The latter in THF (120 ml) was added dropwise to a soln of Ca metal (2 g) in liquid ammonia (200 ml) (cf. Ref. 12). The soln was stirred at room temp until all the ammonia had evaporated. Excess Ca was destroyed with NH_4Cl soln and the mixture concentrated *in vacuo*. The remaining soln was acidified with dil HCl at 0°, the product was taken up in AcOEt and then extracted into Na_2CO_3 soln. The Na_2CO_3 soln was acidified with conc HCl and the organic acid recovered in AcOEt. Chromatography on silica gel and elution with 12·5% AcOEt in light petroleum gave XVI as crystals (850 mg), m.p. 199–202°, which was used without further purification.

The keto-acid XVI (760 mg) in MeOH (70 ml) was reduced by adding $NaBH_4$ (1·8 g) portionwise at 0° during 2 hr and then leaving the soln at room temp overnight. The solvent was removed *in vacuo*, water was added and the product was recovered in AcOEt and chromatographed on silica gel. Elution with 20–25% AcOEt in light petroleum gave [17-^{14}C]*7α-hydroxykaur-16-en-19-oic acid* (XVII) which crystallized from EtOH–light petroleum in needles (424 mg), m.p. 248–249°. (Found: C, 75·6; H, 9·15. $C_{20}H_{30}O_3$ requires C, 75·4; H, 9·5%.) ν_{max} 3400, 1680, 1650 and 862 cm^{-1}.

The methyl ester, prepared with ethereal diazomethane, crystallized from light petroleum in prisms,

m.p. 149–151° (Lit.[16] m.p. 145–146°), τ = 9·17 (10-Me), 8·82 (4-Me), 6·61 (half-band width ~11 c/s, 7β-H), 6·35 (OMe) and 5·22 br (C=CH_2).

Reaction of 7α-hydroxykaur-16-en-19-oic acid (XVII) *with toluene-p-sulphonyl chloride*

The hydroxy acid (270 mg) in pyridine (2 ml) was treated with toluene-*p*-sulphonyl chloride (630 mg) at room temp for 90 hr. The product, isolated in the usual way, was a gum (375 mg) which showed an intense carbonyl band at 1780 cm^{-1}. Chromatography on silica gel and elution with 10% AcOEt in light petroleum gave the toluene-*p*-sulphonate as an oil (74 mg), ν_{max} ($CHCl_3$) 1695, 1656, 1600 and 1178 cm^{-1}.

Preparation of methyl [17-^{14}C]*kaur-6,16-dien-19-oate* (XV)

Compound XVIII (650 mg) in pyridine (4 ml) was treated with toluene-*p*-sulphonyl chloride (1·5 g) at room temp for 90 hr. The mixture was poured into iced dil H_2SO_4 and the toluene-*p*-sulphonate recovered in AcOEt as a gum (1·56 g), ν_{max} (thin film) 1720, 1658 and 1600 cm^{-1}, shown by TLC to be mainly one compound.

The gum was refluxed with collidine (20 ml) overnight, poured into iced dil H_2SO_4 (200 ml) and recovered in AcOEt. The resultant gum was chromatographed on alumina. Elution with 2·5% AcOEt in light petroleum gave XV as an oil (354 mg), ν_{max} (thin film) 1720, 1656, 896 and 690 cm^{-1}.

Treatment of the oily toluene-*p*-sulphonate of XVII with collidine in a similar manner, followed by chromatography and methylation of the product with diazomethane gave an oil identified as XV by its IR spectrum.

[17-^{14}C]*Kaur-6,16-dien-19-ol* (XIV)

The ester XV (350 mg) in dioxan (50 ml) was refluxed with a large excess of LAH for 60 hr. AcOEt was added and the solvents were removed *in vacuo*. Sodium potassium tartrate (20 g in 100 ml water) was added and the product was recovered in AcOEt and chromatographed on silica gel. Elution with 7·5% AcOEt in light petroleum gave [17-^{14}C]*kaur-6,16-dien-19-ol* (XIV) which crystallized from light petroleum in rosettes of needles (158 mg; 2·21 μc), m.p. 119–122° (Found: C, 83·4; H, 10·3. $C_{20}H_{30}O_3$ requires C, 83·8; H, 10·5%.) ν_{max} 3320, 1656, 1032 and 888 cm^{-1}, 8·47 × 10^6 counts/100 sec/mmol).

[17-^{14}C]*Kaur-16-en-19-ol* (II)

Compound XVI (200 mg) (from above) was reduced[12] to IV (194 mg). LAH reduction of the methyl ester of the latter gave II[2] (80 mg; 1·14 μc), m.p. 136–138° (8·90 × 10^6 counts/100 sec/mmol).

Addition of [17-^{14}C]*7-hydroxykaurenolide to a* Gibberella fujikuroi *fermentation*

The fermentation was carried out as described under "general fermentation conditions" (see below) except that a Dextrosol-ammonium nitrate medium was used. [17-^{14}C]7-hydroxykaurenolide (110 mg; 6·8 μc) in ethanol (20 ml) was added and the fermentation continued for a further 72 hr. The acidic and neutral metabolites were isolated and separated as previously described.[8] The gibberellic acid, purified by crystallization from AcOEt–light petroleum, possessed weak activity which could not be removed by repeated crystallization. It was methylated with diazomethane and the product chromatographed on alumina. Elution with 60% AcOEt in light petroleum gave methyl gibberellate (0·59 g; 0·009 μc; 0·1% incorporation). (Found*: r.m.a. × 10^{-4}, 0·13.)

The neutral fraction was chromatographed on celite:silica gel (2:1; 90 g). Elution with 25% AcOEt in light petroleum yielded 7-hydroxykaurenolide (30 mg; 2% of original activity). The fractions eluted with 35, 40 and 45% AcOEt in light petroleum contained 7,18-dihydroxykaurenolide which was crystallized from AcOEt–light petroleum to constant activity (80 mg; 2·9 μc; 43% incorporation) (Found*: r.m.a. × 10^{-4}, 261·4).

General fermentation conditions and isolation procedure

These were used for all labelled substrates except [17-^{14}C]7-hydroxykaurenolide which is described above.

Gibberella fujikuroi ACC 917 was grown on a Cerelose-ammonium tartrate medium (4–5 l) in stirred, aerated culture until the ammonium nitrogen was exhausted. The labelled substrate was then added in ethanol soln and the fermentation continued. At harvest, the mycelium was removed by filtration, the

* Counted as described in Ref. 1.

filtrate and washings were adjusted to pH ~2 with conc HCl, and then extracted with AcOEt (3 × 0·1 volume). The combined AcOEt extracts were extracted several times with $NaHCO_3$ soln and then washed with water. Evaporation of the AcOEt gave the crude neutral metabolites. The $NaHCO_3$ extracts were combined, acidified with dil HCl, and extracted with AcOEt. Concentration of these AcOEt extracts afforded crude crystalline gibberellic acid which was methylated with diazomethane and the methyl gibberellate purified.

Fermentations with ^{14}C-labelled substrates.

(a) [17-^{14}C]7α-*Hydroxykaurenolide.* The fermentation was continued for 112 hr after adding [17-^{14}C]7α-hydroxykaurenolide (340 mg; 1·9 μc). It gave crude gibberellic acid (230 mg) from which methyl gibberellate (needles from benzene–MeOH) (165 mg; 1470 counts/100 sec/mmol; 0·00% incorporation) was obtained.

Chromatography of the gibberellic acid mother liquors on silica gel and elution with 75% AcOEt in $CHCl_3$, gave a fraction which crystallized from AcOEt–light petroleum in prisms of gibberellic acid. The mother liquors deposited gibberellin A_{13}, m.p. 187–191° (decomp) (10 mg; 1940 counts/100 sec/mmol; 0·00% incorporation).

(b) [17-^{14}C]6α,7β,19-*Trihydroxykaurenolide.* The fermentation was run for 69 hr after adding XI (119 mg; 3·69 μc). The methyl gibberellate was purified by chromatography on alumina. Elution with 60–75% AcOEt in light petroleum followed by crystallization from benzene–MeOH gave methyl gibberellate (211 mg; 2210 counts/100 sec/mmol; 0·00% incorporation).

7-Hydroxykaurenolide (100 mg) was added to the neutral fraction from the fermentation which was then chromatographed on alumina. Elution with 20% AcOEt in light petroleum gave a fraction (120 mg) from which 7-hydroxykaurenolide (20 mg) was separated by preparative thick layer chromatography [0·5 mm layer of silica gel G (Merck) developed in benzene–EtOH (9:1)]. The 7-hydroxykaurenolide crystallized from acetone–light petroleum in prisms, m.p. 184–187° [radioactivity (calc. for 100 mg) = $1{\cdot}84 \times 10^{-3}$ μc; 0·05% incorporation].

(c) [17-^{14}C]*Kaur*-16-*en*-19-*ol* (II). The fermentation was run for 59 hr after adding the kaurenol (75 mg; 1·07 μc). The crude methyl gibberellate (94 mg) crystallized from benzene–MeOH in needles (88 mg; $5{\cdot}196 \times 10^{-2}$ μc; 4·9% incorporation).

(d) [17-^{14}C]*Kaur*-6,16-*dien*-19-*ol* (XIV). The dienol (145 mg; 2·03 μc) was fermented for 59 hr. Methyl gibberellate (needles from benzene–MeOH) (92 mg; $0{\cdot}981 \times 10^{-2}$ μc; 0·48% incorporation) was isolated.

Gibberellin A_{13} (49 mg) was added to the gibberellic acid mother liquors which were then chromatographed on silica gel. Elution with 20% AcOEt in light petroleum gave a fraction (61 mg) which crystallized from acetone–light petroleum in prisms of fujenal (XIX), m.p. 165–169° (42 mg; $2{\cdot}41 \times 10^{-2}$ μc; 1·19% incorporation). Elution with 90% AcOEt in light petroleum followed by crystallization of the fraction (116 mg) from AcOEt–light petroleum gave gibberellin A_{13} as prisms (40 mg). It was purified by methylation with diazomethane and chromatography on silica gel. Elution with 20% AcOEt in $CHCl_3$ gave a fraction which crystallized from acetone–light petroleum in needles of gibberellin A_{13} trimethyl ester, m.p. 114–117° [23 mg; $3{\cdot}79 \times 10^{-3}$ μc (calc. for 50 mg); 0·19% incorporation].

Ozonolyses of methyl [^{14}C]gibberellate

(a) *From the kaur*-16-*en*-19-*ol fermentation.* Ozonized oxygen (23 mg/min) was passed through methyl gibberellate (100 mg; 137,400 counts/100 sec/mmol) in AcOH (10 ml) at room temp for 5·5 min. Water (10 ml) was added, and the soln left to stand for 30 min and steam-distilled. The distillate (50 ml) was added to a soln of dimedone (300 mg) in water (100 ml) and left to stand at 5°. After 72 hr formaldehyde dimethone, m.p. 188–191° (17 mg; 133,600 counts/100 sec/mmol = 97% of the activity of methyl gibberellate), was collected.

(b) *From the kaur*-6,16-*dien*-19-*ol fermentation.* Ozonolysis of methyl gibberellate (100 mg; 54,300 counts/100 sec/mmol) as in (a) gave formaldehyde dimethone, m.p. 187–190° (15 mg; 53,600 counts/100 sec/mmol = 98·7% of the activity of the methyl gibberellate).

Transformation of gibberellin A_9 into gibberellin A_{10} by G. fujikuroi

The radioactive acidic gums (10, 26, 53, 57 and 49 mg respectively) reported in Part I[1] were examined by TLC in two solvent systems (di-isopropyl ether–AcOH, 95:5 and benzene–AcOH–water, 8:3:5) followed by autoradiography. The first three gums showed radioactive spots corresponding to gibberellin A_9 and the 1 → 3-lactone[15] (XXIII). In the fourth and fifth gums the main radioactive spot corresponded to gibberellin A_{10}[14] (XXII).

Acknowledgements—We are indebted to Mr. E. G. Jefferys [I.C.I. Ltd. (Pharmaceuticals Division)] for the fermentations, to the Chemical Society for a research grant, and to the Science Research Council for a Research Studentship (to K.N.).

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