Preparation of 13-Hydroxygibberellin A_{12}-7-Aldehyde

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7β- and 7α-Hydroxykaurenolides have been efficiently 13-hydroxylated by cultures of *Rhizopus arrhizus*. The products have been chemically converted into the title compound. In an alternative preparation, gibberellin A_{12}-7-aldehyde, [18-^{2}H]gibberellin A_{12}-7-aldehyde (prepared from 7β,18-dihydroxykaurenolide), and [6-^{3}H]gibberellin A_{12}-7-aldehyde (prepared, by hydrogen–tritium exchange, from gibberellin A_{12}-7-aldehyde), have been 13-hydroxylated by cultures of *Rhizopus arrhizus* to give the unlabelled and the [18-^{2}H]- and [6-^{3}H]-labelled title compound. 13-Hydroxylation of gibberellin A_{12}-7-alcohol by cultures of *Rhizopus arrhizus*, followed by oxidation, also gave 13-hydroxygibberellin A_{12}-7-aldehyde.

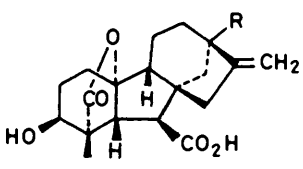
Many 13-hydroxylated gibberellins (GAs), for example GA_1 (1), occur in higher plants and in the fungus *Gibberella fujikuroi*. Present evidence [1] suggests that 13-hydroxylation occurs after ring contraction of *ent*-7α-hydroxykaur-16-en-19-oic acid (3) to GA_{12}-7-aldehyde (5). The conversion of GA_{12}-7-aldehyde (5) into GA_{53} (6) has been observed [2] in enzyme preparations from *Pisum sativum* seeds, and GA_{53} (6) has been shown to occur in seeds of both *Vicia faba* [3] and *Malus sylvestis*,[4] in shoots of *Spinacia oleracea*,[5] and in small tassels of *Zea mays*.[6] In these plants, therefore, the immediate precursor of GA_{53} (6) may be the hitherto unknown 13-hydroxyGA_{12}-7-aldehyde (9).

Working with cultures of *G. fujikuroi*, mutant B1–41a, Bearder *et al.*[7] found that the unnatural substrate steviol (4) was metabolised to GA_{53} (6) and GA_1 (1) but that GA_{53} (6), when refed to the cultures, was not converted into GA_1 (1). By analogy with the normal fungal pathway [8,9] to GAs, in which GA_{12}-7-aldehyde (5) is the precursor of GA_4 (2), Bearder *et al.*[7] suggested that 13-hydroxyGA_{12}-7-aldehyde (9), and not GA_{53} (6), was an intermediate between steviol (4) and GA_1 (1).

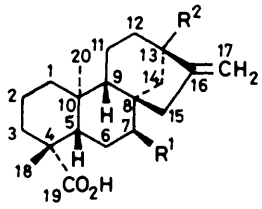
In the light of these facts, unlabelled, 18-deuterio-, and 6-tritio-13-hydroxyGA_{12}-7-aldehyde (9), (10), and (11) have been prepared by the routes shown in the Scheme. The role of this aldehyde in the biosynthesis of 13-hydroxyGAs will be described elsewhere.

Microbiological hydroxylation of 7-hydroxykaurenolides by cultures of *Rhizopus arrhizus* has been studied by Hanson *et al.*[10] These authors reported that the 7β-isomer [(12) and Scheme] gave 7β,13-dihydroxykaurenolide (13) and 7β,11α-dihydroxykaurenolide (20) in 4 and 3% yield, respectively. They also reported that the 7α-isomer (16) gave 7α,13-dihydroxykaurenolide (17) and the 7α,11α-derivative (21) in 20 and 10% yield with minor amounts of 7α,12β-dihydroxykaurenolide (22) and 16,17-dihydro-7α,16α,17-trihydroxykaurenolide (23). Initially, therefore, we reinvestigated the microbial 13-hydroxylation of 7-oxygenated kaurenolides with *Rhizopus* species, followed by the chemical conversion of the 13-hydroxylated products into the required 13-hydroxy-GA_{12}-7-aldehyde (9), using methods analogous to those previously used [11–13] for the preparation of GA_{12}-7-aldehyde (5) from 7-oxygenated kaurenolides.

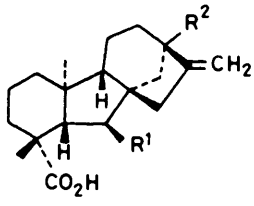
Using shake-cultures, two strains, DP1563 and Z 14a, of *Rhizopus nigricans* and one strain of *Rhizopus stolonifer* were evaluated. They gave qualitatively similar results but *Rh. nigricans*, DP1563, metabolised more substrate and was selected for further study. From the incubation of 7β-hydroxykaurenolide (12) in shake-flask cultures of *Rh. nigricans*, DP1563, the total product was shown by g.l.c.–mass spectrometric analysis to contain 7β,13- and 7β,11α-dihydroxykaurenolide (13) and (20), respectively, in the ratio 95 : 5. From larger scale shake-flask cultures, 7β,13-dihydroxykaurenolide (13) was isolated in *ca.* 35% yield. In the case of 7-oxokaurenolide (14), the total product from shake-flask incubations consisted of a 3 : 2 mixture of the 13-hydroxy derivative (15) and an unidentified trihydroxykaurenolide (by g.l.c.–mass spectrometric analysis) but the low solubility of the 7-ketone (14) precluded large-scale experiments. Metabolism of 7α-hydroxykaurenolide [(16) and Scheme] by *Rh. nigricans*,



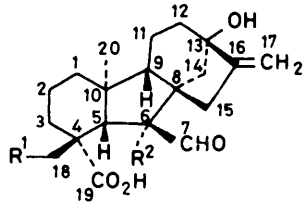
(1) R = OH
(2) R = H



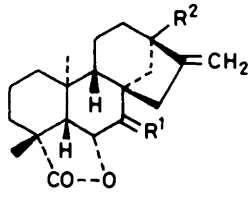
(3) R^1 = OH, R^2 = H
(4) R^1 = H, R^2 = OH



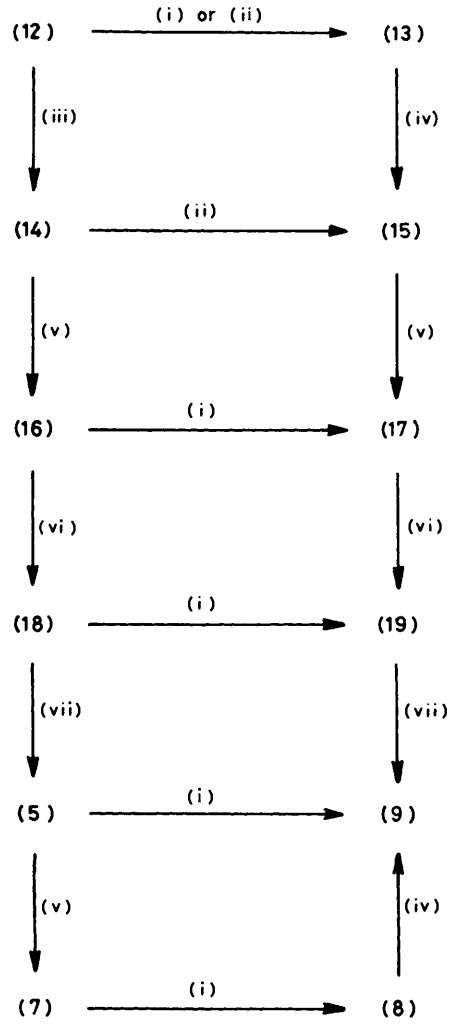
(5) R^1 = CHO, R^2 = H
(6) R^1 = CO_2H, R^2 = OH
(7) R^1 = CH_2OH, R^2 = H
(8) R^1 = CH_2OH, R^2 = OH



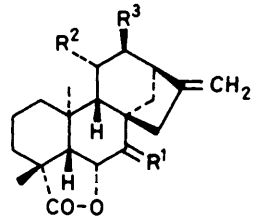
(9) R^1 = R^2 = ^{1}H
(10) R^1 = ^{2}H, R^2 = ^{1}H
(11) R^1 = ^{1}H, R^2 = ^{3}H



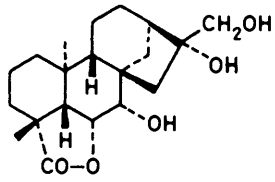
(12) R^1 = H, β-OH, R^2 = H
(13) R^1 = H, β-OH, R^2 = OH
(14) R^1 = O, R^2 = H
(15) R^1 = O, R^2 = OH
(16) R^1 = H, α-OH, R^2 = H
(17) R^1 = H, α-OH, R^2 = OH
(18) R^1 = H, α-$OSO_2C_6H_4Br$-*p*, R^2 = H
(19) R^1 = H, α-$OSO_2C_6H_4Br$-*p*, R^2 = OH



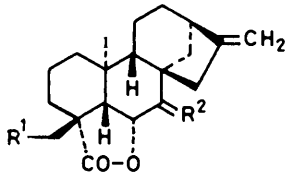
Scheme. Routes to 13-hydroxy GA_{12}-7-aldehyde (9). *Reagents:* (i) Cultures of *Rhizopus arrhizus*; (ii) cultures of *Rhizopus nigricans*; (iii) Jones reagent; (iv) CrO_3–pyridine–CH_2Cl_2; (v) $NaBH_4$ in tetrahydrofuran (THF)–ethanol; (vi) *p*-$BrC_6H_4SO_2Cl$–pyridine; (vii) KOH–Bu^{t}OH–H_2O



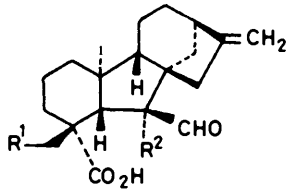
(20) R^1 = H, β–OH, R^2 = OH, R^3 = H
(21) R^1 = H, α–OH, R^2 = OH, R^3 = H
(22) R^1 = H, α–OH, R^2 = H, R^3 = OH



(23)



(24) R^1 = OH, R^2 = H, β–OH
(25) R^1 = Cl, R^2 = H, β–OH
(26) R^1 = ^{2}H, R^2 = H, β–OH
(27) R^1 = ^{2}H, R^2 = O
(28) R^1 = ^{2}H, R^2 = H, α–OH
(29) R^1 = ^{2}H, R^2 = H, α–$OSO_2C_6H_4Br$-*p*



(30) R^1 = ^{2}H, R^2 = ^{1}H
(31) R^1 = ^{1}H, R^2 = ^{3}H

strain DP1563, gave a complex mixture of products, the main components of which were 7α,13-, 7α,11α-, and 7α,12β-dihydroxykaurenolide [(17), Scheme], (21), and (22), respectively.

In the course of these and subsequent studies, the product ratio from the incubation of 7β-hydroxykaurenolide (12) in cultures of *Rh. nigricans*, DP1563, changed. Although the desired 7β,13-dihydroxy compound (13) was still the major product the yield of 7β,11α-dihydroxykaurenolide (21) increased to 40%. Fortunately, a strain of *Rh. arrhizus*, kindly provided by Dr. J. R. Hanson, reproduced the high yield of 7β,13-hydroxykaurenolide (13) originally obtained with strain DP1563. A further useful property of the *Rh. arrhizus* strain was that it metabolised 7α-hydroxykaurenolide (16) to 7α,13-dihydroxykaurenolide (17) in yields of 50—55%, the 7α,11α-isomer (21) being formed only in trace amounts.

Another method of preparing 7β,13-dihydroxykaurenolide (13) was also investigated. Bearder *et al.*[7] reported that steviol (4) was converted into 7β,13-dihydroxykaurenolide (13) in 30% yield (g.l.c.) by cultures of *Gibberella fujikuroi* mutant B1–41a. However, in repeating this work, we could only achieve yields of 7% (g.l.c.).

Conditions for large-scale conversion of the 7β- and 7α-hydroxykaurenolides (12) and (16) into their 13-hydroxy derivatives (13) and (17) by *Rh. nigricans* DP1563 and *Rh. arrhizus* were systematically studied. Corn steep liquor, used by Blunt *et al.*[14] in the growth medium for *Rh. nigricans*, strain DP1563, could not be replaced by Marmite ®, Bovril ®, or Boots malt extract. Canonica *et al.*[15] have reported that lysed cells, but not whole cells, of *Rh. nigricans*, *Rh. arrhizus*, and *Curvularia lunata* metabolise ecdysones. However, when the mycelium of *Rh. nigricans* was resuspended in water, lysis did not occur and the product distribution from added 7β-hydroxykaurenolide (12) was the same as in non-resuspended cultures. Nevertheless, resuspension cultures were used in subsequent work since the products were cleaner.

For preparative-scale experiments using shake-cultures, it was found that 40 ml of medium, containing 10 mg of substrate, in 250-ml conical flasks was a convenient compromise. Under these conditions the mycelium grew in a single clump. With larger volumes of medium in larger flasks the growth form was a mixture of clumps and dispersed mycelial strands. Clump growth was convenient for resuspension in flasks. However, it had disadvantages for stirred cultures where growth occurred mainly on the stirrer and baffle plates even at high stirring rates. However, the mycelium grew, and adhered to, cheese cloth tied round the baffle plates; when this happened the oxygen electrode was coated with mycelium and did not function and the temperature probe was insulated by the adhering mycelium so that the control of temperature oscillated in 5 or 6 °C cycles.

In 10-l stirred cultures of *Rh. arrhizus* the yield of 7α,13-dihydroxykaurenolide (17) from 7α-hydroxykaurenolide (16) was lower than in preparative shake-cultures. In stirred

culture pure 7β-hydroxykaurenolide (12) also gave lower yields of the 13-hydroxy compound (13) because of the low solubility of pure (12); however, an oily sample, obtained from a commercial fermentation of *Gibberella fujikuroi*, gave better yields. Contamination during inoculation and resuspension of the 10-l stirred cultures occurred frequently but their capacity to metabolise up to 2 g of substrate was an overriding advantage.

The chemical preparation of 13-hydroxyGA_{12}-7-aldehyde (9) from the 13-hydroxykaurenolides (13) and (17) was accomplished as shown in the Scheme.

Initially, the direct 13-hydroxylation of GA_{12}-7-aldehyde (5) by *Rhizopus* species had not been considered since Hatton [16] had shown that compound (5) was metabolised by *Rh. nigricans*, strain Z 14a, giving many compounds. However, one of the products (10% by g.l.c.) was tentatively identified as 13-hydroxyGA_{12}-7-aldehyde (9). A re-examination of the microbiological transformation of GA_{12}-7-aldehyde (5) by resuspended shake-cultures of *Rh. arrhizus* on an analytical scale indicated that 13-hydroxyGA_{12}-7-aldehyde (9) was the major product (g.l.c.–mass spectrometry). Accordingly, GA_{12}-7-aldehyde (5), prepared [13] in 50% yield from 7α-hydroxykaurenolide (16) *via* the monobrosylate * (18), was fed to a 10-l resuspension culture of *Rh. arrhizus*. Despite contamination of the culture during resuspension and the consequent curtailment of the period of incubation, 13-hydroxyGA_{12}-7-aldehyde (9) was isolated in 57% yield, together with unmetabolised substrate (5) (27% recovery).

Gibberellin A_{12}-7-alcohol (7) [13,17] was also 13-hydroxylated by resuspended stirred cultures of *Rh. arrhizus* and the product (8) was oxidised by Collins' reagent to 13-hydroxyGA_{12}-7-aldehyde (9) (see Scheme).

Thus all the routes, shown in the Scheme, from 7β-hydroxykaurenolide (13) to 13-hydroxyGA_{12}-7-aldehyde (9) were realised. The best route is probably the chemical preparation of GA_{12}-7-aldehyde (5) from 7β-hydroxykaurenolide (12), followed by its 13-hydroxylation in cultures of *Rh. arrhizus*. This methodology was used to prepare the [18-^{2}H]- and [6-^{3}H]-13-hydroxyGA_{12}-7-aldehydes (10) and (11) as follows.

For the [18-^{2}H]-labelled aldehyde (10) the starting material was 7β,18-dihydroxykaurenolide (24) which was converted into the 18-chloro derivative (25) [18] by treatment with carbon tetrachloride and triphenylphosphine. Reduction of the chloro compound (25) with tri-n-butyl[^{2}H]stannane gave the [18-^{2}H] compound (26) which was converted into [18-^{2}H]-GA_{12}-7-aldehyde (30) *via* the 7-ketone (27), the 7α-alcohol (28), and the brosylate (29). The resultant [18-^{2}H]GA_{12}-7-aldehyde (30) was converted by resuspended shake cultures of *Rh. arrhizus* into 13-hydroxy[18-^{2}H]GA_{12}-7-aldehyde (10) containing 0.75 atom % deuterium.

[6-^{3}H]Gibberellin A_{12}-7-aldehyde (31) (prepared as described by Bearder *et al.*[19]) with specific radioactivity 7.0 mCi mmol^{-1} was converted into 13-hydroxy[6-^{3}H]GA_{12}-7-aldehyde (11) with specific radioactivity 5.9 mCi mmol^{-1}.

Experimental

For general experimental details see ref. 20. Light petroleum refers to that fraction boiling in the range 60—80 °C.

Fungal Cultures.—*Rhizopus nigricans* DP1563, originally obtained from Centralbureau voor Schimmelcultures, Baarn, as *Rh. stolonifer* (Ehrenb. *ex* Fr.) Lind. (Baarn Culture No. 382.52), was supplied by Professor Sir Ewart Jones, Oxford University. *Rhizopus nigricans* Z14a was provided by Dr. M. F. Madelin, University of Bristol. Cultures of *Rhizopus arrhizus* and of *Rh. stolonifer*, of unknown provenance, were a gift from Dr. J. R. Hanson, University of Sussex. These cultures were maintained on 2% agar slopes containing Boots malt extract (5%) at 4 °C.

Conditions for Shake-cultures.—In exploratory experiments the following parameters were systematically varied: crude extracts; volume of medium and volume of flask; length of culture; effect on mycelial growth of 0.2—10% dimethyl sulphoxide (DMSO); concentration of substrate; and resuspension *versus* non-resuspension. The optimum conditions were as follows. Medium B, described by Blunt *et al.*,[14] was adjusted to pH 5.5 with 1M-potassium hydroxide. Sucrose (2 g l^{-1}) was then added and 40 ml of the mixture was dispensed into 250-ml conical flasks which were then sterilised in steam at 120 °C for 20 min. Each flask was inoculated with a small piece of mycelium from the maintenance slopes and grown for 3 d at 25 °C on an orbital shaker. The medium was decanted; the mycelium was washed twice with sterile water (50 ml each) and then resuspended in sterile water (40 ml). Substrate (10 mg) was added in the minimum (usually 100 μl) of acetone or DMSO and the culture was shaken for 5 d at 25 °C.

Conditions for Stirred Cultures.—Cheese cloth was fixed to the baffles of a 20-l fermenter (L and H Engineering Co. Ltd.) with nylon string so that it extended around one-third of the circumference and the baffle was then immersed in 10 l of the following medium: malt extract (20 g, Difco), yeast extract (20 g, Difco), beef extract (20 g, Oxoid), glucose (100 g), sucrose (20 g), corn steep liquor (40 ml), inositol (100 mg), ammonium nitrate (2 g), potassium dihydrogen phosphate (1 g), dipotassium hydrogen phosphate (1 g), 1M-potassium hydroxide (20 ml), and minor-elements solution [21] (30 ml), made up to 10 l with glass-distilled water. The fermenter and medium were autoclaved in steam at 120 °C for 1 h, then inoculated in one of the following ways: (*a*) mycelium from the maintenance slope was placed in the barrel of a dry sterile syringe and transferred to the medium by inserting the needle through a septum on the base of the fermenter, then withdrawing medium, followed by injection of mycelium plus medium; (*b*) a sterilised plastic syringe, modified to take a length of rubber tubing in place of the needle, was used to transfer 10—15 ml of a three-day old shake-culture to the fermenter through an inlet tube on top of the fermenter. For both methods transfer of inoculum was performed in a sterile cabinet and all possible contamination points were washed with methanol. Method (*b*) was the more susceptible to contamination but reduced the culture time by 1 d.

The culture was stirred at 200—250 r.p.m. at 28 °C and aerated at 2 l min^{-1}. When the pH dropped from the initial value of 7.5—8.0 (5 or 6 d) the medium was drained off and replaced by sterile, glass-distilled water (10 l). The substrate (1.0—2.0 g) in acetone or DMSO (10—20 ml) was added and culture was continued for the specified time.

Extraction of Cultures.—The culture filtrates were extracted with an equal volume of ethyl acetate (× 3 for shake cultures and × 2 for stirred cultures) and the ethyl acetate extracts were evaporated on a rotary evaporator. In the case of feeds of GA_{12}-7-aldehyde (5) and GA_{12}-7-alcohol (7) the pH of the culture filtrates were adjusted to pH 2 before extraction.

Shake-culture Incubations with Rhizopus nigricans, *Strain DP1563.*—(a) 7β-*Hydroxykaurenolide* (12). The total product (107 mg) from the incubation of the substrate (10 mg) in each of ten 40 ml resuspension cultures was separated by p.l.c. on

* *p*-Bromobenzenesulphonate.

a silica-gel layer (0.8 mm) with ethyl acetate–light petroleum–acetic acid (70 : 30 : 1) as eluant. The band at R_F 0.35 yielded 7β,13-dihydroxykaurenolide (13) as needles (40 mg), m.p. 261—263 °C (lit.,[22] 261—263 °C) (from acetone–light petroleum) (Found: C, 72.5; H, 8.9. Calc. for $C_{20}H_{28}O_4$: C, 72.3; H, 8.5%), identified by n.m.r. and mass spectrometry. Elution of the band at R_F 0.5 gave traces of 7β,11α-dihydroxykaurenolide (20), identified by g.l.c.–mass spectrometric comparison with an authentic specimen.[23]

The above experiment was typical of the early incubations in which the ratio of 13- to 11α-hydroxylation was 95 : 5. Over several months this ratio changed to 55 : 45 with poor recovery at each product.

When shake-cultures of *Rh. nigricans*, strain Z 14a, were used, g.l.c. of the crude extract, before and after trimethylsilylation, showed that only 50% of the substrate was metabolised [to a 2 : 1 ratio of compounds (13) and (20)]. Even less conversion of the substrate (12) occurred with *Rh. stolonifer*.

(b) *7-Oxokaurenolide* (14). The substrate (14) (5 mg) was added to each of two 40-ml cultures of strain DP1563. After incubation for 5 d the crude product was methylated and trimethylsilylated. G.l.c.–mass spectrometric analysis indicated that 90% of the substrate had been metabolised to a 3 : 2 mixture of 13-hydroxy-7-oxokaurenolide (15) (see later for characterisation) and unidentified dihydroxylated products.

(c) *7α-Hydroxykaurenolide* (16). The substrate (16) (10 mg) in each of two 40-ml cultures of strain DP1563 was incubated for 5 d. The total product was analysed by g.l.c.–mass spectrometry after methylation and trimethylsilylation, and was shown to consist of a mixture of four major and two minor products. The major products were identified from their mass spectra as the 7α,11α-, 7α,12β-, and 7α,13-dihydroxykaurenolide (21), (22), and (17) and probably 7α,16α,17-trihydroxykaurenolide (23).

Incubations with Shake-cultures of Rhizopus arrhizus.—(a) *7β-Hydroxykaurenolide* (12). This substrate was metabolised in a manner identical with that described for the original experiments using shake-cultures of *Rhizopus nigricans* DP1563.

(b) *7α-Hydroxykaurenolide* (16). The following results are typical. The substrate (10 mg) in acetone (100 μl) was added to each of 45 conical flasks containing 40 ml of resuspension culture. After 5 d the crude product was recovered from the culture filtrate, adsorbed on silica gel (6 g) from acetone solution, and placed on a column (30 × 3 cm) of silica gel, made up in light petroleum. Elution with increasing amounts of ethyl acetate in light petroleum gave 7α,11α-dihydroxykaurenolide (21) (97 mg), identified by g.l.c.–mass spectrometry. Further elution gave a gum (368 mg) from the 60% ethyl acetate and later fractions. Crystallisation of the gum from acetone–light petroleum gave 7α,13-dihydroxykaurenolide (17) (245 mg), m.p. 220—221 °C (lit.,[10] 220—222 °C) (Found: C, 72.1; H, 8.8. Calc. for $C_{20}H_{28}O_4$: C, 72.3; H, 8.5%); δ-values as in ref. 10; *m/z* 332 (M^+, 100%), 152 (45), 137 (43), 122 (24), 121 (21), and 109 (89); *m/z* for bis-trimethylsilyl ether: 476 (M^+, 82%), 267 (22), 208 (47), 207 (14), 109 (40), and 73 (100).

(c) [18-^{2}H]*Gibberellin A_{12}-7-aldehyde* (30). The substrate (26.5 mg) in acetone (0.5 ml) was added in five equal portions to five 50 ml shake-flask resuspension cultures. After incubation for 3 d, the product was purified by flash chromatography using 30% ethyl acetate in light petroleum as eluant to give 13-hydroxy[18-^{2}H]gibberellin A_{12}-7-aldehyde (10) as a gum (8 mg) [Found (for Me ester $SiMe_3$ ether): M^+ 419.2564. $C_{24}{}^1H_{37}{}^2H_1O_4Si$ requires *M*, 419.2604], containing 0.75 atoms of deuterium per molecule by mass spectroscopic determination on the M^+ cluster; ^{2}H n.m.r. δ 1.13; ^{1}H n.m.r. (60 MHz) δ 0.79 (s, 20-H_3), 1.13 (br s, 18-H_2), 3.26 (dd, *J* 5 and 12 Hz, 6-H), 4.85 and 5.12 (each br s, together 17-H_2), and 9.66 (d, *J* 5 Hz, 7-H); *m/z* 333 (M^+, 10%), 332 (11), 331 (27), 330 (12), 305 (22), 304 (35), 303 (38), 302 (22), 287 (81), 286 (35), 258 (93), 257 (43), 256 (35), 136 (43), 129 (36), 121 (65), 110 (40), 109 (32), 101 (47), 95 (33), 91 (38), 73 (70), 71 (38), 69 (48), 60 (68), 59 (100), and 41 (80); *m/z* (Me ester $SiMe_3$ ether) 419 (M^+, 17%), 418 (5), 391 (60), 390 (45), 235 (29), 221 (28), 208 (76), 207 (57), 193 (100), 180 (26), and 73 (72).

(d) [6-^{3}H]*Gibberellin A_{12}-7-aldehyde* (31). The substrate (21.5 mg, 7.0 mCi mmol^{-1}), prepared as described by Bearder *et al.*,[19] was added in acetone (0.4 ml) to four 50 μl resuspension cultures. After 3 d, the product was recovered in the usual way. Purification by preparative t.l.c. on silica gel with ethyl acetate–light petroleum–acetic acid (50 : 50 : 1) as developer gave 13-hydroxy[6-^{3}H]gibberellin A_{12}-7-aldehyde (11) as a gum (5 mg, 5.9 mCi mmol^{-1}).

Stirred Culture Incubations with Rhizopus arrhizus.—(a) *7β-Hydroxykaurenolide* (12). To a resuspended 6-d old stirred culture (13 l) was added 7β-hydroxykaurenolide (2 g, oily material described later) in acetone (20 ml). After 7 d, the crude product (1.5 g) was extracted from the medium and chromatographed on a column (20 × 2.5 cm) of silica gel. Elution with 60—80% ethyl acetate in light petroleum gave 7β,13-dihydroxykaurenolide (13) (750 mg), identified by spectroscopic data (see earlier). In this culture, metabolism of the substrate was almost complete (>95%). In several cultures, metabolism of substrate varied from 0% (contaminated cultures) to over 95%; yields of crude product varied from 69 to 87%; and yields of isolated 7β,13-hydroxykaurenolide (13) varied from 17 to 50% of the crude product. Pure substrate gave lower yields, owing to its low solubility, than did the oily crystalline material.

(b) *7α-Hydroxykaurenolide* (16). The substrate (2.0 g) in acetone (20 ml) was added to a 5-d old culture and the mixture was resuspended in distilled water (10 l) and incubated for 6 d. The crude extract (2.3 g) was chromatographed on a column (30 × 2.5 cm) of silica gel. Elution with 50—60% ethyl acetate in light petroleum gave a gum (906 mg) which was crystallised from acetone–light petroleum to give 7α,13-dihydroxykaurenolide (17) (550 mg, 26%) as prisms, identified by m.p. and spectroscopic data. In two similar experiments in one of which the mycelium was not resuspended, and in the other the substrate was added in DMSO, the yields were *ca.* 25%.

(c) *Gibberellin A_{12}-7-aldehyde* (5). The substrate (1.1 g) in acetone (15 ml) was added to a 6-d old resuspended culture (10 l). After 1 d the culture was heavily contaminated and after 2 d it was worked up. Extraction of the culture filtrate at pH 2.0 with ethyl acetate gave an emulsion which was filtered through Celite. The crude product (2.8 g), recovered from the ethyl acetate extract, was chromatographed on a column (24 × 4 cm) of silica gel, made up in light petroleum, and was eluted with increasing amounts of ethyl acetate in light petroleum. The solvents were de-aerated immediately before use. Elution with 50—60% ethyl acetate gave *13-hydroxygibberellin A_{12}-7-aldehyde* (9) as a gum (663 mg) which was pure by g.l.c. and n.m.r. but which was intractable (Found: M^+ 332.199. $C_{20}H_{28}O_4$ requires *M*, 332.199); $\nu_{max.}$ 3 582, 3 450, 2 960, and 1 705 cm^{-1}; δ 0.80 (s, 20-H_3), 1.18 (s, 18-H_3), 3.30 (dd, *J* 5 and 12 Hz, 6-H), 4.92 (br, 17-H), 5.18 (br, 17-H), and 9.72 (d, *J* 5 Hz, 7-H); *m/z* 332 (M^+, 20%), 304 (12), 303 (26), 286 (82), and 257 (100); *m/z* (Me ester $SiMe_3$ ether) 418 (M^+, 21), 208 (64), 207 (42), and 193 (100).

Attempts to crystallise the gum led to oxidation to gibberellin A_{53} (6). A portion of the gum (40 mg) was fractionated by h.p.l.c. on C_{18}–silica gel using a potassium hydrogen

phosphate–potassium dihydrogen phosphate buffer (pH 3.0) and methanol (9 : 1) as eluant; the resultant gum was crystallised with difficulty from acetone–light petroleum to give a few prisms, m.p. 235—238 °C.

ent-6β,7α-*Dihydroxykaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (7β-*Hydroxykaurenolide*) (12).—This compound, m.p. 186—187 °C (lit.,[24] 186—187 °C), was isolated from the neutral extract of a large-scale fermentation of *Gibberella fujikuroi*, strain TP70, as described by Hedden *et al.*,[23] and was identified by its i.r.[11] and n.m.r.[23] spectra. As well as pure crystalline lactone (12), some fractions from the chromatography column produced a semi-crystalline gum, which contained essentially pure lactone (12), in an oil which was probably derived from the non-synthetic culture medium. This oily material was used in some shake-flask and stirred fermentations.

ent-6β-*Hydroxy*-7-*oxokaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (7-*Oxokaurenolide*) (14).—This compound, m.p. 270—271 °C (lit.[11] 264—265 °C), was prepared by literature methods [11] and identified by its spectroscopic properties.[11,25]

ent-6β,7β-*Dihydroxykaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (7α-*Hydroxykaurenolide*) (16).—This compound, m.p. 179—180 °C (lit.,[11] 178—179 °C), was prepared by literature methods [11] and identified by its spectroscopic properties.[11,25]

13-*Hydroxy*-7-*oxokaurenolide* (15).—7β,13-Dihydroxykaurenolide (13) (50 mg) in the minimum volume of dichloromethane was added to chromium trioxide (100 mg) in dichloromethane (3 ml) containing pyridine (150 μl). After being stirred for 20 min at 18 °C the reaction mixture was diluted with ethyl acetate (2 vol) and was filtered through a short column of Celite. The product, recovered from ethyl acetate, was purified by preparative t.l.c. to give the required *ketone* (15) m.p. 235—239 °C (from acetone–light petroleum) (Found: M^+, 330.184. $C_{20}H_{26}O_4$ requires M, 330.183); δ 0.72 (s, 20-H_3), 1.32 (s, 18-H_3), 4.87 (d, J 7 Hz, 6-H), 4.92 (s, 17-H), and 5.08 (s, 17-H); *m/z* 330 (M^+, 38%), 302 (7), 137 (13), 117 (46), 115 (21), 109 (17), 99 (23), and 59 (100); *m/z* (trimethylsilyl ether) 402 (M^+, 35%), 208 (55), 207 (65), 193 (25), 167 (96), 137 (82), 109 (100), and 73 (98); $\nu_{max.}$ 3 596, 1 782, and 1 724 cm^{-1}.

Reduction of 13-*Hydroxy*-7-*oxokaurenolide* (15).—The preceding ketone (36 mg) in THF–ethanol (10 ml, 1 : 1) was reduced in the usual way with sodium borohydride (15 mg) for 1 h. Preparative t.l.c. of the product gave 7α,13-dihydroxykaurenolide (17) (29 mg), m.p. 220—221 °C (from ethyl acetate–light petroleum), identical (mass and n.m.r. spectra) with the compound obtained earlier from microbiological hydroxylation of 7α-hydroxykaurenolide (16).

7α-(p-*Bromophenylsulphonyloxy*)-13-*hydroxykaurenolide* (19).—A solution of 7α,13-dihydroxykaurenolide (17) (125 mg) and *p*-bromobenzenesulphonyl chloride (125 mg) in the minimum volume of pyridine was left at 18 °C for 4 d. The usual work-up and preparative t.l.c. gave the required 7α-*brosylate* (19) as prisms, m.p. 223—226 °C (decomp.) (from toluene–light petroleum) (Found: C, 56.0; H, 5.7. $C_{26}H_{31}BrO_6S$ requires C, 56.6; H, 5.6%); $\nu_{max.}$ 3 590, 1 785, 1 582, 1 360, 1 192, 1 095, 1 075, 971, 892, and 840 cm^{-1}; δ 1.02 (s, 20-H_3), 1.22 (s, 18-H_3), 4.85 (m, 6-, 7-, and 17-H), 5.16 (br, 17-H), and 7.7 (m, 4 × ArH).

Ring Contraction of 7α-(p-*Bromophenylsulphonyloxy*)-13-*hydroxykaurenolide* (19).—The *p*-bromobenzenesulphonate (19) (100 mg) was dissolved in t-butyl alcohol–water (40 : 1, 6 ml) and the solution was flushed with nitrogen for 5 min. Potassium hydroxide pellets (320 mg) were then added and the mixture was refluxed for 1 h under nitrogen. Water (15 ml) was then added and the t-butyl alcohol was removed on a rotary evaporator. Further dilution with water, adjustment of the pH to 2.0, and extraction with ethyl acetate gave a product (70 mg) which, after preparative t.l.c. gave 13-hydroxygibberellin A_{12}-aldehyde (9) (27 mg), identical (mass and n.m.r. spectra) with the product obtained from microbiological hydroxylation of gibberellin A_{12}-7-aldehyde (5) (see above).

Oxidation of 13-*Hydroxygibberellin* A_{12}-*Alcohol* (8).—A mixture of chromium trioxide (22 mg) and pyridine (35 ml) in dichloromethane (0.5 ml) was stirred at 18 °C for 20 min. A solution of 13-hydroxygibberellin A_{12}-alcohol (8) (12 mg) in pyridine–dichloromethane (1 : 10, 100 μl) was then added and the reaction mixture was stirred for a further 30 min. The usual work-up and preparative t.l.c. of the crude product gave 13-hydroxygibberellin A_{12}-7-aldehyde (9) (4 mg).

ent-18-*Chloro*-6β,7α-*dihydroxykaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (18-*Chloro*-7β-*hydroxykaurenolide*) (25).—7β,18-Dihydroxykaurenolide (24) (7.57 g) and triphenylphosphine (15 g) were refluxed for 2 h in a mixture of carbon tetrachloride (300 ml) and pyridine (15 ml). The residue, obtained by evaporation of the solvents under reduced pressure, was chromatographed on a column (60 × 5 cm) of silica gel, and eluted with increasing concentrations of ethyl acetate in light petroleum. After elution of triphenylphosphine in the early fractions, elution with 20—25% ethyl acetate gave the required chloro compound (25) (6.0 g), m.p. 196—200 °C (from ethyl acetate–light petroleum) (lit.,[18] 193—195 °C); $\nu_{max.}$ 3 530, 1 760, 895, and 735 cm^{-1}; δ 0.84 (s, 20-H_3), 2.15 (d, J 6 Hz, 5-H), 3.52 and 3.68 (both d, J 12 Hz, together 18-H_2), 4.39 (d, J 6 Hz, 7-H), 4.65 (t, J 6 Hz, 6-H), 4.87 and 5.00 (both br s, together 17-H_2); *m/z* 352 [(M^+ + 2), 0.5%], 350 (M^+, 1.6), 334 (34), 332 (100), 319 (8), 317 (20), 291 (8), 289 (16), 283 (13), 269 (12), 239 (13), 147 (13), 143 (22), 107 (12), 105 (11), 93 (16), 91(21), 79 (13), and 77 (9).

ent-6β,7α-*Dihydroxy*[18-^{2}H]*kaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (26).—A solution of 18-chloro-7β-hydroxykaurenolide (25) (979 mg) and sodium borodeuteride (170 mg) in absolute ethanol (80 ml) was deoxygenated by bubbling dry, oxygen-free, nitrogen through it. To this solution under nitrogen was added tri-n-butyltin chloride (0.5 ml) and the solution was irradiated using a mercury lamp for 3 h. The solvent was removed under vacuum, water was added, and the product was recovered in ethyl acetate. Flash chromatography of the gum on silica gel and elution with 30% ethyl acetate in light petroleum gave 18-*deuterio*-7β-*hydroxykaurenolide* (26) (850 mg), m.p. and mixed m.p. with 7β-hydroxykaurenolide 187—190 °C (from acetone–light petroleum) [Found: (M^+ − 18), 299.1971; $C_{20}{}^1H_{25}{}^2H_1O_2$ requires *m/z* 299.1995] containing 0.80 atoms of deuterium per molecule by mass spectrometric measurement on the (M^+ − 18) ion cluster; ^{1}H n.m.r. δ 0.87 (s, 20-H_3), 1.30 (br, s, 18-H_2), 1.79 (d, J 6 Hz, 5-H), 4.39 (d, J 6 Hz, 7-H), 4.68 (t, J 6 Nz, 6-H), and 4.86 and 5.01 (each br, together 17-H_2); ^{2}H n.m.r. δ 1.28; ^{13}C n.m.r. δ-values as for lit.[10] values except for C-18 at δ 25.35 p.p.m. (t, J_{CD} 28 Hz); *m/z* 317 (M^+, 3%), 299 (100), 271 (13), 138 (33), 137 (14), 110 (24), and 109 (13).

ent-6β-*Hydroxy*-7-*oxo*[18-^{2}H]*kaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (27).—Jones reagent (3.0 ml) was added dropwise to a solution of 18-deuterio-7β-hydroxykaurenolide (26) (1.39 g) in acetone (50 ml), and the mixture was stirred for 20 min.

Methanol (10 ml) and water (20 ml) were then added and the solution was concentrated under reduced pressure. The aqueous residue was extracted with ethyl acetate and the extract was evaporated to give 18-deuterio-7-oxokaurenolide (27) (1.05 g) as a gum (Found: M^+, 315.1938; $C_{20}H_{25}{}^2H_1O_3$ requires M, 315.1945); ^{1}H n.m.r. δ 0.70 (s, 20-H_3), 1.31 (br, s, 18-H_2), 4.86 (d, J 6.6 Hz, 6-H), and 4.9 and 5.05 (both br s, together 17-H_2); m/z 315 (M^+, 94%), 314 (24), 275 (18), 166 (19), 147 (16), 138 (100), 110 (81), 109 (26), 93 (15), 91 (26), 79 (23), 77 (17), and 41 (27).

ent-6β,7β-*Dihydroxy*[18-^{2}H]*kaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (28).—The ketone (27) (1.05 g) and sodium borohydride (0.5 g) in ethanol–THF (1 : 1, 200 ml) were stirred for 2 h. Water was then added and the solution was concentrated under reduced pressure. The product was recovered in ethyl acetate and purified by flash chromatography. Elution with 20% ethyl acetate in light petroleum gave 18-deuterio-7α-hydroxykaurenolide (28) (916 mg) as a gum (Found: M^+, 317.2101; $C_{20}{}^1H_{27}{}^2H_1O_3$ requires M, 317.2101); ^{1}H n.m.r. (60 MHz) δ 1.13 (s, 20-H_3), 1.35 (br s, 18-H_2), 1.70 (d, J 6 Hz, 5-H), 4.07 (d, J 8 Hz, 7-H), 4.86 (t, J 7 Hz, 6-H), and 4.85 and 4.98 (each br, together 17-H_2); m/z 317 (M^+, 100), 316 (34), 315 (17), 299 (12), 110 (75), 109 (28), and 43 (11).

ent-7β-(p-*Bromophenylsulphonyloxy*)-6β-*hydroxy*[18-^{2}H]-*kaur*-16-*en*-19-*oic Acid* 19,6-*Lactone* (29).—*p*-Bromobenzenesulphonyl chloride (4.0 g) and 18-deuterio-7α-hydroxykaurenolide (28) (850 mg) were dissolved in pyridine (4 ml) and the mixture was stirred for 48 h. The pyridine was evaporated off under reduced pressure, water was added, and the residue was extracted with ethyl acetate. Purification of the product (recovered from the extract) by flash chromatography with 15% ethyl acetate in light petroleum as eluant gave 18-deuterio-7α-(*p*-bromophenylsulphonyloxy)kaurenolide (29) (1.23 g), m.p. 151—153 °C (acetone–light petroleum) (lit.,[13] 158—161 °C); ^{1}H n.m.r. (60 MHz) δ 0.89 (s, 20-H_3), 1.25 (br s, 18-H_2), 4.77 (d, J 8 Hz, 7-H), 4.88 and 5.01 (each br, together 17-H_2), 5.02 (t, J 6 Hz, 6-H), 7.63 (d, J 9 Hz, 2 × ArH), and 7.88 (d, J 9 Hz, 2 × ArH); m/z 537 [(M^+ + 2), 1%], 535 (M^+, 1), 299 (100), 298 (34), 271 (12), 138 (24), 137 (11), 110 (18), 109 (14), and 43 (15).

ent-6-*Formyl*[18-^{2}H]-7-*norgibberell*-16-*en*-19-*oic Acid* (30).—Potassium hydroxide (350 mg) was dissolved in a mixture of t-butyl alcohol (10 ml) and water (0.25 ml) which had been thoroughly deoxygenated. The brosylate (29) (128 mg) was added to this solution which was then refluxed for 1 h. The t-butyl alcohol was evaporated off under reduced pressure, water was added, and the pH was adjusted to 2.0. The product, recovered in ethyl acetate, was purified by flash chromatography on silica gel. Elution with 25% ethyl acetate in light petroleum gave 18-*deuteriogibberellin* A_{12}-7-*aldehyde* (30) (38.6 mg), m.p. 161—165 °C (lit.,[12] 159—163 °C) (from acetone–light petroleum) (Found: C, 75.8; H, 9.3. $C_{21}{}^1H_{29}{}^2H_1O_3$ requires C, 75.7; H, 9.2%) [Found (for methyl ester): M^+, 331.2251; $C_{21}{}^1H_{29}{}^2H_1O_3$ requires M, 331.2258] containing 0.80 atoms of deuterium per molecule by mass spectrometric measurements on the (M^+ − 60) and (M^+ − 90) clusters of the methyl ester; ^{2}H n.m.r. δ 1.12; ^{1}H n.m.r. (60 MHz) δ 0.78 (s, 20-H_3), 1.17 (br s, 18-H_2), 3.24 (dd, J 5 and 12 Hz, 6-H), 4.92 and 5.02 (each br s, together 17-H_2), and 9.80 (d, J 5 Hz, 7-H); m/z 317 (M^+, 2%), 299 (8), 271 (58), 270 (22), 256 (17), 242 (100), 241 (38), 105 (12), 91 (15), and 43 (16); m/z (Me ester) 331 (M^+, 11%), 288 (14), 271 (51), 270 (12), 242 (100), 241 (29), 240 (21), 228 (11), 110 (14), 105 (10), and 93 (10).

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