Fungal Products. Part XVI.[1] Conversion of Isosteviol and Steviol Acetate into Gibberellin Analogues by Mutant B1–41a of *Gibberella fujikuroi* and the Preparation of [^{3}H]Gibberellin A_{20}

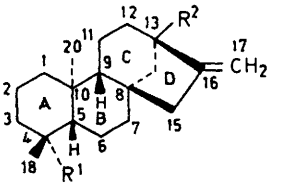
By **John R. Bearder, Valerie M. Frydman, Paul Gaskin, Jake MacMillan,*** and **Colin M. Wels,** School of Chemistry, The University, Bristol BS8 1TS
Bernard O. Phinney, Department of Biology, University of California, Los Angeles, California 90024, U.S.A.

Isosteviol (*ent*-16-oxobeyeran-19-oic acid) and steviol acetate (*ent*-13-acetoxykaur-16-en-19-oic acid) are efficiently metabolised by cultures of resuspended mycelium of *Gibberella fujikuroi*, mutant B1–41a. Isosteviol is converted, *inter alia*, into ring-CD-rearranged derivatives of gibberellins A_{17}, A_{19}, A_{20}, and 13-hydroxygibberellin A_{12}. Steviol acetate is metabolised mainly to the 7β-hydroxy- and 6β,7β-dihydroxy-derivatives and to the 13-acetates of gibberellins A_{17} and A_{20}. The preparation of [^{3}H]steviol acetate and its conversion into [^{3}H]gibberellin A_{20} of high specific activity is described. These results provide further evidence that the fungal enzymes for gibberellin biosynthesis from *ent*-kaurenoic acid lack substrate specificity. They also reveal that structural changes in the CD ring system of *ent*-kaurenoid substrates suppress the 3-hydroxylating enzyme.

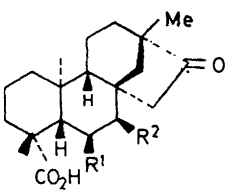
THE mutant B1–41a of *Gibberella fujikuroi* has been shown [2] to be blocked for gibberellin (GA) biosynthesis at the step between *ent*-kaur-16-en-19-al (1) and *ent*-kaur-16-en-19-oic acid (2). By investigating the metabolism of known metabolites of wild-type strains of *G. fujikuroi* when fed to cultures of the mutant B1–41a, metabolic sequences from *ent*-kaurenoic acid (2) to the known fungal gibberellins have been determined.[3] It was subsequently shown [1] that steviol (3), the 13-hydroxy-analogue of the natural intermediate *ent*-kaurenoic acid (2), was metabolised by the mutant to 13-hydroxy-GAs, hitherto unknown as fungal metabolites and only found in vascular plants. This apparent lack of substrate specificity of the enzymes in the GA pathway prompted the present study of the metabolism, by the mutant B1–41a, of isosteviol (4) and steviol acetate (13) (Scheme), two other ring-CD derivatives of *ent*-kaurenoic acid (2).

Isosteviol (4), obtained by acid-catalysed ring-CD rearrangement of steviol (3), was fed to re-suspended mycelium of B1–41a which was cultured for a further 1 and 5 days. The products, extracted from the culture medium, were analysed by g.l.c.–mass spectrometry with computer data processing as the methylated (Me) and methylated trimethylsilylated (MeTMSi) derivatives. From the 5-day incubation the metabolites (5—11) were identified by direct comparison with the derivatives of the products obtained by rearrangement of the appropriate GA or *ent*-13-hydroxykaurenoid with dilute hydrochloric acid. No seco-ring-B metabolites such as (12) were detected. Thus although the *ent*-6α,7α-dihydroxy-derivative (6) was formed in high yield it does not appear to be converted into seco-ring-B compounds, unlike the unrearranged *ent*-6α,7α,13-trihydroxykaurenoic acid.[1]

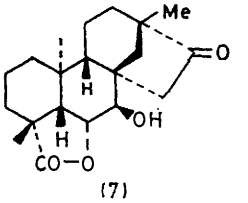
Even in the 1-day cultures isosteviol (4) was completely metabolised, mainly to the *ent*-7α-hydroxy-derivative (5), although all the metabolites detected after 5 days were present in small amounts. The significance of this rapid and complete metabolism of isosteviol (4) is discussed later. Of particular interest was the absence of 3-hydroxylated metabolites from isosteviol (4). A further example of this effect was found in the following study of the metabolites of steviol acetate (13) (see Scheme).



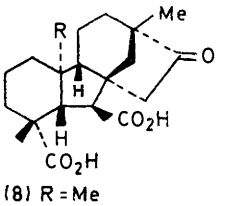
(1) R^1 = CHO, R^2 = H
(2) R^1 = CO_2H, R^2 = H
(3) R^1 = CO_2H, R^2 = OH



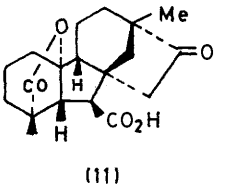
(4) R^1 = R^2 = H
(5) R^1 = H, R^2 = OH
(6) R^1 = R^2 = OH



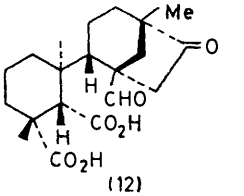
(7)



(8) R = Me
(9) R = CHO
(10) R = CO_2H



(11)



(12)

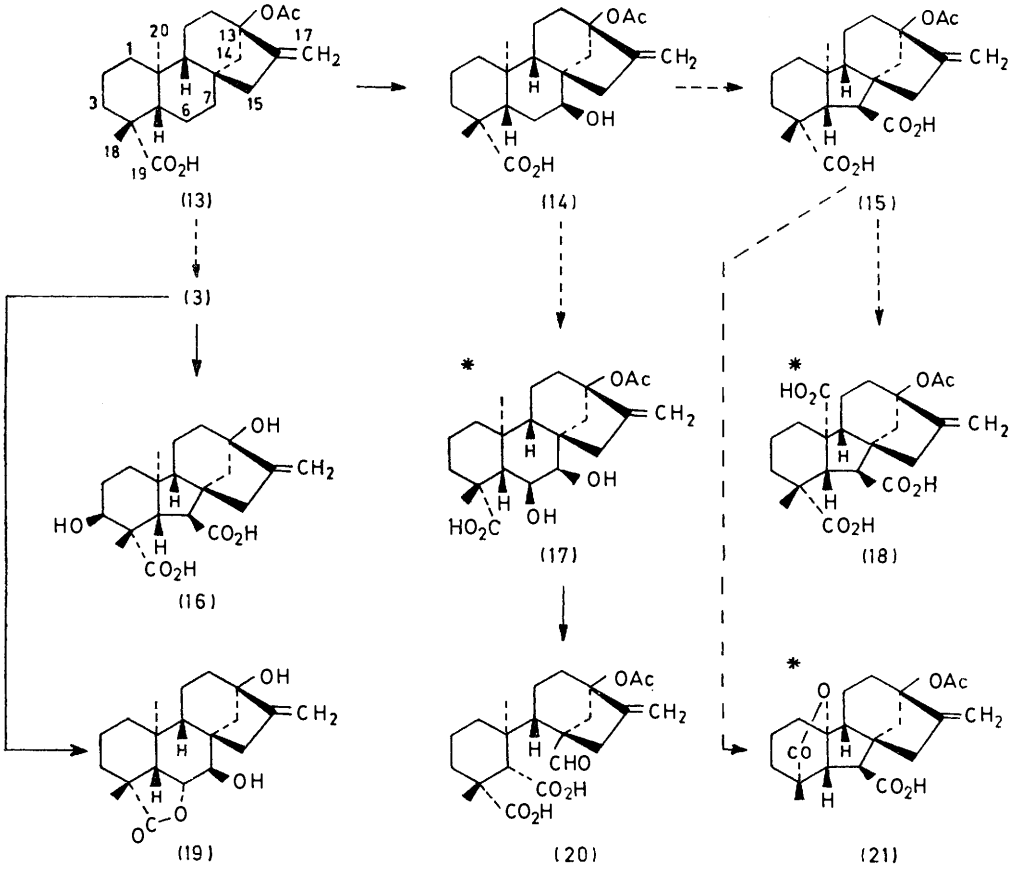
Small-scale feeds of steviol acetate (13) were conducted under the conditions used for steviol (3) [1] and isosteviol (4). After 5 days the metabolites shown in the Scheme were identified by g.l.c.–mass spectrometry of the Me and MeTMSi derivatives of the acetates extracted from the culture filtrate and of the free alcohols obtained by alkaline hydrolysis of this extract. Some unmetabolized steviol acetate was found both in the

[1] Part XV, J. R. Bearder, J. MacMillan, C. M. Wels, and B. O. Phinney, *Phytochemistry*, 1975, **14**, 1741.
[2] J. R. Bearder, J. MacMillan, C. M. Wels, M. B. Chaffey, and B. O. Phinney, *Phytochemistry*, 1974, **13**, 911.
[3] J. R. Bearder, J. MacMillan, and B. O. Phinney, *J.C.S. Perkin I*, 1975, 721.

culture filtrate and in the mycelium. Traces of non-acetylated GA_{18} (16) and of 7β,13-dihydroxykaurenolide (19) were also detected in the culture filtrate. Both these compounds are known [1] metabolites of steviol (3) and are probably derived from it after hydrolysis of steviol acetate (13) as indicated in the Scheme. As in the metabolism of steviol (3), the main metabolite after 1 day was the *ent*-7α-hydroxy-derivative (14), which had been almost completely metabolised after 5 days.

A large-scale feed of steviol acetate (13) was performed under conditions similar to those of the small-scale feed except that a longer incubation time was used. After 7 days the major metabolites were isolated by crystallisation and by p.l.c. of the extract from the culture filtrate. Appreciable amounts of unmetabolised steviol acetate (13) were present in mycelial extracts and the yields of metabolites were lower than in the small-scale feeds.

ent-13-Acetoxy-6α,7α-dihydroxykaurenoic acid (17), isolated in *ca.* 10% yield, showed the expected spectroscopic properties. The n.m.r. spectrum was similar to that [1] of the non-acetylated triol except for the acetate-methyl singlet and for the shift of 0.5 p.p.m. to higher field of the signal of one of the 17-protons. The mass spectrum of the methyl ester contained ions at *m/e* 137 and 109, associated with kaurenoids unsubstituted in ring A, and that of the MeTMSi derivative contained intense peaks at *m/e* 269, 209, and 147, assigned structures (22)—(24) and characteristic of 6,7-dihydroxy-kaurenoids. When re-fed to cultures of the mutant B1–41a, compound (17) was slowly metabolised to the seco-ring-B compound (20), which was obtained in 25% yield after 5 days incubation. Thus in both steviol (3) and steviol acetate (13) metabolism, the seco-ring-B derivatives are produced *via* the *ent*-6α,7α-dihydroxy-derivatives.



SCHEME Metabolism of steviol acetate (13); * major metabolites; (——) established steps; (– – –) presumed steps

Gibberellin A_{17} acetate (18), obtained in *ca.* 4% yield, showed unexceptional n.m.r. and mass spectra and gave the known GA_{17} on alkaline hydrolysis. Like C_{20} GAs with a 10-carboxy-group,[1,3,4] GA_{17} acetate (18) was not metabolised when re-fed to cultures of the mutant B1–41a.

Gibberellin A_{20} acetate (21), obtained in *ca.* 20% yield, showed the expected spectroscopic properties and yielded the known GA_{20} on alkaline hydrolysis. When re-fed to cultures of B1–41a, GA_{20} acetate was mainly unmetabolised but gave a trace of a compound which may be the 2-epimer (25) of gibberellin A_{29} acetate (26) since the mass spectra of the MeTMSi derivative of the alkaline hydrolysis product and of GA_{29} showed similar fragmentation patterns. 2α-Hydroxylation of GA_{20}

[4] B. E. Cross, K. Norton, and J. C. Stewart, *J. Chem. Soc.* (*C*), 1968, 1054.

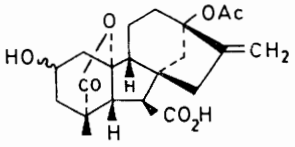
acetate by the mutant B1–41a would be analogous to the recently observed [5] conversion of GA_9 (27) into GA_{40} (28) by this mutant.

(22)

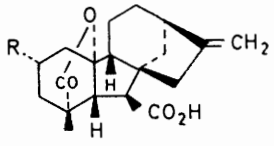
(23)

$Me_3Si-\overset{+}{O}=SiMe_2$

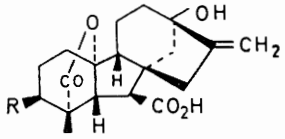
(24)



(25) 2α-OH
(26) 2β-OH



(27) R = H
(28) R = OH

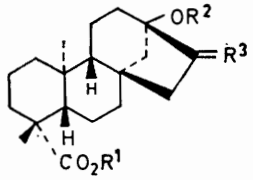


(29) R = OH
(30) R = H

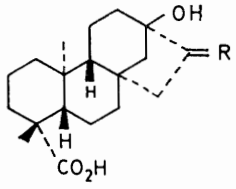
Comparison of the metabolic products obtained from steviol acetate (13) with those previously obtained [1] from steviol (3) and from *ent*-kaurenoic acid (2) [3] show that the presence of a 13-acetoxy-group has completely suppressed 3β-hydroxylation. Thus while the major GAs from steviol (3) were GA_{18} (16) and GA_1 (29), those from steviol acetate (13) were GA_{17} acetate (18) and GA_{20} acetate (21). This inhibition of 3β-hydroxylation is not only of theoretical interest (see later) but also of practical importance since metabolism of steviol acetate (13) by the mutant B1–41a provides a preparative route to GA_{17} and GA_{20} which were originally obtained by large-scale extractions of higher plant materials. For example the amount of steviol acetate derived *via* stevioside from 5 g of dried leaves of *Stevia rebaudiana* is converted by the mutant cultures *via* GA_{20} acetate (21) into the same quantity (7 mg) of GA_{20} (30) isolated [6] from 60 kg of immature seed of *Pharbitis nil*. This preparative route has been used to make [^{3}H]GA_{20}, of high specific activity, in the following way.

Stevioside (31) was oxidised with sodium periodate–osmium tetraoxide. The iodate and excess of periodate were then removed by ion-exchange resins before hydrolytic removal of the oxidised carbohydrate residues. The resultant steviol norketone (32) was treated with potassium t-butoxide and [^{3}H]methyltriphenylphosphonium bromide; the methyl protons in the salt were exchanged with ($^3H_2$)O in acetonitrile containing triethylamine, a procedure which is more convenient than previously described methods.[7] The Wittig reaction gave equal amounts of the required [^{3}H]steviol and the [^{3}H]-stereoisomer (34).[8] The latter was presumably formed by base-catalysed isomerisation of steviol norketone (32), followed by reaction of the isomer with the ylide. Acetylation of the [^{3}H]steviol gave the corresponding acetate with specific activity 40.7 mCi mmol^{-1}. The yield of olefinic products from steviol norketone (32) and methyltriphenylphosphonium bromide varied from 0% (with n-butyl-lithium or t-butyl-lithium) to 100% (with potassium t-butoxide).[9] Stabilisation of the intermediate betaine in Wittig reactions by lithium ions has been observed previously.[10] For steviol norketone and related norketones [5,11] we have found the use of essentially salt-free ylides, prepared [12] in tetrahydrofuran from the bromide and an excess of sodium hydride, to be convenient and efficient.

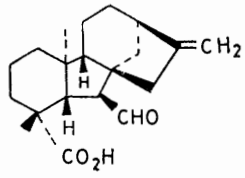
As expected,[13] scrambling of the label occurred in the Wittig reaction between steviol norketone (32) and [^{3}H]methylenetriphenylphosphorane. Ozonolysis of the [^{3}H]steviol acetate gave [^{3}H]formaldehyde containing only one-third of the total radioactivity. This result suggests both that ^{3}H–^{1}H exchange occurs at C-15 with



(31) R^1 = Glu, R^2 = GluGlu, R^3 = CH_2
(32) R^1 = R^2 = H, R^3 = O



(33) R = O
(34) R = CH_2



(35)

the [^{3}H]ylide acting as the base and as the source of ^{3}H, and that C-15 and C-14 in steviol norketone (32)

[5] J. R. Bearder, V. M. Frydman, P. Gaskin, W. E. Harvey, I. Hatton, J. MacMillan, and B. O. Phinney, following paper.
[6] N. Murofushi, N. Takahashi, T. Yokota, and S. Tamura, *Agric. and Biol. Chem.* (*Japan*), 1968, **32**, 123.
[7] P. W. Khong, B.Sc. Thesis, University of Western Australia, 1969; D. H. R. Barton, D. M. Harrison, G. P. Moss, and D. A. Widdowson, *J. Chem. Soc.* (*C*), 1970, 775; H. J. Bestmann, O. Kratzer, and H. Simon, *Chem. Ber.*, 1962, **95**, 2750.
[8] I. F. Cook and J. R. Knox, *Tetrahedron Letters*, 1970, 4091; E. Mossetig, U. Beylinger, F. Dolder, H. Lichti, P. Quitt, and J. A. Waters, *J. Amer. Chem. Soc.*, 1963, **85**, 2305.
[9] Y. Nakahara, K. Mori, and M. Matsui, *Agric. and Biol. Chem.* (*Japan*), 1971, **35**, 918.
[10] M. Schlosser and K. F. Christmann, *Angew. Chem. Internat. Edn.*, 1964, **3**, 636.
[11] L. J. Beeley and J. MacMillan, unpublished work.
[12] H. Schmidbauer, H. Stühler, and W. Vonberger, *Chem. Ber.*, 1972, **105**, 1084.
[13] For leading references see D. Hasselmann, *Chem. Ber.*, 1974, **107**, 3486.

become equivalent through rapid equilibration of steviol norketone (32) and its isomer (33). Such an equilibration, which is being investigated, would mean that the ^3H label in steviol acetate (13) is equally distributed amongst C-17, C-15, and C-14.

Finally, incubation of [^3H]steviol acetate in re-suspended cultures of the mutant B1–41a, as previously described, gave [^3H]GA_{20} acetate, which was hydrolysed to [^3H]GA_{20}. The radiochemical purity of the [^3H]GA_{20} was established by t.l.c.–radio-counting and by g.l.c.–radio-counting [14] of the methyl ester on a 2% SE-33 column and of the MeTMSi derivative on 2% QF-1 and 2% SE-33 columns.

The efficient metabolism of the unnatural substrates steviol (3),[1] isosteviol (4), and steviol 13-acetate (13) by *G. fujikuroi*, mutant B1–41a, may mean that the enzymes necessary for the conversion of these substrates into GA analogues can be rapidly induced. It is more likely, however, that most of the enzymes, including those responsible for ring B contraction and for the removal of C-20, are insensitive to structural changes in rings C and D of the natural substrate, *ent*-kaurenoic acid (2). However, the 3-hydroxylating enzyme appears to be more discriminating, accepting a 13-hydroxy-group [see (3)] but rejecting the bulkier 13-acetoxy-group [see (13)] and also the gross structural change of ring-CD rearrangement [see (4)] in its substrate, which, by analogy with the natural pathway,[3] would be the corresponding derivative of GA_{12} aldehyde (34).

EXPERIMENTAL

General experimental details are described in Part V.[15] Re-suspended cultures were prepared as described in Parts XI [2] and XIV [3] except that pigmented mycelium of B1–41a was obtained from cultures grown on 40% I.C.I. medium as defined by Geissman *et al.*[16] Also the re-suspension medium contained potassium dihydrogen phosphate at a concentration of only 5 g l^{-1} so that the pH of the re-suspended cultures remained at 4.8 throughout the experiments.

Small-scale Feed of Isosteviol (4).—To Erlenmeyer flasks (100 ml) containing hot water, isosteviol (2 mg) in acetone (0.1 ml) was added. The acetone was allowed to evaporate, re-suspended mycelium culture (10 ml) was added, and the flasks were returned to the shaker for 1 or 5 days.

After incubation, the medium was centrifuged at 2 500 rev. min^{-1} for 20 min. The supernatant was added to 1M-potassium hydrogen phosphate (pH 2.5; 5 ml), which was then extracted with ethyl acetate (10 ml). The gum recovered from the ethyl acetate was analysed by g.l.c.–mass spectrometry as the Me and MeTMSi derivatives. The following compounds were identified as the Me ester or MeTMSi derivative by direct comparison of mass spectra with the reference spectra detailed below: (a) ent-7α-*hydroxy*-16-*oxobeyeran*-19-*oic acid* (5); (b) ent-6α,7α-*dihydroxy*-16-*oxobeyeran*-19-*oic acid* (6); (c) ent-13-*methyl*-16-*oxo*-17-*nor*-13β-*gibberellane*-7,19-*dioic acid* (8); (d) ent-6β,7α-*dihydroxy*-16-*oxobeyeran*-19-*oic acid* 19,6-*lactone* (7); (e) ent-13-*methyl*-16-*oxo*-17-*nor*-13β-*gibberellane*-7,19,20-*trioic acid* (10); (f) ent-10β-*hydroxy*-13-*methyl*-16-*oxo*-17-*nor*-13β-*gibberellane*-7,19-*dioic acid* 19,10-*lactone* (11); and (g) ent-13-*methyl*-16,20-*dioxo*-17-*nor*-13β-*gibberellane*-7,19-*dioic acid* (9).

Reference compounds were prepared by refluxing the appropriate GA or *ent*-13-hydroxykaurenoid (100 μg) for 1 h with 2N-hydrochloric acid. The product was extracted with ethyl acetate, and derivatives were formed and subjected to g.l.c.–mass spectrometry. The metabolites (a)—(g) listed in the previous paragraph, were identified by the following data for the authentic derivatives: (a) MeTMSi derivative of (5), *m/e* 420 (M^+, 14%), 405 (20), 330 (59), 271 (100), 270 (42), 255 (23), 239 (18), 216 (44), 173 (24), 129 (42), 121 (24), 116 (30), 109 (19), 107 (19), 105 (17), 75 (52), and 73 (76); (b) MeTMSi derivative of (6), *m/e* 508 (M^+, 0.2), 493 (42), 418 (4), 375 (5), 269 (100), 209 (20), 151 (6), 147 (11), and 73 (29); (c) Me ester of (8), *m/e* 376 (M^+, 6), 344 (100), 316 (11), 301 (27), 284 (33), 273 (30), 257 (29), 242 (11), 241 (12), 213 (11), and 109 (12); (d) MeTMSi derivative of (7), *m/e* 404 (M^+, 11), 389 (23), 361 (88), 271 (10), 247 (15), 165 (41), 137 (100), 129 (20), 116 (14), 109 (27), 75 (22), and 73 (62); (e) Me ester of (10), *m/e* 420 (M^+, 14), 402 (7), 388 (100), 360 (35), 328 (86), 318 (48), 301 (45), 300 (62), 286 (45), 259 (34), 257 (52), 241 (48), 109 (62), 107 (38), and 105 (35); (f) Me ester of (11), *m/e* 346 (M^+, 20), 302 (15), 270 (20), 259 (52), 243 (43), 242 (100), 218 (23), 217 (18), 199 (28), 197 (21), 191 (20), and 159 (31); and (g) Me ester of (9), *m/e* 390 (M^+, 0), 362 (10), 330 (100), 302 (29), 301 (19), 281 (17), 270 (21), 259 (29), 243 (28), 242 (26), 241 (21), 195 (46), 194 (23), 168 (54), 136 (36), 135 (23), and 134 (45).

Large-scale Feed of Steviol Acetate (13).—Steviol acetate (281 mg) was distributed in re-suspended mycelium (30 × 100 ml) and incubated for 7 days. Work-up by filtration, extraction of the culture filtrate with ethyl acetate at pH 2.5, and evaporation gave an 'acidic' extract (376 mg). A trace of unmetabolised steviol acetate was detected in the mycelial extract. Crystallisation of the acidic extract from acetone and recrystallisation from acetone–light petroleum gave ent-13-*acetoxy*-6α,7α-*dihydroxykaur*-16-*en*-19-*oic acid* (17) (28 mg), m.p. 209—216° (Found: M^+, 392.220. $C_{22}H_{32}O_6$ requires M, 392.220), $\nu_{max.}$ 3 480, 3 310, 1 740, and 1 692 cm^{-1}, τ ([$^2H_5$]pyridine) 8.64 (s, 20-H_3), 8.16 (s, 18-H_3), 7.99 (s, COMe), 6.03 (d, J 2 Hz, 7-H), 5.08 (dd, J 2 and 12 Hz, 6-H), 4.96br (17-H), and 4.86br (17-H), *m/e* 392 (M^+, 7), 374 (14), 356 (23), 332 (23), 328 (22), 314 (50), 299 (21), 296 (31), 286 (25), 268 (38), 137 (65), 109 (83), and 43 (100); Me ester, *m/e* 406 (M^+, 1), 346 (9), 328 (45), 313 (7), 299 (14), 296 (19), 286 (9), 268 (26), 137 (50), 109 (100), and 43 (51); MeTMSi derivative *m/e* 550 (M^+, 0.5), 535 (32), 460 (4), 400 (6), 269 (100), 209 (17), 147 (6), 75 (5), and 73 (15).

P.l.c. of the mother liquors on silica gel with ethyl acetate–chloroform–acetic acid (15 : 5 : 1) gave two compounds as follows. (*a*) The material eluted from the band R_F 0.4—0.6 was rechromatographed on silica gel layers with ethyl acetate–light petroleum–acetic acid (50 : 50 : 1) to give *GA_{17} acetate* (18) (12 mg), m.p. 216—218°, τ ([$^2H_5$]pyridine) 8.24 (s, 18-H_3), 8.12 (s, COMe), 5.11 (d, J 12 Hz, 6-H), 4.97br (17-H), and 4.75br (17-H), *m/e* of Me ester 430 (M^+, 33), 370 (76), 342 (100), 328 (14), 310 (37), 300 (25), 287 (39), 282 (36), 241 (13), 223 (33), and 43 (58); hydrolysis with 2N-potassium hydroxide for 1 h at

[14] J. MacMillan and C. M. Wels, *Phytochemistry*, 1974, **13**, 1413.
[15] J. MacMillan and T. J. Simpson, *J.C.S. Perkin I*, 1973, 1487.
[16] T. A. Geissman, A. J. Verbiscar, B. O. Phinney, and G. Cragg, *Phytochemistry*, 1966, **5**, 933.

100 °C gave a gum identified as GA_{17} by g.l.c.–mass spectrometry of the Me and MeTMSi derivatives.[17] (*b*) The material (151 mg) eluted from the band R_F 0.6—0.8 was rechromatographed (p.l.c.) on silica gel with ethyl acetate–light petroleum–acetic acid (30 : 70 : 1) to give *GA_{20} acetate* (21) (58 mg), m.p. 255—261° (from acetone–light petroleum), $\nu_{max.}$ 3 510, 1 770, 1 730, 1 665, and 880 cm^{-1}, τ ([$^2H_5$]-pyridine) 8.70 (s, 18-H_3), 8.04 (s, COMe), 4.98br (17-H), and 4.72br (17-H), *m/e* 374 (M^+, 26), 356 (4), 332 (78), 328 (14), 314 (32), 289 (17), 286 (49), 270 (34), 268 (22), 258 (13), 244 (32), and 43 (100); *m/e* for Me ester 388 (M^+, 8), 357 (8), 356 (6), 346 (100), 342 (4), 328 (39), 314 (42), 303 (20), 300 (40), 286 (44), 284 (44), 268 (32), 257 (32), 244 (38), and 43 (56). Hydrolysis of a portion (10 mg) with 2N-potassium hydroxide for 1 h at 100 °C gave GA_{20} (6 mg), identified by g.l.c.–mass spectrometry of the Me ester and MeTMSi derivative.[16]

The following metabolites were not isolated but were characterised by g.l.c.–mass spectrometry from both large- and small-scale feeds: (i) *ent*-13-acetoxy-7α-hydroxykaurenoic acid (14), *m/e* for MeTMSi derivative 462 (M^+, 0), 447 (3), 420 (3), 418 (7), 402 (7), 372 (85), 330 (22), 312 (100), 269 (22), 253 (41), 252 (37), 193 (33), 121 (56), 75 (33), 73 (52), and 43 (26); (ii) the seco-ring-B diacid acetate (20) *m/e* for dimethyl ester 434 (M^+, 0.1), 227 (41), 195 (100), 167, (72), 135 (13), 109 (27), (107 91), and 43 (19); and (iii) 13-acetoxy-GA_{12} (15), *m/e* for dimethyl ester 386 (M^+, 7), 358 (11), 298 (100), 239 (11), and 43 (11).

Feed of [^{3}H]*Steviol Acetate; Preparation of* [^{3}H]*Gibberellin A_{20}*.—[^{3}H]Steviol acetate (50 mg) was distributed equally to pigmented mycelium of the mutant B1–41a, re-suspended in unbuffered 0% I.C.I. medium (100 ml) in each of ten conical flasks (500 ml). Work-up as for the cold feed gave a red acidic oil (173 mg; 81% of the radioactivity), which was fractionated by p.l.c. on silica gel HF developed twice with ethyl acetate–chloroform–acetic acid (5 : 15 : 1). The radioactive band at R_F 0.75 was eluted to give a gum (10.8 mg), shown to contain gibberellin A_{20} acetate by g.l.c. of a methylated sample. This gum was refluxed for 1 h with aqueous 2N-sodium hydroxide. Potassium dihydrogen phosphate was added and the solution was adjusted to pH 2.5 with 3N-hydrochloric acid. Extraction with ethyl acetate (3 × 30 ml) afforded crude [^{3}H]GA_{20} which was purified by p.l.c. on silica gel G, developed with ethyl acetate–chloroform–acetic acid (15 : 5 : 1). Recovery from the band at R_F 0.5 gave [^{3}H]GA_{20} (8.4 mg), characterised by g.l.c.–mass spectrometry of the MeTMSi derivative. Radiochemical purity (>95%) was established by g.l.c.–radio-counting[14] of the methyl ester on 2% SE-33 (programmed from 192 °C at 3° min^{-1}) and of the MeTMSi derivative on 2% SE-33 (from 185 °C at 3° min^{-1}) and on 2% QF-1 (from 188 °C at 3° min^{-1}). Recrystallisation was not attempted. The [^{3}H]GA_{20} was stored in sealed vials containing *ca.* 1 mg in ethyl acetate (4 ml). Radio-counting of a sample from one of these vials gave a specific activity of 33 mCi $mmol^{-1}$.

Steviol Acetate (ent-13-*Acetoxykaur*-16-*en*-19-*oic Acid*) (13).—Steviol (600 mg), pyridine (0.2 ml), and acetic anhydride (10 ml) were heated at 80 °C for 18 h. The mixture was evaporated under vacuum and the residue was redissolved in methanol–water to decompose some mixed anhydride. The recovered gum was chromatographed on a column of silica gel (50 g) and the material eluted with 25—30% ethyl acetate in light petroleum was crystallised from methanol–water to give *steviol acetate* (560 mg, 83%), m.p. 199—201° (Found: M^+, 360.231. $C_{22}H_{32}O_4$ requires *M*, 360.230); $\nu_{max.}$ 1 720, 1 690, and 880 cm^{-1}; τ 9.02 (s, 18-H_3), 8.76 (s, 20-H_3), 8.00 (s, COMe), and 5.10br (17-H_2); *m/e* 360 (M^+, 95), 318 (57), 300 (100), 285 (23), 262 (23), 146 (41), 132 (58), 121 (71), and 43 (89); the methyl ester had *m/e* 374 (M^+, 16), 332 (30), 314 (58), 254 (25), 146 (58), 121 (100), and 43 (23).

[^{3}H]*Steviol Acetate*.—The product from [^{3}H]steviol (100 mg), pyridine (1 ml), and acetic anhydride (9 ml) was purified by p.l.c. on silica gel G, developed with ethyl acetate–light petroleum–acetic acid (50 : 50 : 1). Recovery of the band at R_F 0.5 by elution with ethyl acetate gave [^{3}H]steviol acetate (62 mg), m.p. 200—202°, which was crystallised from ethyl acetate–light petroleum to constant activity (40.7 mCi $mmol^{-1}$). Subsequently higher yields were obtained by replacing the pyridine by catalytic amounts of toluene-*p*-sulphonic acid [18] at room temperature.

Ozonolysis of [^{3}H]*Steviol Acetate*.—The acetate (48 mg; 399.4 × 10^6 disint. min^{-1} $mmol^{-1}$) in acetic acid (5 ml) was treated with ozonised oxygen for 12 min. The solution was steam-distilled and saturated aqueous dimedone (50 ml) was added to the distillate. After 3 days at 4 °C, the formaldehyde dimedone derivative (15.3 mg), m.p. 191—192°, was collected and dried (3 h; 100 °C); specific activity 129.8 × 10^6 disint. min^{-1} $mmol^{-1}$ (*i.e.* 32.5% of the activity of the [^{3}H]steviol acetate).

Steviol Norketone (ent-13-*Hydroxy*-16-*oxo*-17-*norkauran* 19-*oic Acid*) (32).—Stevioside (31) (900 mg) in water (60 ml) was stirred with osmium tetraoxide (5 mg) for 15 min at 18 °C. Powdered sodium periodate (3 g) was added over 10 min and stirring was continued for 15 h. The solution was then treated with a mixture of Amberlite IRA-120 (H^+) (5 g) and IRA-400 (OH^-) (5 g) resins and, after 5 min, the filtrate was washed through a column of the same resins (10 g) with water (180 ml). The eluate (*ca.* 250 ml) was acidified with concentrated hydrochloric acid (25 ml) and heated under reflux for 2 h. Extraction with ethyl acetate gave steviol norketone (300 mg), m.p. 237—241° (lit.,[8] 221—222°) (from methanol–water), identified by g.l.c. and g.l.c.–mass spectrometric comparison of the methyl ester with an authentic specimen.

[^{3}H]*Methyltriphenylphosphonium Bromide*.—Methyltriphenyl phosphonium bromide (1.78 g) was dried at 100 °C for 3 h over phosphorus pentaoxide under vacuum and dissolved in acetonitrile (10 ml; distilled from calcium hydride). Triethylamine (2.5 ml; distilled from calcium hydride) and tritiated water (0.25 ml; *ca.* 5 Ci ml^{-1}) were added. After 24 h at 20 °C the solvent was removed under a stream of nitrogen gas and the residual salt was dried at 100 °C for 3 h over phosphorus pentaoxide. Conditions for the exchange were established by using deuterium oxide and observing the disappearance of the methyl doublets (τ 6.76; *J* 14 Hz) in the n.m.r. spectrum.

[^{3}H]*Steviol*.—A 1.24M-solution (4.5 ml) of sublimed potassium t-butoxide in tetrahydrofuran (distilled from calcium hydride) was added to a suspension of [^{3}H]methyltriphenylphosphonium bromide (1.68 g) in tetrahydrofuran (10 ml). After 1 h steviol norketone (32) (430 mg) in tetrahydrofuran (10 ml) was added. The mixture was stirred for 48 h then more potassium t-butoxide solution (5 ml) was added. After a further 140 h with stirring,

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water (50 ml) was added and the tetrahydrofuran was evaporated off. The product, recovered from an ethyl acetate extract (4 × 50 ml) of the aqueous residue was chromatographed on a column of silica gel eluted with increasing concentrations of ethyl acetate in light petroleum. The radioactive fractions (30—50% ethyl acetate) were bulked and fractionated by p.l.c. on 0.4 mm silica gel layers developed (× 3) with ethyl acetate–light petroleum–acetic acid (30 : 70 : 1) to give two radioactive bands at R_F 0.5 and 0.45. [^{3}H]Steviol (154 mg), recovered from the R_F 0.45 band was recrystallised from aqueous methanol to constant activity (40.7 mCi mmol^{-1}; m.p. 212—214.5°) and was identified by g.l.c.–mass spectrometric comparison of the MeTMSi derivative with an authentic specimen.

Recovery from the band at R_F 0.5 gave a gum (131 mg), the presumed [^{3}H]episteviol (34). The MeTMSi derivative gave a single peak on g.l.c.–mass spectrometry; the mass spectrum was similar to that of steviol MeTMSi derivative.

The same results were obtained when steviol norketone was treated with an essentially salt-free solution of [^{3}H]-methylenetriphenyl phosphorane, prepared from a resuspension of [^{3}H]methyltriphenyl phosphonium bromide in tetrahydrofuran and an excess of sodium hydride.

We thank the S.R.C. for research studentships (to J. R. B. and C. M. W.) and for a research grant (to J. MacM.); I.C.I. Ltd. for a Fellowship (to V. M. F.); and the N.S.F. for a research grant (to B. O. P.).

[5/1341 *Received, 7th July,* 1975]