Studies in Terpenoid Biosynthesis. Part X.[1] Incorporation of (5*S*)-[5-$^3H_1$]Mevalonic Acid into Gibberellic Acid

By **Roger Evans** and **James R. Hanson,*** The School of Molecular Sciences, The University of Sussex, Brighton BN1 9QJ
Larry J. Mulheirn, Shell Research Ltd., Milstead Laboratory, Sittingbourne, Kent

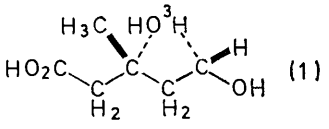
Incorporation of four 5-*pro-S* mevalonoid hydrogen atoms into gibberellic acid and location of two of these at C-10 and C-11 respectively has been shown by tritium labelling. The *exo*-9-H of methyl gibberate has been shown to exchange more rapidly than the *endo*-H and this has been used to demonstrate that the C(11β)- (*exo*) H of gibberellic acid is derived from a 5-*pro-S* of mevalonic acid.

THE distribution of radioactivity from variously labelled mevalonates in (—)-kaurene (2), the kaurenolides, and the gibberellins has been used to clarify stages in the biosynthesis of the tetracyclic diterpenoids.[2,3] Recently (5*S*)-[5-$^3H_1$]mevalonic acid (1) has become available [4] and we have been able to use it to determine the stereochemistry of labelling at C-10 and C-11 in gibberellic acid (3). We originally established [3] the origin of the kauranoid 6-hydrogens and their subsequent fate at C-10 of the gibberellins by the difference between the stereospecifically labelled (5*R*)-[5-$^3H_1$]mevalonic acid labelling pattern and that from the non-stereospecifically labelled [1-$^3H_1$,2-^{14}C]geranyl pyrophosphate.

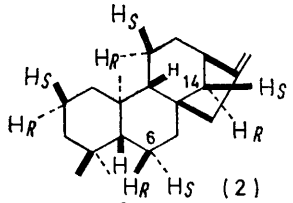
(—)-Kaurene (2) was prepared [5] enzymatically from (5*S*)-[5-$^3H_1$]mevalonic acid and [2-^{14}C]mevalonic acid. The (—)-kaurene (^{3}H : ^{14}C, 3·76 : 1) was fed to *Gibberella fujikuroi* and the metabolites were isolated after a further 60 h growth. Gibberellic acid (3) (1·4% incorporation) was purified as its methyl ester, which showed a ^{3}H : ^{14}C ratio of 3·79 : 1, corresponding to the retention of all four tritium labels. This is in contrast to the (5*R*)-[5-$^3H_1$]mevalonic acid incubation in which only two labels remained in the gibberellic acid. The methyl gibberellate was converted into methyl allogibberate (4) (^{3}H : ^{14}C,3·75 : 1) and this was epimerized [5] with base to form methyl 10-*epi*-allogibberate (5) (^{3}H : ^{14}C, 2·91 : 1). This showed a loss of one quarter of the tritium. When this reaction was carried out in deuterium oxide–sodium deuterioxide, the mass spectrum of the methyl 10-*epi*-allogibberate showed that only one deuterium label was introduced. Hence the tritium label that was exchanged was at C-10. The methyl gibberellate was also converted into methyl gibberate (6) (^{3}H : ^{14}C, 2·78 : 1) with the loss of one label and this was then epimerized [6] to form methyl 10-*epi*-gibberate (7) (^{3}H : ^{14}C, 1·87 : 1) with the loss of a second tritium label (in this rearrangement 11-H_2 of methyl gibberellate becomes 9-H_2 of methyl gibberate). [$^2H_3$]-Methyl 10-*epi*-gibberate was obtained by carrying out this sequence in deuteriated base. The mass spectral fragmentation pattern (Table 1) showed an ion at *m/e* 254 (ion B) (Scheme) corresponding to the loss of C_2H_4O ($C_2H_2D_2O$ in the deuteriated material) representing the loss of ring D. Consequently the additional label lost in the degradation to methyl 10-*epi*-gibberate was at C-11 of gibberellic acid (3).

[1] Part IX, J. R. Hanson, J. Hawker, and A. F. White, *J.C.S. Perkin I*, 1972, 1892.
[2] J. R. Hanson and A. F. White, *J. Chem. Soc.* (*C*), 1969, 981.
[3] R. Evans, J. R. Hanson, and A. F. White, *J. Chem. Soc.* (*C*), 1970, 2601.
[4] J. W. Cornforth and F. P. Ross, *Chem. Comm.*, 1970, 1395.
[5] R. Evans and J. R. Hanson, *J.C.S. Perkin I*, 1972, 2382.
[6] J. F. Grove and T. P. C. Mulholland, *J. Chem. Soc.*, 1960, 3007.

There is a marked difference in the ease of exchange of the *endo*- and *exo*-9-H in methyl gibberate (6). This has enabled us to assign the stereochemistry of the mevalonoid labels at this centre, which corresponds to C-11 in

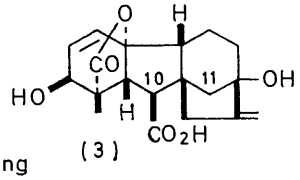


(1)

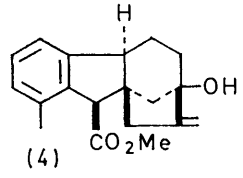


(2)

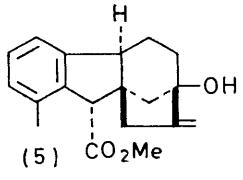
the [5-^{3}H]MVA labelling pattern of (−)-kaurene



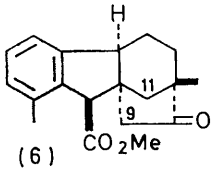
(3)



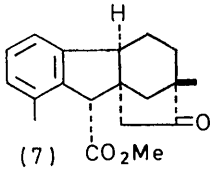
(4)



(5)



(6)



(7)

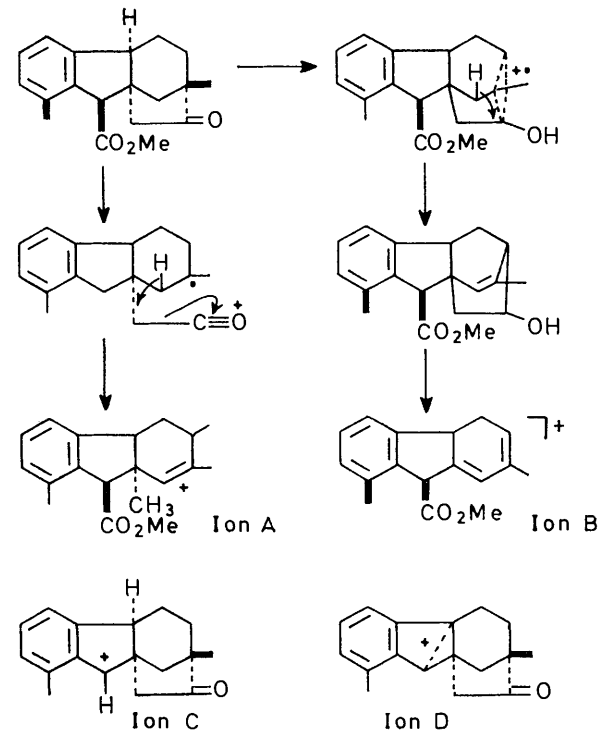
gibberellic acid. The mass spectrum of methyl gibberate which had been heated under reflux with 2N-deuteriochloric acid for 1 h showed that it contained 5·0 [$^2H_0$], 86 [$^2H_1$], and 9% [$^2H_2$] species. After 20 h at room temperature in 2N-sodium deuterioxide another sample of methyl gibberate showed negligible epimerization at C-10 but it contained 3·8 [$^2H_0$], 89·1 [$^2H_1$], and 7·1% [$^2H_2$] species. The mass and n.m.r. spectra of the two deuteriated samples were identical. Thus only one of the two protons at C-9 exchanges easily. Whereas conversion of the gibberellic acid (3) biosynthesized [3] from (5*R*)-[5-$^3H_1$]mevalonic acid into methyl gibberate (6) did not result in the loss of tritium, conversion of the gibberellic acid from the (5*S*)-[5-$^3H_1$]mevalonic acid led to the loss of tritium under the acidic conditions. This led us

TABLE 1

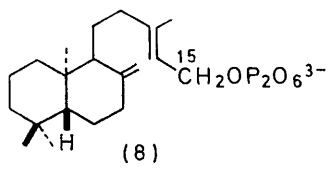
Mass spectra (*m/e* values) of methyl gibberate and its epimer

	M^+	Ion A	Ion B	Ion C	Ion D
Methyl gibberate	298	270	254	239	
[$^2H_1$]Methyl gibberate	299	271	254	240	
Methyl 10-*epi*-gibberate	298	270	254		238
[$^2H_3$]Methyl 10-*epi*-gibberate	301	273	255		241

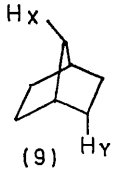
to repeat our earlier degradation [2] of methyl gibberellate biosynthesized from non-stereospecifically labelled (−)-[15-$^3H_1$]labda-8,13-dien-15-yl pyrophosphate (8). This had been carried out with singly labelled material which had apparently retained all its label in the conversion into methyl gibberate. Samples of methyl gibberellate biosynthesized from (−)-[15-$^3H_1$]labda-8,13-dien-15-yl pyrophosphate and [2-^{14}C]mevalonic acid were co-crystallized to constant activity (^{3}H : ^{14}C, 5·04 : 1). This material was then converted into methyl gibberate (^{3}H : ^{14}C, 2·38 : 1) with a 54% loss of tritium.



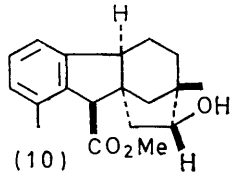
SCHEME Fragmentation pattern of methyl gibberate and its epimer



(8)



(9)



(10)

The stereochemistry of this exchange was elucidated as follows. The n.m.r. spectrum of methyl gibberate (see Table 2) shows the two-proton signal from the $-CH_2{\cdot}CO-$ bridge as an AB system at τ 7·34 and 7·54 (J_{AB} 18 Hz). The signal at τ 7·54 also shows a long-range coupling of 3·0 Hz. Only the *endo*-proton has a W-type configura-

tion relative to another proton (at C-11). This long-range coupling is of the same order of magnitude as that found in analogous simpler systems [*e.g.* in (9), J_{XY} 3 Hz].[7] In bicyclo[2.2.1]heptanes the *endo*-protons resonate at higher fields than the *exo*-protons. Deuteriation of methyl gibberate under both acidic and basic conditions resulted in the loss of the low-field proton, and the signal at τ 7·54 then appeared as a broad doublet. Consequently it is the *exo*-proton that is exchanged. Camphor and isofenchone both exchange the 3-*exo*-hydrogen for deuterium in preference to the 3-*endo*-hydrogen.[8] We suggest that this selectivity may be explained in terms of the torsional strain in the transition state.[9] In the enol there is a small torsion angle between the C(9)–H bond and the 10,10a-bond. Deuteriation from the β face (*exo*) moves the C-9 hydrogen towards the α face increasing this torsion angle and diminishing the torsional strain. Molecular models suggest that there is a marginal difference between ease of access to the two protons in favour of the β (*exo*)-proton. A similar explanation has been advanced for the stereospecific enolization of the 15-ketones of (—)-kaurene and phyllocladane.[10]

In order to confirm this assignment we attempted to reduce methyl gibberate to obtain the C-8 epimeric alcohols. However, reduction with sodium borohydride or with aluminium isopropoxide in propan-2-ol gave the same alcohol, characterized as its crystalline acetate. This alcohol was also obtained by reduction of gibberic acid with sodium in liquid ammonia using ammonium chloride as the proton donor, followed by methylation. Bicyclo[2.2.1]heptanones on reduction with metal hydrides or with sodium in liquid ammonia, afford the *endo*-alcohols.[11] By analogy this alcohol is assigned the 8α stereochemistry (10). Furthermore the C-8 proton resonance at τ 6·1 showed $J_{8,9}$ values of 4·5 and 10 Hz corresponding to *exo–endo* and *exo–exo* couplings. Addition of the shift reagent, Eu(fod)$_3$,* led to a greater downfield shift of the C-9 *endo*-proton signal compared to the C-9 *exo*- proton signal. Reduction of the methyl [9-^{2}H$_1$]gibberate gave the [9-^{2}H$_1$]alcohol (10) in which the C-8 proton resonance showed $J_{8,9}$ 4·5 Hz corresponding to an *endo–exo* coupling. The C-9 *exo*-proton resonance at τ 7·4 was absent.

We conclude that it is the C-9 *exo*-hydrogen that exchanges easily and that this is derived from a 5-*pro-S* mevalonoid hydrogen. The formation of methyl gibberate from methyl gibberellate does not disturb this centre. Consequently the C-11α proton of gibberellic acid is derived from a 5-*pro-R* mevalonoid hydrogen and the C-11β proton from a 5-*pro-S* mevalonoid hydrogen. These correspond to the C-14 protons of (—)-kaurene and the terminal protons of (—)-labda-8,13-dien-15-yl (copalyl) pyrophosphate. These results also support our previous conclusion[3] that the ring contraction stage involves the retention of the 5-*pro-S* mevalonoid hydrogen atom at C-10 in gibberellic acid. This corresponds to the kauranoid C-6α (axial) hydrogen.

TABLE 2

N.m.r. spectra of methyl gibberate (6) and the alcohol (10)

Chemical shift (τ)

	C(7)Me	C(1)Me	CO_2Me	C(10)H	C(9)H H_A	C(9)H H_B	C(8)H	C(2)H, C(3) H, C(4)H
Methyl gibberate	8·97	7·84	6·23	5·85	7·34 [d]	7·54(q) [a]		2·76—3·10
Methyl[9-^{2}H$_1$]gibberate	8·98	7·84	6·22	5·85		7·55		2·76—3·10
Methyl 10-*epi*-gibberate	9·06	7·77	6·37	6·27	7·52	7·52		2·80—3·10
Alcohol(10) (i)	8·98	7·89	6·27	6·01	7·4	8·0	6·10(q) [b]	2·85—3·15
(ii) [c]	8·23	7·73	6·15	5·61	6·5	6·5	4·15(q)	2·60—2·95
(iii) [d]	7·49	7·60	6·02	5·20	5·6	5·1	2·00(q)	2·50—2·90
[9-^{2}H$_1$]Alcohol(10)	8·98	7·89	6·28	6·02		8·0	6·10	2·85—3·15

[a] J_{AB} 18·0, J_{BE} 3·0 Hz. [b] $J_{8,9}$ 10 and 4·5 Hz. [c] Alcohol(10) (40 mg) + Eu(fod)$_3$ (20 mg). [d] Alcohol(10) (40 mg) + Eu(fod)$_3$ (40 mg).

EXPERIMENTAL

General experimental and fermentation details have been described.[1,5]

Preparation of Gibberellic Acid from (5S)-[5-^{3}H$_1$]*Mevalonic Acid.*—(—)-Kaurene, biosynthesized[5] separately from [2-^{14}C]- and (5S)-[5-^{3}H$_1$]mevalonic acid, was crystallized to give a ^{3}H : ^{14}C ratio of 3·76 : 1 [^{3}H, 2,010,045 d.p.m. (disint. min^{-1}), ^{14}C, 534,870 d.p.m.]. The doubly-labelled (—)-kaurene in ethanol (1 ml) and 10% aqueous Tween 80 (1 ml) was added to *Gibberella fujikuroi* (1 l of culture) 48 h after inoculation. The fermentation was harvested after a further 60 h. The metabolites were recovered in ethyl acetate and separated into acidic and neutral fractions with aqueous sodium hydrogen carbonate. The gibberellic acid was purified by preparative layer chromatography (p.l.c.) on silica with chloroform–ethyl acetate–acetic acid (5 : 5 : 1) as eluant. The gibberellic acid was then methylated with diazomethane and rechromatographed in the same system. The methyl gibberellate was diluted with inactive material (15 mg) and then crystallized to constant activity. The product (12·5 mg) had m.p. 207—208° (lit.,[12] 209—210°)

* Eu(fod)$_3$ = tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionato)europium(III). See R. E. Rondeau and R. E. Sievers, *J. Amer. Chem. Soc.*, 1971, **93**, 1522, for applications.

[7] J. C. Davis and T. V. Van Auken, *J. Amer. Chem. Soc.*, 1965, **87**, 3900.

[8] A. F. Thomas, R. A. Schneider, and J. Meinwald, *J. Amer. Chem. Soc.*, 1967, **89**, 68.

[9] P. von R. Schleyer, *J. Amer. Chem. Soc.*, 1967, **89**, 699.

[10] J. MacMillan and E. R. H. Walker, *J.C.S. Perkin I*, 1972, 986.

[11] S. Beckmann and R. Mazger, *Chem. Ber.*, 1956, **89**, 2738; H. C. Brown and H. R. Deck, *J. Amer. Chem. Soc.*, 1965, **87**, 5620.

[12] B. E. Cross, *J. Chem. Soc.*, 1954, 4670.

(^{3}H, 2294 d.p.m. mg^{-1}; ^{14}C, 604 d.p.m. mg^{-1}; ^{3}H : ^{14}C, 3·79 : 1).

Conversion of [^{3}H,^{14}C]*Methyl Gibberellate into* [^{3}H,^{14}C]-*Methyl Allogibberate.*—The methyl gibberellate (6 mg) was stirred with 2N-hydrochloric acid (5 ml) for 18 h. Methyl allogibberate (2·5 mg) was recovered in ethyl acetate and purified by p.l.c. on silica with n-hexane–ethyl acetate as eluant to give needles (from n-hexane), m.p. 96—97° (lit.,[13] 98—99°) (^{3}H, 2736 d.p.m. mg^{-1}; ^{14}C, 730 d.p.m. mg^{-1}; ^{3}H : ^{14}C, 3·75 : 1).

Epimerization of [^{3}H,^{14}C]*Methyl Allogibberate.*—Methyl allogibberate (1·5 mg) was heated under reflux in 2N-sodium hydroxide (5 ml) for 90 min. The solution was acidified and the product was recovered in ethyl acetate and remethylated with diazomethane. Methyl 10-*epi*-allogibberate was purified by chromatography on silica in n-hexane–ethyl acetate (1 : 1). It co-chromatographed with authentic material and appeared as one component on g.l.c. (on 1% OV-17 at 220° with t_R 6·7 min). A sample of gum had (^{3}H, 2124 d.p.m. mg^{-1}; ^{14}C, 730 d.p.m. mg^{-1}; ^{3}H : ^{14}C, 2·91 : 1).

Conversion of [^{3}H,^{14}C]*Methyl Gibberellate into Methyl Gibberate.*—[^{3}H,^{14}C]Methyl gibberellate (6 mg) was heated under reflux in 2N-hydrochloric acid (5 ml) for 1 h. Methyl gibberate (3·1 mg) was recovered in ethyl acetate and purified by p.l.c. on silica with n-hexane–ethyl acetate (1 : 1) as eluant to give prisms (from ethyl acetate–n-hexane), m.p. 113—114° (lit.,[11] 113—115°) (^{3}H, 2030 d.p.m. mg^{-1}; ^{14}C, 730 d.p.m. mg^{-1}; ^{3}H : ^{14}C, 2·78 : 1).

Epimerization of [^{3}H,^{14}C]*Methyl Gibberate.*—[^{3}H,^{14}C]-Methyl gibberate (1·7 mg) was heated under reflux with 2N-sodium hydroxide (5 ml) for 90 min. The solution was acidified and the product was recovered in ethyl acetate, remethylated with diazomethane, and purified by p.l.c. to give methyl 10-*epi*-gibberate as a gum (^{3}H, 1365 d.p.m. mg^{-1}; ^{14}C, 730 d.p.m. mg^{-1}; ^{3}H : ^{14}C, 1·87 : 1).

Feeding of (−)-[15-^{3}H$_1$]*Labda*-8,13-*dien*-15-*yl Pyrophosphate.*—The pyrophosphate (15 mg, 1·54 × 10^5 d.p.m. mg^{-1}) in ethanol (1 ml) was added to *Gibberella fujikuroi* (1 l of culture) 5 days after inoculation. After a further 5 days, the metabolites were isolated and the gibberellic acid was purified by p.l.c. first as the free acid and then as the methyl ester. Methyl gibberellate (12·8 mg) crystallized as needles (from ethyl acetate–light petroleum), m.p. 208—209° (^{3}H, 2736 d.p.m. mg^{-1}). The [^{3}H]methyl gibberellate was diluted with [^{14}C]methyl gibberellate (prepared from [2-^{14}C]mevalonic acid). The mixture was crystallized to constant activity (^{3}H, 2189 d.p.m. mg^{-1}; ^{14}C, 434 d.p.m. mg^{-1}; ^{3}H : ^{14}C, 5·04 : 1). It was converted into methyl gibberate (3H, 1198 d.p.m. mg^{-1}; ^{14}C, 504 d.p.m. mg^{-1}; ^{3}H : ^{14}C, 2·38 : 1) as described above.

Exchange Reactions of Methyl Gibberate.—(*a*) Methyl gibberate (58 mg) was heated under reflux with 2N-deuteriochloric acid (12·5 ml) for 1 h. The methyl gibberate (51 mg) was recovered in ethyl acetate and crystallized from ethyl acetate–n-hexane as prisms, m.p. 113—114°.

(*b*) Methyl gibberate (60 mg) in dioxan (1 ml) was stirred at room temperature with sodium deuterioxide [from sodium metal (80 mg) in deuterium oxide (1 ml)] for 20 h. The methyl gibberate (45 mg) was recovered in ethyl acetate and crystallized from ethyl acetate–n-hexane as prisms, m.p. 112—114°. The two samples showed identical mass and n.m.r. spectra.

Reduction of Methyl Gibberate.—(a) *With sodium borohydride.*—Methyl gibberate (90 mg) in methanol (5 ml) was treated with sodium borohydride (20 mg) at room temperature for 3 h. Water (5 ml) was added and the product was recovered with ethyl acetate and purified by p.l.c. on silica with chloroform–ethyl acetate–light petroleum (5 : 5 : 1) as eluant. The gummy alcohol (80 mg) ($\nu_{max.}$ 3450, 1740, and 1600 cm^{-1}, *m/e* 300, 282, and 241) had t_R 6·9 min on g.l.c. (1% OV-17 at 220°). The *acetate*, prepared with acetic anhydride and sodium acetate, crystallized from aqueous methanol as needles, m.p. 123—125°, $\nu_{max.}$ 1725, 1600, and 1240 cm^{-1} (Found: C, 72·7; H, 7·7. $C_{21}H_{26}O_4$ requires C, 73·6; H, 7·6%). The [9-^{2}H$_1$]methyl gibberate was reduced under similar conditions.

(b) *With aluminium isopropoxide.* Methyl gibberate (10 mg) in propan-2-ol (2 ml) was added to a solution of aluminium isopropoxide (100 mg) in propan-2-ol (15 ml). The mixture was slowly distilled over a period of 3 h and then cooled. Water was added and the gummy product recovered in ethyl acetate. It showed identical R_F on t.l.c. and t_R on g.l.c. with the alcohol described above.

Reduction of Gibberic Acid.—Gibberic acid (65 mg) in ether (2 ml) was added to a solution of sodium metal (500 mg) in liquid ammonia (20 ml). After 10 min, excess of ammonium chloride was added and the ammonia was allowed to evaporate. Water (15 ml) was added and the solution was acidified. The product was recovered in ethyl acetate and methylated with diazomethane. The ester was purified by p.l.c. on silica with chloroform–ethyl acetate–light petroleum (5 : 5 : 1) as eluant to give an alcohol (38 mg) which showed an identical i.r. spectrum and retention tine on g.l.c. (1% OV-17) with the alcohol described above.

We thank Professor J. W. Cornforth F.R.S., for helpful discussion, Mr. P. Ross for a gift of (5*S*)-[5-^{3}H$_1$]-mevalonic acid, and Shell Research Ltd. for hospitality.

[2/2511 *Received*, 6*th November*, 1972]

[13] T. P. C. Mulholland, *J. Chem. Soc.*, 1958, 2693.