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PREPARATION OF [^{14}C]-GIBBERELLIC ACID

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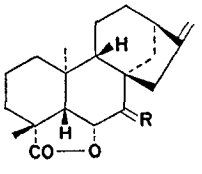
Key Word Index—*Gibberella fujikuroi*; Fungi; [^{14}C]-gibberellic acid; gibberellin biosynthesis.

Abstract—A combination of a chemical and a microbiological method is described for the preparation of [^{14}C]-gibberellic acid.

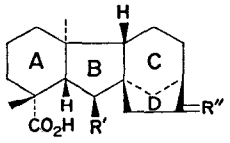
THE BIOLOGICAL activity of gibberellic acid (VII) has been widely studied and it has a number of commercial applications.[1] In order to study its metabolism, a viable route to specifically-labelled [^{14}C]-gibberellic acid was required. The ring-D exocyclic methylene group has been a useful site for labelling tetracyclic diterpenoids with C-14.[2] However, with the more highly hydroxylated gibberellins the Wittig reaction, which is used to insert this group, requires a large excess of the reagent and it can be accompanied by base-catalysed isomerization products.[3] Consqeuently we studied a combination of chemical and microbiological methods. The label was introduced into a less-highly oxygenated gibbane (V) which was then converted into gibberellic acid (VII) by the fungus, *Gibberella fujikuroi*.



(I) R = $\overset{OH}{H}$

(II) R = $\overset{H}{OH}$

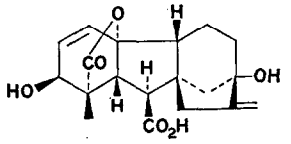
(III) R = $\overset{H}{OSO_2C_6H_4Br}$



(IV) R′ = CHO ; R″ = CH_2

(V) R′ = CH_2OH ; R″ = CH_2

(VI) R′ = CH_2OH ; R″ = O



(VII)

The gibbane skeleton may be formed by rearrangement of the toluene-*p*-sulphonate of 7α-hydroxykaurenolide (II) with base.[4] 7α-Hydroxykaurenolide (II)[5] which is readily available from the fungal metabolite, 7β-hydroxykaurenolide (I), was converted to its *p*-bromobenzenesulphonate (III). The optimum conditions for ring contraction utilized potassium hydroxide in refluxing wet *t*-butyl alcohol for 1 hr. This afforded the aldehyde (IV)[6] in 76% yield. The aldehyde was reduced with sodium borohydride to the alcohol (V).

[1] LANG, A. (1970), *Ann. Rev. Plant Physiol.* **21**, 537.
[2] CROSS, B. E., GALT, R. H. B. and HANSON, J. R. (1964), *J. Chem. Soc.* 295.
[3] ALDRIDGE, D. C., HANSON, J. R. and MULHOLLAND, T. P. C. (1965), *J. Chem. Soc.* 3539.
[4] GALT, R. H. B. and HANSON, J. R. (1965), *J. Chem. Soc.* 1565.
[5] CROSS, B. E., GALT, R. H. B. and HANSON, J. R. (1963), *J. Chem. Soc.* 2944.
[6] CROSS, B. E., NORTON, K. and STEWART, J. C. (1968), *J. Chem. Soc.* C, 1054.

Ozonolysis in pyridine and carbon tetrachloride gave the nor-ketone (VI) in 64% yield. The best conditions for the Wittig reaction using ^{14}C-triphenylphosphonium methiodide in dimethylsulphoxide, afforded the gibbane alcohol (V) in 63% yield. This was used as a substrate for microbiological conversion to gibberellic acid (VII). In the first experiment the alcohol was incorporated in 28·5% yield. The incorporation was shown to be specific by ozonolysis. The formaldehyde which was isolated as its dimedone derivative, contained 98·9% of the activity of the gibberellic acid. In order to optimize the conversion, the gibbane alcohol (V) was fed at different concentrations to parallel fermentations. The results are given in Table 1. In the range which was examined the specific activity of the gibberellic acid rose with concentration but the overall incorporation passed through a maximum. The percentage incorporation also increased with time and was 4·8% after 17 hr, 28·5% after 6 days and 31·4% after 10 days. These conditions have been used to prepare a total of 8 mCi gibberellic acid.[7]

TABLE 1. EFFECT OF SUBSTRATE CONCENTRATION ON INCORPORATION

Wt of substrate (mg)	Wt of gibberellic acid (mg from 1 l.)	Sp. act. of gibberellic acid (dpm/mg × 10^5)	Incorporation (%)
4·5	61·7	0·079	27·2
13·8	67·3	0·2314	28·3
28·3	61·7	0·4285	23·5
57·3	63·6	0·5258	14·6

Substrate = [^{14}C]-gibbane alcohol (V) (sp. act. 3·98 × 10^5 dpm/mg).

Since the gibbane alcohol (V) was such an efficient precursor of gibberellic acid, we have looked for it as a normal metabolite of *Gibberella fujikuroi*. A careful search of a fermentation to which [2-$^3H_2$, 2-^{14}C]-mevalonic acid of known ^3H:^{14}C ratio had been fed, was made by dilution analysis with the gibbane alcohol (V). The gibbane alcohol when re-isolated did not have a discrete ^3H:^{14}C atomic ratio and we were unable to crystallize it to constant radioactivity. Furthermore, in a microsomal preparation in which the conversion of (−)-kaurene to the gibbane aldehyde (IV) could be detected, no radioactivity was associated with the gibbane alcohol (V) fraction. Hence although we cannot exclude the alcohol as a transient intermediate or in an enzyme-bound form, we have obtained no evidence for it as yet on the biosynthetic pathway and consequently what we are observing may be an extremely efficient microbiological conversion. This area of the biosynthesis is under further examination and will be reported in full at a later date.

Two general points emerge from this. First that the percentage incorporation in this fermentation can be dependent upon substrate levels and period of feeding and consequently considerable care must be exercized in drawing conclusions from relative efficiencies of biosynthesis that are of the same order of magnitude. Second a high level of incorporation may not necessarily be associated with a biosynthetic intermediate.

EXPERIMENTAL

M.ps were determined on a Kofler hot-stage apparatus. IR spectra were recorded as Nujol mulls. NMR spectra were determined for solutions in $CDCl_3$ with tetramethylsilane as internal standard. Radioactive

[7] Available from the Radiochemical Centre, Amersham, Bucks.

compounds were crystallized to constant activity and counted by liquid scintillation (Koch-Light KL 355 liquid scintillator) on a Beckmann LS 100 counter with a preset error of ±1%. *Gibberella fujikoroi* (CMI 58290) was grown as a shake culture (120 rpm) in Erlenmeyer flasks (250 ml) at 24° on the following medium: glucose (80 g), NH_4NO_3 (0·48 g), KH_2PO_4 (5·0 g) and $MgSO_4$ (1·0 g) per l. of dist. H_2O. A trace elements solution (2 ml) was added. This contained $FeSO_4$ (0·1 g), $CuSO_4$ (0·015 g), $ZnSO_4$ (0·161 g), $MnSO_4$ (0·01 g), and ammonium molybdate (0·1 g) per 100 ml dist. H_2O. The main fermentation (100 ml/flask) was inoculated with seed (1 ml) grown on the same medium for 4–5 days. The fermentations were harvested by filtration of the mycelium, acidification of the culture filtrate to pH 2 and extraction with EtOAc. The extract was dried over Na_2SO_4 and the solvent was evaporated off *in vacuo* to give a gum from which the metabolites were isolated by chromatography.

The gibbane aldehyde (IV). 7-Hydroxykaurenolide (II)[5] was converted to its p-*bromobenzenesulphonate* (III) with *p*-bromobenzenesulphonyl chloride in pyridine. It crystallized from Et_2O–light petrol. as needles, m.p. 158–161° (Found C, 58·6; H, 5·9. $C_{26}H_{31}O_5SBr$ requires: C, 58·45; H, 5·8%), ν_{max}. 1758, 1665, 1580 cm^{-1}. τ 9·10 (3H, *s*), 8·73 (3H, *s*) 5·0 (3H, *m*), 2·25 (4H, *m*). The *p*-bromobenzenesulphonate (6·2 g) was dissolved in *t*-BuOH (400 ml) containing KOH (20 g) in H_2O (20 ml) and heated under reflux under N_2 for 1 hr. The solution was concentrated *in vacuo* and the residue acidified with dil. HCl and extracted with boiling light petrol. (×3). The extract was dried and concentrated. The aldehyde (IV) (2·78 g) crystallized from light petrol. as needles, m.p. 175–176° (lit.,[6] 159–163°), ν_{max} 2600, 1712, 1692, 1667 cm^{-1}, τ 9·23 (3H, *s*), 8·80 (3H, *s*), 6·72 (1H, *q*, *J* 6 and 13 Hz), 5·17 (1H,) 5·06 (1H,), 0·20 (1H, *d*, *J* 6 Hz).

The alcohol (V). The aldehyde (IV) (1·0 g) in EtOH (250 ml) was treated with $NaBH_4$ (250 mg) at room temp. for 2 hr. The solution was concentrated *in vacuo* and the residue acidified with dil. HOAc and the product recovered in EtOAc. The *gibbane alcohol* (V) (0·78 g) crystallized from EtOAc as needles, m.p. 185–187° (Found: C, 75·3; H, 9·3. $C_{20}H_{30}O_3$ requires: C, 75·4, H, 9·5%), ν_{max} 3310, 2600 (br), 1700, 1660, 883 cm^{-1}, τ 9·26 (3H, *s*), 8·55 (3H, *s*), 6·16 (2H, *m*), 5·20 (1H, *m*), 5·05 (1H, *m*).

Ozonolysis of the alcohol (V). The above alcohol (0·70 g) in pyridine (5 ml) and CCl_4 (5 ml) was cooled with an acetone–solid CO_2 freezing mixture and a slow stream of ozonized O_2 was passed through for 1 hr. The solvent was removed and the product was purified by preparative TLC on silica in diisopropyl ether–5% HOAc to afford the *nor-ketone* (VI) (450 mg) which crystallized from acetone–light petrol. as needles, m.p. 215–216° (Found: C, 70·9; H, 8·85. $C_{19}H_{28}O_4$ requires: C, 71·2; H, 8·8%), ν_{max} 3395, 2600 (br), 1780, 1700 cm^{-1}, τ 9·26 (3H, *s*), 8·55 (3H, *s*), 6·16 (2H, *m*).

Labelling of the gibbane alcohol (V). NaH (50% dispersion in oil) (100 mg) was washed under N_2 in *n*-pentane and dissolved in freshly purified dimethylsulphoxide (2·5 ml) at 60°. ^{14}C-Methyl triphenylphosphonium iodide (sp. act. 44·40 mCi/mmol) (600 mg) in dry dimethyl sulphoxide (2·5 ml) was added and the solution was stirred under N_2 at room temp. for 1 hr. The nor-ketone (VI) (300 mg) was added and the reaction was stirred under N_2 at 60° for 17 hr. 2 N HCl (5·0 ml) was added and the reaction was then stirred for a further 1 hr at room temp. The product was recovered in EtOAc and purified by preparative TLC on silica in diisopropyl ether–5% HOAc. The [^{14}C]-gibbane alcohol (V) (184 mg sp. act. 44·36 mCi/mmol) crystallized from Et_2O as needles, m.p. 186–188°, identical (IR, m.m.p. and R_f to the authentic alcohol.

Incubation of the [^{14}C]-*gibbane alcohol with* Gibberella fujikuroi. The [^{14}C]-gibbane alcohol (180 mg) as its potassium salt, in 50% aq. EtOH (50 ml) was evenly distributed between 50 flasks of a 6-day-old *Gibberella fujikuroi* fermentation. The metabolites were recovered in EtOAc after a further 10 days and purified by chromatography on Sephadex G25, filtration through charcoal in acetone and crystallization to constant activity. [^{14}C]-Gibberellic acid (490 mg) crystallized from EtOAc–Me_2CO as prisms, m.p. 226–230° (sp. act. 5·58 mCi/mmol incorp. 31·4%). The chromatography and radiochemical purity were followed by radio-TLC on silica with diisopropyl ether–5% HOAc and $CHCl_3$–EtOAc–HOAc (5:5:1) as the solvents.

Specificity of labelling. (a) *Gibbane alcohol.* The [^{14}C]-alcohol (1·6 mg) was diluted with inactive material (19·2 mg) and dissolved in HOAc (0·5 ml). Ozonized oxygen was passed through the solution for 15 min. H_2O (10 ml) was added and the solution was steam distilled into a solution of dimedone in H_2O. The formaldehyde dimethone (m.p. 190°) had 98·2% of the activity of the gibbane alcohol. (b) *Gibberellic acid.* Gibberellic acid (10·4 mg) when ozonized as above gave formaldehyde dimethone, m.p. 192° (sp. act. 5·52 mCi/mmol, 98·9% of the activity of the gibberellic acid).

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