Phytochemistry, 1975, Vol 14, pp 2593–2597 Pergamon Press Printed in England

CYCLIZATION AND HYDROXYLATION STEREOCHEMISTRY IN THE BIOSYNTHESIS OF GIBBERELLIC ACID

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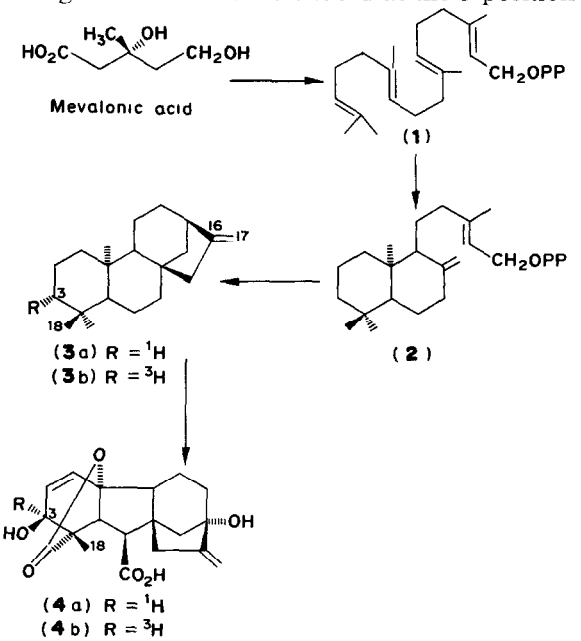
(*Received* 11 *April* 1975)

Key Word Index—*Gibberella fujikuroi*, *ent*-[3β-^{3}H,17-^{14}C]kaurene, gibberellic acid

Abstract—In *Gibberella fujikuroi* cultures, *ent*-[3β-^{3}H,17-^{14}C]kaurene is converted to gibberellic acid with retention of the tritium label at the 3α-position. This evidence for the stereochemistry of 3-hydroxylation also permits the stereochemistry of the 'proton-initiated' cyclization step in gibberellic acid biosynthesis to be deduced.

INTRODUCTION

The biosynthesis of GA (**4a**) in *Gibberella fujikuroi* (Scheme 1) [1,2] has separate steps for the cyclization of the polyene precursor (geranylgeranyl pyrophosphate) and for the introduction of the 3-hydroxyl group. According to biogenetic theory [3], in the cyclization step a hydrogen from the biological medium is introduced at the 3-position of copalyl pyrophosphate (**2**) [and ultimately of *ent*-kaurene (**3a**)] whereas the other 3-hydrogen derives from the 4-pro-R hydrogen of mevalonic acid [4]. Hanson and White [5] have observed that GA derived from 4R[4-^{3}H,2-^{14}C] mevalonic acid in *G. fujikuroi* has ^{3}H labels at the 5- and the 9-positions but not at the 3-position (or the 13-position) whereas *ent*-7α-hydroxykaurenoic acid 19→6 lactone and *ent*-7α,18-dihydroxykaurenoic acid 19→6 lactone have the full complement of ^{3}H labels (four) to be anticipated from cyclization without loss of label. This shows that the conversion of *ent*-kaurene into GA involves loss of the label at the 3-position but the stereochemistry of the cyclization and hydroxylation steps remain unidentified.



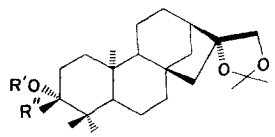
Scheme 1 Intermediates in the biosynthetic pathway to gibberellic acid

In this paper we are able to define the stereochemistry of these steps from our observations using *ent*-[3β-^{3}H,17-^{14}C] kaurene as a precursor of GA.

DISCUSSION

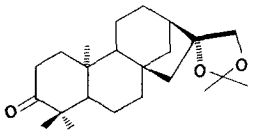
ent-[3β-^{3}H]Kaurene (**3b**) was prepared by an unexceptional route in which the stereospecific labelling was introduced through $LiAlH_4$ reduction of the ^{3}H-tosylate (**6c**). The labelled tosylate was obtained from the previously-described acetonide derivative [6] *ent*-3β,16β,17-trihydroxykaurane (**5a**) by oxidation to the ketone (**7**) fol-

lowed by reduction with NaB^3H_4. This formed almost entirely the equatorial alcohol (**5c**) which was then converted to the labelled tosylate (**6c**) before purification was carried out

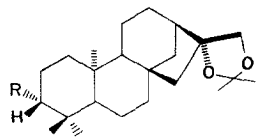


(**5**) R′ = H (**6**) R′ = Ts

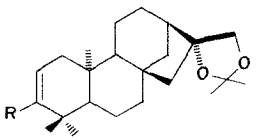
(a) R″ = ^{1}H
(b) R″ = ^{2}H
(c) R″ = ^{3}H



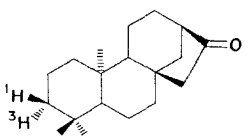
(**7**)



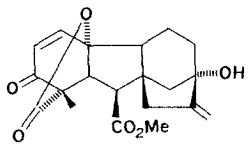
(**8a**) R = ^{1}H
(**8b**) R = ^{2}H
(**8c**) R = ^{3}H



(**9a**) R = ^{1}H
(**9b**) R = ^{3}H



(**10**)



(**11**)

In trial experiments with the unlabelled tosylate (**6a**), $LiAlH_4$ treatment was found to give not only the required product of reductive displacement of the tosyloxy group (**8a**) but also the products of reductive hydrolysis and elimination (**5a** and **9a** respectively). Whereas **5a** was readily separated from the mixture for identification, **8a** and **9a** were recovered only in admixture, their identification rests on NMR spectral and TLC (Si gel-$AgNO_3$) comparison with the product of dehydration of the alcohol (**5a**) and the product of further catalytic hydrogenation. For the purpose of obtaining the 3H-labelled saturated compound (**8c**), the corresponding mixture from reduction of the labelled tosylate (**6c**) was treated with excess diborane and then oxidized with H_2O_2. This converted the unsaturated compound (**9b**) completely to a mixture of alcohols which were then readily removed by chromatography affording the required labelled compound (**8c**) in pure form.

The label in this compound is assigned to the 3α-position (*ent*-3β) because $LiAlH_4$ reductions of sulfonate esters are known to proceed predominantly or exclusively by rearside attack of the reagent at the centre of displacement [7–9]. Furthermore, the corresponding reductive displacement of the tosyloxy groups of **6b** and **6a** using $LiAlH_4$ and $LiAl^2H_4$ respectively gave deuterated species (**8b** and the 3-epimer) which had IR characteristics indicative of [9] equatorially-located (C-D stretch 2145 cm^{-1}) and axially-located (C-D stretch 2125 cm^{-1}) D atoms with essentially no intermixing; this is in accord with the stereospecific labelling that was expected.

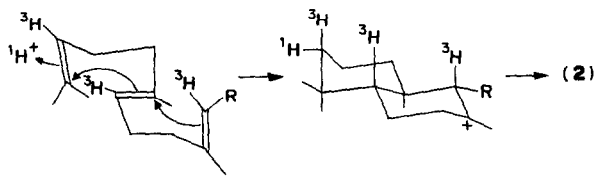
Oxidation of the 3H-labelled compound (**8c**) with Jones reagent produced the norketone (**10**) which was transformed into the required *ent*-[3β-^{3}H] kaurene (**3b**) by reaction with triphenylmethylene phosphorane. Admixture with *ent*-[17-^{14}C] kaurene then gave a doubly-labelled sample (^{3}H.^{14}C = 15·0). The radiochemical purity of this material was demonstrated by scanning TLC and of the product of isomerization with I_2–C_6H_6 [10, 11]. The product from isomerization showed the anticipated two radioactive areas (*ent*-kaurene and the Δ^{15}-isomer) which had, as required, unchanged ^{3}H.^{14}C ratios.

The *ent*-[3β-^{3}H, 17-^{14}C] kaurene sample was added to a culture of *G. fujikuroi* and after 4·5 days GA was recovered from the medium as the methyl ester. The ^{3}H ^{14}C ratio of this material (14·0) showed that more than 93% of the ^{3}H was retained in the metabolic conversion to GA. Furthermore, this label was located almost entirely at the 3-position because MnO_2 oxidation of the labelled ester afforded the ketone (**11**) which had essentially no ^{3}H (^{3}H ^{14}C = 0·4). This shows that the 3α-hydrogen (*ent*-3β) of *ent*-kaurene retains its position in the metabolic conversion to GA, the departures from ideal ^{3}H ^{14}C ratios are presumably due to counting inaccuracies and incomplete specificity of labelling in the chemical synthesis of the precursor.

Several lines of evidence suggest that in the biosynthetic pathway to GA [1,2] a 3-hydroxyl group is introduced by the conversion of GA_{12} to GA_{14} (possibly as the 7-aldehydes). Direct hydroxylations of this type typically proceed with

retention of configuration at the substituted carbon [12, 13a] and apparent exceptions to this rule in the elaboration of the allylic alcohol grouping of lycorine [14] and in the introduction of the 14-hydroxyl group of cardenolides [15] may involve different mechanisms. The above evidence for retention of configuration in forming GA is thus in accord with the suggested biosynthetic pathway; furthermore this evidence excludes the possibility of intervention of 3-keto intermediates and related species which would lead to loss of the ^3H label from the *ent*-[3β-^{3}H,17-^{14}C]kaurene precursor.

The evidence for retention of configuration in the hydroxylation also permits an assignment for the stereochemistry of the cyclization of geranylgeranyl pyrophosphate leading to copalyl pyrophosphate (**2**) and *ent*-kaurene (**3**). Because the H derived from the 4- pro R position of mevalonic acid is lost in the conversion of *ent*-kaurene into GA [5], it follows that this H adopts the 3β-position in **2** and **3**. Furthermore, it is known that C-18 of GA derives from C-2 of mevalonic acid [16] and the best interpretation of the available evidence is that it is this C atom which is the source of the *trans*-Me group of the terminal isopropylidene group of polyene isoprenoid intermediates [13b]. Consequently, the cyclization of geranylgeranyl pyrophosphate in this pathway must result from antiplanar addition to the terminal double bond with the polyene in an incipient chair conformation for the A-ring (Scheme 2).



Scheme 2 A-ring stereochemistry in the cyclization of geranylgeranyl pyrophosphate derived from [4R-^{3}H]mevalonic acid

Proton-initiated cyclizations are postulated for the biosynthesis of a wide range of other terpenoids [17] and the theory of these cyclizations [18] predicts antiplanar addition pathways but evidence for the stereochemistry of these conversions has been lacking until this time.

EXPERIMENTAL

General procedures have been described [19]l Ir spectra were measured in CS_2 solns

ent-16β,17-*Isopropylidenedioxy*-3β-*tosyloxykaurane* (**6a**). Tosyl chloride (2 g) was added to *ent*-16β,17-isopropylidenedioxykauran-3β-ol (**5a**, 766 mg) in C_5H_5N (12 ml) After 22 hr, ice was added and the mixture worked up in the usual way to give the tosylate (**6a**, 1·01 g) which crystallized from petrol as needles; mp 148–148·5° (d) $[\alpha]_D$ −36·9° (Found: C, 69·4, H, 8·4, S, 6·3 $C_{30}H_{44}SO_5$ requires C, 69·7, H, 8·6, S, 6·2%) PMR (δ). *s* at 0·78 (6H), 1·00 (3H), 1·34 (6H), 2·42 (3H); AB *q* at 3·86, 4·01 (*J* 9 Hz), *br t* at 4·18 (1H)

LiAlH$_4$ reduction of ent-16β,17-*isopropylidenedioxy*-3β-*tosyloxykaurane* (**6a**) The tosylate (**6a**, 75 mg) was reduced with $LiAlH_4$ in refluxing solvent (10 ml) in the various ways summarized in the following table

Solvent	Molar ratio ($LiAlH_4$: **6a**)	Time (hr)	% Yield 'Hydrocarbon'	**6a**	**5a**
Dioxan	5:1	18	69	—	12
	1:1	35	63	30	1
THF	20:1	66	28	—	48
	5:1	42	34	23	20
	1:1	84	45	44	5

The product was separated by chromatography on Al_2O_3 into a 'hydrocarbon' fraction, *ent*-16β,17-isopropylidenedioxykauran-3β-ol (**5a**) and (in some cases) unreduced material The 'hydrocarbon' fraction from reactions with THF solvent was a mixture of the saturated and unsaturated acetonides (**8a**) and (**9a**) which ranged in proportion from very little of **8a** with the equimolar quantity of $LiAlH_4$ up to *ca* 2:3 with the 4-fold excess of $LiAlH_4$ (NMR, TLC) Several additional substances were present in the 'hydrocarbon' fraction from the reactions in dioxan solvent

ent-16β,17-*Isopropylidenedioxykaur*-2-*ene* (**9a**) Phosphorous oxychloride (1 ml) was added carefully to a cooled soln of *ent*-16β,17-isopropylidenedioxykauran-3β-ol (**5a**, 920 mg) in C_5H_5N (18 ml) After 16 hr at room temp., the soln was heated with steam for 1 hr then cooled and poured onto ice After the usual workup the recovered material was chromatographed on Al_2O_3 to give *ent*-16β,17-isopropylidenedioxykaur-2-ene (**9a**, 601 mg) which crystallized from MeOH as needles, mp 131–2°, $[\alpha]_D$ −61·8° (Found C, 79·9, H, 10·3 $C_{23}H_{36}O_2$ requires: C, 80·2, H 10·5%) PMR (δ): *s* at 0·89 (3H), 0·96 (3H), 1·05 (3H), 1·39 (6H); AB *q* at 3·94, 4·08 (*J* 9 Hz), *br* 5·42 (2H, $W_{\frac{1}{2}}$ 3·5 Hz)

ent-16β,17-*Isopropylidenedioxykaurane* (**8a**) *ent*-16β,17-Isopropylidenedioxykaur-2-ene (**9a**, 289 mg) in HOAc (25 ml) was hydrogenated over Adam's catalyst for 3 days After filtration and evaporation of the solvent, the residue was filtered through Al_2O_3 in petrol The product, ent-16β,17-*isopropylidenedioxykaurane* (**8a**, 285 mg) crystallized from MeOH as needles, mp 125·6°, $[\alpha]_D$ −40·3° (Found C, 79·4, H, 11·0 $C_{23}H_{38}O_2$ requires: C, 79·7, H, 11·1%) PMR (δ) *s* at 0·80 (3H), 0·84 (3H) 1·01 (3H), 1·36 (6H), AB *q* at 3·88 4·04 (*J* 9 Hz).

ent-[3β-^{3}*H*]-16β,17-*Isopropylidenedioxykaurane* (**8c**) [^{3}H]-$NaBH_4$ (1·9 mg, 5 mCi) was added to a dil soln of $NaBH_4$ in diglyme (2 mM, 2 ml) An aliquot (1 ml) of this soln was added to *ent*-16β,17-isopropylidenedioxykauran-3-one (**7**, 41 mg), the soln stood for 1 hr at room temp and then heated for 1 hr at 100° To ensure complete reduction, 0·1 M $NaBH_4$ in diglyme (0·6 ml) was then added and heating continued for 1 hr at 100° Excess $NaBH_4$ was destroyed by the addition of H_2O and HOAc and the reaction mixture

then diluted with H_2O and extracted 2× with Et_2O. Combined Et_2O extracts were washed with dil $NaHCO_3$ soln, dried and evaporated to give a residue ($2{\cdot}52 \times 10^9$ dpm) which showed one radioactive spot corresponding with *ent*-16β,17-isopropylidenedioxykauran-3β-ol (**5a**) on TLC. This material was diluted with the unlabelled alcohol (**5a**, 75 mg) and converted to the tosylate (**6c**) as for the unlabelled substance (above). The product was chromatographed on Al_2O_3 and crystallized to give pure *ent*-[3α-^{3}H]-16β,17-isopropylidenedioxy-3β-tosyloxykaurane (**6c**, 147 mg, $2{\cdot}1 \times 10^9$ dpm). The labelled tosylate was then reduced with $LiAlH_4$ (56 mg) in refluxing THF (2 ml) for $5\frac{1}{2}$ days. The product was chromatographed on Al_2O_3 and a mixture of the labelled saturated and unsaturated acetonides (**8c**, **9b**; $3{\cdot}1 \times 10^7$ dpm) recovered by elution with petrol; further elution with Et_2O yielded the labelled alcohol (**5c**) and a small amount of the labelled tosylate (**6c**). The Et_2O fraction was treated with tosyl chloride to regenerate the tosylate which was again reduced with $LiAlH_4$ in THF. This cycle was repeated several times to give a combined 'hydrocarbon' fraction (**8c**, **9b**: $1{\cdot}3 \times 10^9$ dpm) and a residual alcohol fraction (**5c**, $4{\cdot}1 \times 10^8$ dpm). The 'hydrocarbon' fraction was dissolved in THF and treated with externally-generated diborane for 1 hr. H_2O was then cautiously added followed by 1 M NaOH and 30% H_2O_2. The mixture was stirred for 2 hr at room temp. and then worked up in the usual way and the product chromatographed on Al_2O_3. action eluted with petrol was identified as *ent*-[3β-^{3}H]-16β,17-isopropylidenedioxykaurane (**8c**, 35 mg, $3{\cdot}6 \times 10^8$ dpm) from the PMR spectrum and TLC comparison (Si gel–$AgNO_3$). No unsaturated compound (**9b**) remained. Elution of the column with Et_2O gave a mixed alcohol fraction (69 mg, $8{\cdot}7 \times 10^8$ dpm).

Preparation of deuterated compounds. ent-16β,17-Isopropylidenedioxykauran-3-one (**7**, 235 mg) was reduced with [^{2}H]-$LiAlH_4$ (90 mg) in refluxing Et_2O (8 ml) for 2 hr. The crude product (239 mg) was converted to the toxylate (**6b**, 329 mg) as for the unlabelled material; this compound was identified by the TLC and PMR. It was then reduced with $LiAlH_4$ and the mixture of products separated as for the ^{3}H-labelled compound to give *ent*-[3β-^{2}H]-16β,17-isopropylidenedioxykaurane (**8b**, 53 mg). ν_{max} 2145 cm^{-1} (equatorial C-^{2}H stretch). *ent*-16β,17-Isopropylidenedioxy-3β-tosyloxykaurane (**6a**, 337 mg) was similarly converted by reaction with [^{2}H]-$LiAlH_4$ into *ent*-[3α-^{2}H]-16β,17-isopropylidenedioxykaurane (3-epimer of **8b**, 44 mg). ν_{max} 2125 cm^{-1} (axial C-^{2}H stretch).

ent-[3β-^{3}*H*]-17-*Norkauran*-16-*one* (**10**). *ent*-[3β-^{3}H]-16β,17-Isopropylidenedioxykaurane (**8c**, 17·5 mg, $1{\cdot}8 \times 10^8$ dpm) from the above preparation was dissolved in Me_2CO (10 ml) and oxidized with Jones reagent (0·5 ml) for 2 hr. Chromatography of the product on Al_2O_3 afforded nor-ketone (**10**, 10 mg, $1{\cdot}7 \times 10^8$ dpm) which was identified by TLC and NMR comparison [20]. Oxidation of a second batch of **10** yielded further labelled nor-ketone (4 mg, $6{\cdot}5 \times 10^7$ dpm). The radiochemical homogeneity of this material was demonstrated by dilution of an aliquot ($1{\cdot}5 \times 10^6$ dpm) with unlabelled nor-ketone (109 mg). The sp act was essentially unchanged after 2 crystallizations from aq. MeOH.

ent-[3β-^{3}*H*,17-^{14}*C*]*Kaur*-16-*ene* (17-^{14}*C-labelled* **3b**). The ^{3}H-labelled nor-ketone (**10**, $2{\cdot}3 \times 10^8$ dpm) in dry Et_2O (20 ml) was added under N_2 to a stirred Et_2O soln (15 ml) of triphenylmethylenephosphorane (prepared from 360 mg of methyltriphenylphosphonium iodide and 0·3 ml of 2·6 M butyllithium in pentane). After 21 hr at room temp. the product was isolated and purified by column chromatography and preparative TLC. The *ent*-[3β-^{3}H]kaur-16-ene (**3b**, 7 mg, $7{\cdot}2 \times 10^7$ dpm) thus obtained was identified by TLC and PMR spectral comparison. Admixture of this material with *ent*-[17-^{14}C]kaur-16-ene gave the doubly-labelled compound (17-^{14}C-labelled **30**, 27 mg, ^{3}H = $1{\cdot}0 \times 10^7$ dpm/mg, ^{14}C = $6{\cdot}7 \times 10^6$ dpm/mg, ^{3}H:^{14}C = 15·0). This showed only a single radioactive spot corresponding with **3a** on TLC (Si gel–$AgNO_3$; petrol–$CHCl_3$, 4:1). An aliquot was heated in C_6H_6 under reflux with a few crystals of I_2 for $5\frac{1}{2}$ hr. After cooling, the soln was diluted with Et_2O, washed with aq. $Na_2S_2O_3$ and H_2O, dried and evaporated. The product showed 2 radioactive spots corresponding to *ent*-kaur-16-ene and *ent*-kaur-15-ene using the same TLC system as above. The material extracted from the 2 radioactive zones had ^{3}H:^{14}C ratios of 15·3 and 15·6 respectively.

Metabolism of ent-[3β-^{3}*H*,17-^{14}*C*]*kaur*-16-*ene* (**3b**). Half of the sample of *ent*-[3β-^{3}H,17-^{14}C]kaur-16-ene (13·5 mg) was added in EtOH (1 ml) to a fermentation of *G. fujikuroi* which had exhausted the inorganic nitrogen. After $4\frac{1}{2}$ days, the acidic fraction was recovered from the filtrate of the culture and subjected to methylation (CH_2N_2). Chromatography on Al_2O_3 then gave methyl [3α-^{3}H,17-^{14}C] GA, (17-^{14}C labelled methyl ester of **4b**, 17 mg, ^{3}H = $2{\cdot}0 \times 10^6$ dpm/mg, ^{14}C = $1{\cdot}4 \times 10^5$ dpm/mg, ^{3}H:^{14}C = 14·6) which was identified by TLC and NMR comparison. After dilution with unlabelled methyl GA (101 mg) the sp act and isotope ratios were essentially unchanged during 3 crystallizations from EtOAc–petrol (^{3}H = $1{\cdot}51 \times 10^4$ dpm/mg, ^{14}C = $1{\cdot}1 \times 10^3$ dpm/mg, ^{3}H:^{14}C = 14·1).

Oxidation of the metabolite methyl ester. The 3× crystallized Me ester (20 mg) from the above metabolism was stirred and heated under reflux with MnO_2 (215 mg) in $CHCl_3$ (12 ml) for 20 hr [21]. The product was purified by preparative TLC (Si gel, $CHCl_3$–petrol–C_6H_6–HOAc, 10:4:4:1). The 3-keto compound (**11**, 11 mg) thus obtained was diluted with the unlabelled substance (65 mg) and crystallized from EtOAc–petrol, a constant sp act and isotope ratio was reached after 1 crystallization (^{3}H = 51 dpm/mg, ^{14}C = 118 cpm/mg, ^{3}H:^{14}C = 0·43).

Acknowledgement—This work was supported in part by a grant from the Australian Research Grants Committee.

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