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BIOSYNTHESIS OF *ENT*-KAURANES: EVIDENCE FOR A C-17, C-16 HYDROGEN 1,2-SHIFT

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Abstract—The incorporation of *ent*-kaurenoic acid into the derived hydroxy acid and dihydroxy acid by *Beyeria calycina* has been studied. The biosynthesis of the hydroxy acid involves a hydrogen 1,2-shift from the C-17 position of *ent*-kaurenoic acid.

INTRODUCTION

The biosynthesis of diterpenes in whole plants has received relatively little attention in the past. This partly can be ascribed to the difficulty of incorporating mevalonic acid into plant diterpenes which imposes limitations on the biosynthetic processes that can be investigated. More recently some attempts have been made to study secondary transformations in diterpene biosynthesis [1, 2] and in continuation of our work on this topic we have investigated the incorporation of labelled *ent*-kaurenoic acid into the tetracyclic diterpenes of *Beyeria calycina*. Previous investigations had shown that *B. calycina* contains *ent*-kaurenoic acid (**1**), the hydroxy acid (**2**) and the diol acid (**3**) as well as a number of bicyclic diterpenes and a flavone [3]. It was of particular interest to us to see if *ent*-kaurenoic acid could be incorporated into the other tetracyclic diterpenes by direct application to the leaves of the plant and, more importantly, to study the mechanism of conversion of **1** into **2**.

RESULTS AND DISCUSSION

For the incorporation studies samples of *ent*-kaurenoic acid (**1**) labelled with ^{14}C- and ^{3}H- at C-17 were prepared. *ent*-Kaurenoic acid-[17-^{14}C] (2.2×10^6 dpm/mg) was obtained by a Wittig reaction of the norketo acid (**4**) with methyltriphenylphosphonium iodide-[^{14}C].

ent-Kaurenoic acid-[17-$^{3}H_2$] (1.87×10^7 dpm/mg) was obtained similarly using the tritiated Wittig salt, prepared by exchange of the unlabelled salt in dioxan with tritiated water and triethylamine.

In a preliminary experiment *ent*-kaurenoic acid-[17-$^{3}H_2$] in acetone was applied as microdrops to the underside of the leaves of *B. calycina* seedlings over a period of 6 days. Two days after the last application the seedlings were harvested and the major acidic components, isolated as described previously [3], were recrystallized to constant specific activity. The results are shown in Table 1 (experiment 1) and indicate that *ent*-kaurenoic acid (**1**) was incorporated in significant amounts into the hydroxy acid (**2**) and the dihydroxy acid (**3**).

Consideration of the likely mechanisms for the conversion of **1** to **2** suggests four possibilities and these are summarised below (Scheme 1). Mechanism (a) is essentially an anti-Markownikoff addition of water to the double bond and has been postulated [4] for a similar conversion of *ent*-kaurene in barley seeds. Mechanisms (b_1) and (b_2) require formation of an epoxide and epoxide ring opening with transfer of one of the hydrogens originally vinylic (C-17) leading to an aldehyde, directly (b_1) or by way of the diol (b_2).

These mechanisms have a number of chemical analogies in the acid catalysed reactions of epoxides and 1,2-diols [5]. Reduction of the aldehyde to the alcohol is unexceptional and has adequate precedents. Mechanism (c) is analogous to that observed for the dioldehydrase reaction and is distinguishable from (b) since in the coenzyme B_{12} dependent reaction only a small amount of H_1 is transferred to the substrate due to exchange of this proton on the enzyme complex [6, 7]. (It is well known that vitamin B_{12} does not occur in plants and mechanism (c) is proposed only as an analogy without specification of the coenzyme involved). Mechanism (d) involves a dehydratase reaction leading to an enol which would tautomerize accepting a proton from the medium. A distinction between the four mechanisms can be made by considering the fate of the vinylic protons in **1** in the transformation to the hydroxy acid (**2**).

Doubly-labelled *ent*-kaurenoic acid for experiments 2 and 3 (Table 1) was prepared by mixing the two singly-labelled species to obtain a ^{3}H:^{14}C ratio of 42.5:1 and 5.7:1 respectively. Experiment 2 was designed to determine if a pathway analogous to that shown for mechanism (c) was operating. The results of the incorporation experiments are shown in Table 1.

ent-Kaurenoic acid was found to be consistently incorporated into the hydroxy acid (**2**) and the dihydroxy acid (**3**) the two major diterpenoid constituents of the resin of *B. calycina*. Although the levels of incorporation differ this is expected in view of the known seasonal variations of resin production in the species of this genus [8].

Table 1. Incorporation of labelled *ent*-kaurenoic acid

Experiment	Compound	Weight (mg)	Specific activity (dpm/mg)		Ratio $^3H:^{14}C$	% Incorporation
			^3H	^{14}C		
1. (July)	*ent*-kaurenoic acid (**1**)					
	administered	12.9	1.2×10^5			
	recovered	20	2.7×10^4			(30% recovered)
	hydroxy acid (**2**)	6	9.6×10^3			2.5
	dihydroxy acid (**3**)	5	8×10^2			0.4
2. (September)	*ent*-kaurenoic acid (**1**)					
	administered	2.5	1.87×10^7	4.4×10^5	42.5	
	recovered	3.8	2.4×10^6	5.8×10^4	41.4	(20% recovered)
	hydroxy acid (**2**)	14.2	8.9×10^3	1.9×10^2	46.8	0.3
	dihydroxy acid (**3**)	6	1.3×10^4	2.2×10^2	59.0	0.14
	diester (**6**) from **2**		5×10^2	2.3×10^1	21.7	
3. (May)	*ent*-kaurenoic acid (**1**)					
	administered	5	1.24×10^7	2.2×10^6	5.7	
	recovered	6	1.4×10^6	2.6×10^5	5.4	(15% recovered)
	hydroxy acid (**2**)	6.4	3.9×10^3	5.8×10^2	6.7	0.04
	dihydroxy acid (**3**)	3	2.8×10^4	5×10^3	5.6	0.16
	diester (**6**) from endogenous **5**	1.5	3×10^3	1.1×10^3	2.7	0.02
	diester (synthetic) from **2**		7.6×10^2	2.3×10^2	3.3	

More importantly experiments 2 and 3 clearly show that in the conversion of **1** to **2** no tritium label was lost. These results can be taken as evidence against mechanisms (c) and (d) for which a loss of some of the tritium label is predicted.

Oxidation of the labelled hydroxy acid (**2**) yielded the diacid (**5**) which was characterized as the dimethyl ester (**6**). The $^3H:^{14}C$ ratio of this compound (experiment 2 and 3) indicated a loss of ~50% of the tritium which must have been located at C-17.

In experiment 3 the diacid (**5**), a minor constituent of the plant, was also isolated and purified through its diester derivative. After recrystallization to constant specific activity the $^3H:^{14}C$ ratio (2.7:1) showed that 50% of the tritium was retained in a position other than C-17. Mechanism (a) which requires retention of all the label at C-17 therefore can be excluded.

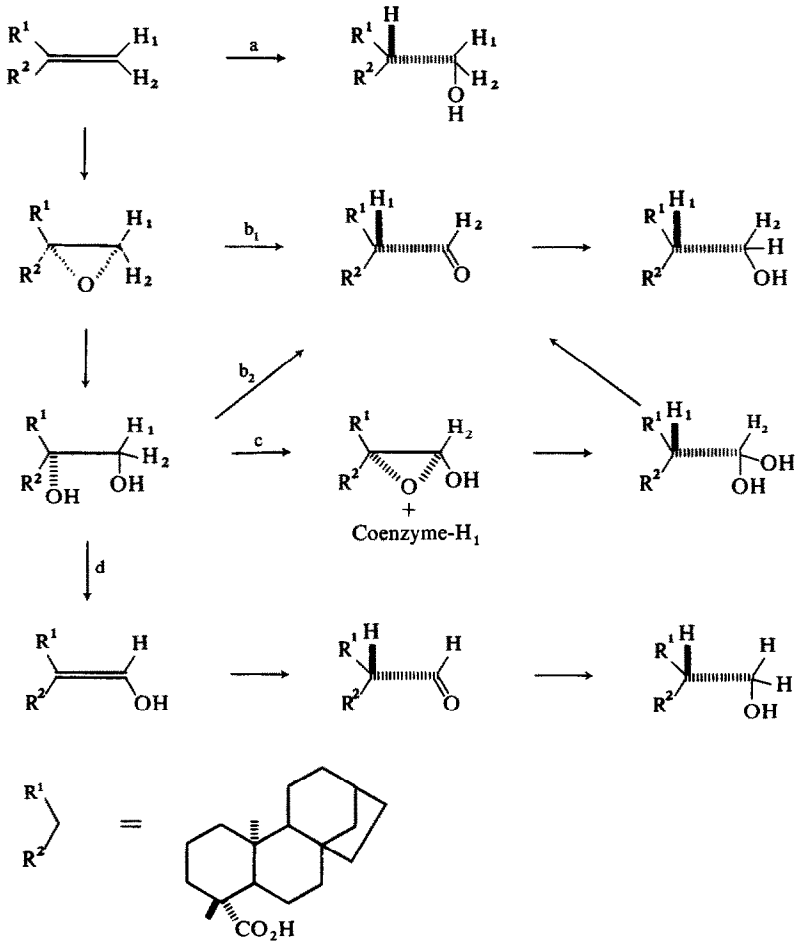
To determine if a mechanism of type (b) was operating we attempted to establish the presence of tritium at C-16 in the labelled hydroxy acid and the diester. Chemical conversion of the hydroxy acid from experiment 2 to *ent*-kaurenoic acid via an established sequence [9] gave a sample of the latter which showed a $^3H:^{14}C$ ratio of 8:1 instead of the expected ratio (22:1). This result could be explained by assuming an isotope effect in the elimination step of the reaction sequence. Equilibration of the diester (**6**), obtained from the labelled hydroxy acid (experiment 2), with potassium *t*-butoxide in *t*-BuOH resulted in the loss of only *ca* 50% of the tritium originally present in the diester. Again the operation of an isotope effect would not favour complete exchange of the tritium at C-16 but this result also suggested that tritium was present at positions other than C-16 and C-17.

Although an initial check on the tritium labelled *ent*-kaurenoic acid used in experiment 1 had shown that a negligible amount of tritium was present at C-15 a similar check on a sample prepared subsequently showed that some tritium scrambling occurs during the Wittig reaction used for the preparation of the labelled substrate. Similar observations have since been reported [10, 11] and in our case the amount of tritium which can be exchanged into the C-15 position has been found to vary from almost 0 to ~20%. The method used to determine this involved an $OsO_4/NaIO_4$ oxidation in neutral conditions [12] of the labelled *ent*-kaurenoic acid to the norketone (**4**).

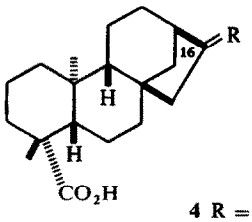
In another attempt to determine the amount of tritium at C-16 the hydroxy acid was methylated and the hydroxy ester was oxidized to the aldehyde (**8**) with pyridinium chlorochromate. [13]. Bayer–Villiger oxidation of the aldehyde gave an intermediate formate which was hydrolysed to the norhydroxy ester (**9**). Oxidation of the latter with pyridinium chlorochromate in a buffered solution [13] gave the keto ester (**10**). A comparison of the specific activity of the labelled hydroxy acid (**2**; 5×10^3 dpm/mg) norhydroxy ester (**9**, 3.26×10^3 dpm/mg) and keto ester (**10**, 1×10^3 dpm/mg) used in this sequence indicated that similar levels of tritium were present at C-17 and C-16. These results indicate that approximately half of the tritium label originally located at C-17 in *ent*-kaurenoic acid was transferred to C-16 in the transformation of this compound to the hydroxy acid (**2**) and provide evidence that a mechanism of type (b) is operating.

The mechanism requires an aldehyde as an intermediate in the conversion. However, attempts to detect this compound as a labelled species in the crude extract were unsuccessful. In fact an authentic sample of aldehyde, prepared as a standard, was found to be rapidly air oxidized to the corresponding acid.

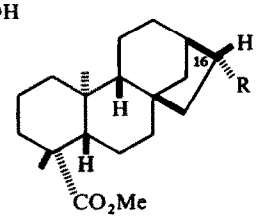
A further point requiring clarification is whether the epoxide and/or diol are intermediates. The formation of diols from epoxides is now well recognised in plant metabolism [14]. However, the diol could also be an



Scheme 1.



1 R = CH_2
2 R = CH_2OH
3 R = CH_2OH, OH
4 R = O
5 R = CO_2H
11 R = CH_2, O



6 R = CO_2Me
7 R = CH_2OH
8 R = CHO
9 R = OH
10 16-ketone

intermediate in the conversion (mechanism b_2). 16,17-Dihydroxy-*ent*-kaurenoic acid-[17-$^3H_2$] was fed to the plant but no incorporation into the hydroxy acid was observed, a result which could be rationalized by considering the insolubility of the dihydroxy acid.

Given the instability of the epoxide acid (**11**) which is predisoposed to polymerization, no attempt was made to test this compound as a precursor. Thus it appears that a distinction between mechanism b_1 and b_2 will require the use of a homogenate preparation.

In one experiment we had observed that the dihydroxy acid accumulated ^3H compared to the hydroxy acid. Furthermore, the ^3H:^{14}C ratio of the *ent*-kaurenoic acid recovered was significantly lower than that of the acid administered to the plant. These preliminary results suggest that the distribution of label in the products may be subject to isotope effects and/or other controls, e.g. varying (season-dependent) rates of formation of the compounds. It is noteworthy that the total incorporation of tritium in the hydroxy acid and dihydroxy acid varied from 1:3 to 14:1. The nature of these controls as well as the problem of determining whether the hydrogen transfer is stereospecific are presently under investigation.

At the commencement of this work the mechanism proposed for the conversion of **1** → **2** involving a 1,2-hydrogen shift had been observed only in the biological hydroxylation of aromatic compounds (NIH shift) [15]. Since then it has been shown that a similar hydrogen shift occurs in the transformation of mevalonic acid to verrucarinic acid. [12].

EXPERIMENTAL

General experimental details were as described previously [1].

Preparation of methyltriphenylphosphonium iodide[^3H]. Methyl triphenylphosphonium iodide (120 mg)– was dried over P_2O_5 under vacuum for 12 hr and dissolved in dry dioxan (1 ml). Triethylamine (1 drop) and ^3H_2O (50 μl, ~5 m Ci/μl) were added and the mixture heated at 100° for 24 hr in a sealed ampoule. HI (2 drops) was added and the solvents removed under vacuum. The residual salt was dried over P_2O_5. Conditions for the exchange reaction were established by using D_2O and observing the disappearance of the methyl doublet (δ 3.2, J = 14 Hz) in the NMR spectrum.

ent-*Kaurenoic acid*-[*17-*$^3H_2$]. Butyl lithium (0.1 ml, 2.8 × 10^{-3} mol/ml) was added to a suspension of methyltriphenylphosphonium iodide-[^3H] (100 mg) in THF (10 ml) under N_2. After 15 min the norketo acid (**4**, 25 mg) in THF (5 ml) was added and the soln stirred for 18 hr then refluxed for 3 hr. The mixture was diluted with H_2O, acidified (5% HCl), extracted with Et_2O, and the Et_2O layer extracted with 3% NaOH. The NaOH extract was acidified (5% HCl) and extracted with Et_2O. The organic layer was washed, dried and evapn of the Et_2O gave a residue (20 mg) which was purified by PLC to yield *ent*-kaurenoic acid-[17-$^3H_2$] (**1**, 5 mg). This was diluted with unlabelled acid (5 mg) and recrystallized to constant sp. act. (1.87 × 10^7 dpm/mg; 5.65 × 10^9 dpm/mol).

ent-*Kaurenoic acid*-[*17-*^{14}C]. Methyltriphenylphosphonium iodide-[^{14}C] was prepared by adding methyl iodide-[^{14}C] (55 μCi) to triphenylphosphine (200 mg) in Et_2O (10 ml). After 3 hr at room temp. excess MeI was added and the soln was left for 3 hr. The Et_2O was removed and the residual salt dried over P_2O_5 under vacuum. Reaction of this salt (250 mg) with the norketo acid (**4**, 50 mg) under the conditions described above yielded *ent*-kaurenoic acid-[17-^{14}C] which was recrystallized from Me_2CO–petrol to constant sp. act. 2.2 × 10^6 dpm/mg).

16,17-Dihydroxy-ent-*kaurenoic acid*-[*17-*$^3H_2$]. *ent*-Kaurenoic acid-[17-$^3H_2$] (15 mg) in Py (3 ml) was stirred with OsO_4 (20 mg) for 16 hr. Sodium metabisulphite (150 mg) in H_2O (2.5 ml) was added and the soln stirred for 30 min. The compound recovered with Et_2O was purified by PLC to give the dihydroxy acid (9 mg) which was recrystallized from MeOH–EtOAc to constant sp. act. (1.3 × 10^5 dpm/mg).

Incorporation of ent-*kaurenoic acid* (**1**) *into* Beyeria calycina. The methods for experiment 2 are given here as representative of all the incorporation experiments. Doubly labelled *ent*-kaurenoic acid (**1**, 2.5 mg; ^3H 1.87 × 10^7 dpm/mg; ^{14}C 4.4 × 10^5 dpm/mg, ^3H: ^{14}C = 42.5:1) in Me_2CO (60 μl) was applied as microdrops to the underside of the leaves of *B. calycina* seedlings over a period of 6 days. The plant (1.12 g) was harvested on the eighth day and extracted with Et_2O. The crude extract (450 mg) was partitioned into satd $NaHCO_3$, 5% NaOH and neutral fractions. The $NaHCO_3$ fraction contained mainly the dihydroxy acid (**3**) whereas the NaOH fraction contained **1** (3.8 mg) and the hydroxy acid (**2**, 14.2 mg). The major components were separated by TLC and recrystallized to constant ^3H:^{14}C ratio. The dihydroxy acid (**3**, 6 mg) was methylated with ethereal CH_2N_2 and the ester formed recrystallized from C_6H_6–petrol to constant ^3H:^{14}C ratio. In experiment 3 a small quantity (1.5 mg) of the diacid (**5**) was isolated by PLC of the residue from the $NaHCO_3$ fraction. The sample was diluted with unlabelled diacid and characterized as the dimethyl ester (**6**). The results obtained from this, and all the other incorporation experiments, are given in Table 1.

Jones oxidation of the hydroxy acid (**2**). After dilution with unlabelled compound, a sample of hydroxyacid (**2**, 23 mg, ^3H:^{14}C = 46.8:1) from experiment 2 was treated with excess Jones reagent in Me_2CO for 15 min at room temp. The excess reagent was destroyed by the addition of a few drops of MeOH. The compound recovered with Et_2O was treated with CH_2N_2 and the diester (**6**, 20 mg) obtained after PLC was recrystallized from Me_2CO–petrol to constant ^3H:^{14}C ratio (21:7) and constant mp (107–108°; lit. [16] 108–109°).

Preparation of the norhydroxy ester (**9**). Hydroxy acid (**2**, 1.95 g) was treated with CH_2N_2 to give the ester (**7**), mp 115° (lit. [3] 115–116°) in quantitative yield. The ester was added to a stirred soln of pyridinium chlorochromate (1.5 g) in dry CH_2Cl_2 (30 ml). After 1.5 hr Et_2O (30 ml) was added and the solvent decanted and passed through a column of florisil. Evapn of the solvent gave the aldehyde (**8**, 450 mg), mp 100–103° (lit. [3] 101–103°). The aldehyde (450 mg) was stirred with *m*-chloroperbenzoic acid (600 mg) and *p*-toluene sulphonic acid (15 mg) in CH_2Cl_2 (20 ml) for 48 hr. The compound recovered with Et_2O was dissolved in alcoholic 5% NaOH and heated under reflux for 30 min. The soln was diluted, acidified and the organic material extracted with Et_2O. Evapn of the solvent gave a residue which was recrystallized from $CHCl_3$–petrol as needles of the norhydroxy ester (**9**, 250 mg), mp 174–175° (Found: C, 75.0; H, 9.7%. $C_{20}H_{32}O_3$ requires: C, 75.0; H, 10.1%). NMR (60 MHz; $CDCl_3$): δ 0.84 and 1.19 (*s*, tertiary methyls), 3.69 (*s*, $-CO_2\underline{CH}_3$); 4.13 (1H, *m*, $W_{1/2}$ = 10 Hz, 16-H).

Oxidation of the norhydroxy ester (**9**). The compound (100 mg) was added to a stirred suspension of pyridinium chlorochromate (250 mg) and NaOAc (25 mg) in dry CH_2Cl_2 (10 ml). After 2 hr the mixture was worked up as described above. The compound recovered was recrystallized from petrol as needles of the keto ester (**10**, 80 mg), mp 142–143° (lit. [3] 143°). Following the procedure outlined above, a sample of labelled hydroxy acid (**2**, 60 mg; ^3H, 5 × 10^3 dpm/mg) was converted to the norhydroxy ester (**9**, 45 mg; ^3H, 3.26 × 10^3 dpm/mg) which in turn was oxidized to the keto ester (**10**, 25 mg; ^3H, 1 × 10^3 dpm/mg).

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