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THE BIOSYNTHESIS OF 12α-HYDROXYLATED GIBBERELLINS IN A CELL-FREE SYSTEM FROM *CUCURBITA MAXIMA* ENDOSPERM

PETER HEDDEN*, JAN E. GRAEBE, MICHAEL H. BEALE†, PAUL GASKIN† and JAKE MACMILLAN†

Pflanzenphysiologisches Institut der Universität, 3400 Göttingen, Federal Republic of Germany; †A. R. C. Research Group, School of Chemistry, The University, Bristol BS8 1TS, U.K.

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; pumpkin; biosynthesis; cell-free system; gibberellins; 12α-hydroxylation.

Abstract—A previously unknown pathway for the biosynthesis of 12α-hydroxylated gibberellins was found in a cell-free system from *Cucurbita maxima* endosperm. The microsome fraction converts the gibberellin precursor GA_{12}-aldehyde simultaneously to GA_{12} and 12α-hydroxy-GA_{12}-aldehyde. The ratio of these products is pH-dependent: above pH 6.5, the production of GA_{12} is favoured, whilst below pH 6.5, 12α-hydroxy-GA_{12}-aldehyde is the predominant product. 12α-Hydroxy-GA_{12}-aldehyde is converted further by soluble enzymes to 12α-hydroxy-GA_{14}, 12α-hydroxy-GA_{15}, 12α-hydroxy-GA_{37} and several unidentified products. This conversion is optimal between pH 6.0 and 6.5 in contrast to the previously known conversion of GA_{12}-aldehyde to GA_{43} by soluble enzymes, which is optimal at pH 7.5. GA_{58}, a major 12α-hydroxylated endogenous constituent of *C. maxima* endosperm, was not obtained when 12α-hydroxy-GA_{12}-aldehyde was used as a substrate, but it was obtained together with GA_4 when GA_9 was incubated with a preparation containing both microsomal and soluble enzymes.

INTRODUCTION

The *in vitro* biosynthesis of gibberellins (GAs) from mevalonate has previously been shown in preparations endosperm of *Cucurbita maxima L.* This biosynthesis includes the conversion of the hydrocarbon *ent*-kaurene to GA_{12}-aldehyde by a series of microsomal mono-oxygenase-catalysed oxidations [1, 2] and the further conversion of GA_{12}-aldehyde to C_{20}- and C_{19}-GAs by soluble, α-ketoglutarate-dependent dioxygenases [3–5].

Identification of the GAs occurring endogenously in *C. maxima* [6] showed good agreement with the products obtained in the cell-free system with one exception: several 12α-hydroxylated GAs were found as major endogenous components in the endosperm. No 12α-hydroxylated GAs were obtained as products in the cell-free system although 7β,12α-dihydroxykaurenolide was [7]. This paper describes the biosynthesis of several of the naturally occurring 12α-hydroxylated GAs in the cell-free system under conditions that were not used in the previous studies. Some of the results have been mentioned in a symposium report [8].

RESULTS

Incubations with the microsomal system

We have previously shown that GA_{12}-aldehyde (**1**) is converted to GA_{12} (**2**) as sole product when it is incubated with the microsomal fraction from *C. maxima* endosperm at pH 7.5 [2]. This reaction requires NADPH. Preliminary experiments now showed that the same kind of incubation yields three products if it is done at pH 6.3.

In order to obtain sufficient quantities for the identification of the products, [^{14}C]GA_{12}-aldehyde was incubated on a semi-preparative scale with the 200000 *g* pellet, NADPH, magnesium chloride, phosphate buffer and ancymidol at pH 6.3. Ancymidol, which specifically inhibits the oxidation steps between *ent*-kaurene and *ent*-kaurenoic acid [9], was included to prevent the conversion of endogenous *ent*-kaurene and thus dilution of ^{14}C-label in the products [7]. The products were extracted, separated by TLC and analysed by GC/MS.

The most polar product, Fraction A (R_f 0.1, 5% of the products) was identified as GA_{53} (**3**) by comparison of its mass spectrum with that of an authentic sample. The molecular ion was accompanied by an $[M+8]^+$ ion, showing that GA_{53} was ^{14}C-labelled and thus a true product of the [^{14}C]GA_{12}-aldehyde. The relative heights of $[M]^+$ and $[M+8]^+$ were the same as for the substrate, showing that no dilution of the label had occurred. Fraction B (R_f 0.25, 29%) was the major product. Its MeTMSi derivative had a molecular ion at *m/z* 418, corresponding to a monohydroxylated GA_{12}-aldehyde, and also showed losses of 28 and 29 amu, characteristic of an aldehyde. The mass spectrum did not correspond to those of derivatized 3β-hydroxyGA_{12}-aldehyde (GA_{14}-aldehyde) [10] or 13-hydroxy-GA_{12}-aldehyde (GA_{53}-aldehyde) [11]. Since the microsomes from *C. maxima* endosperm were known to 12α-hydroxylate 7β-hydroxy-kaurenolide [7] and since 12α-hydroxylated GAs are present in the endosperm [6], fraction B was assumed to be 12α-hydroxy-GA_{12}-aldehyde (**4**). Proof for its identity will be given later, but it will be referred to henceforth by this name for convenience. Fraction C at R_f 0.4 (10%) was identified as GA_{12} (**2**) and fraction D at R_f 0.7 (56%) was

*Present address: East Malling Research Station, East Malling, Maidstone, Kent ME19 6BJ, U.K.

shown to be unconverted GA_{12}-aldehyde (**1**). Like GA_{53}, the other products also contained ^{14}C and were undiluted by endogenous products.

When GA_{12} and 12α-hydroxy-GA_{12}-aldehyde were reincubated with the 200000 *g* pellet, NADPH and $MgCl_2$ at pH 6.3, only GA_{12} was converted and to GA_{53}. Thus the pathway shown in Scheme 1 is proposed for the metabolism of GA_{12}-aldehyde by the microsomal fraction at low pH. 12α-Hydroxylation occurs at the stage of GA_{12}-aldehyde, whereas GA_{12} becomes hydroxylated at C-13. In order to determine whether 12α-hydroxylation might occur at an earlier stage yet in the biosynthetic sequence, 12α-hydroxy-[17-^{13}C,15,17-^{3}H]kaurenoic acid (**5**) was incubated with a 5000 *g* supernatant fraction of *C. maxima* endosperm. The substrate was 7β-hydroxylated in high yield to *ent*-7α,12β-dihydroxykaurenoic acid (**6**), but no ring contraction to the gibberellane skeleton occurred although the same preparation converted *ent*-kaurenoic acid to GA_{12}-aldehyde. Thus the presence of a 12α-hydroxy group prevents ring contraction and 12α-hydroxylation of intermediates earlier than GA_{12}-aldehyde is of no importance in the biosynthesis of 12α-hydroxylated GAs in this system.

Figure 1 shows the pH-dependence for the oxidation of GA_{12}-aldehyde by the microsomal enzymes. Above pH 7.0, the overall conversion of GA_{12}-aldehyde is high and the predominant product is GA_{12}. Below pH 6.5, the major metabolite is 12α-hydroxy-GA_{12}-aldehyde, but the total conversion is lower. The yield of 12α-hydroxy-GA_{12}-aldehyde was optimal at pH 6.0–6.5.

Some further properties of the 12α-hydroxylation were also examined. The reaction was dependent on NADPH.

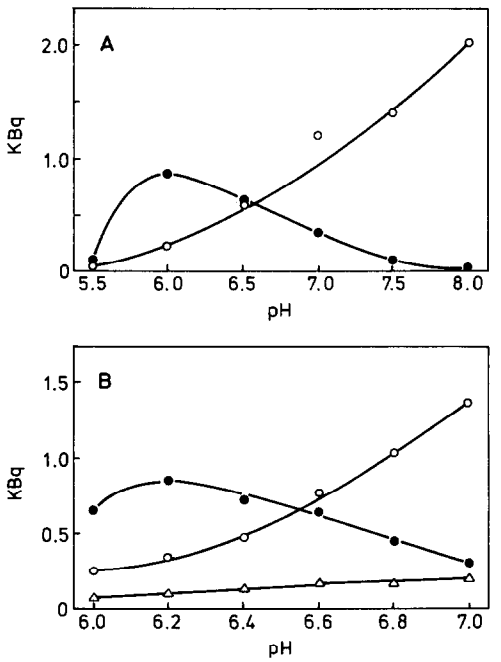


Fig. 1. Influence of pH on the microsomal conversion of GA_{12}-aldehyde to 12α-hydroxy-GA_{12}-aldehyde (●—●), GA_{12} (○—○) and GA_{53} (△—△). A, Only the two main products measured. B, Repeat at a different range of pH-values and with all products measured.

When GA_{12}-aldehyde was incubated with resuspended microsomes at pH 6.3 in an $^{18}O_2$-containing atmosphere, ^{18}O was incorporated into 12α-hydroxy-GA_{12}-aldehyde as shown by GC/MS. The mass spectrum of the MeTMSi derivative contained an $[M+2]^+$ ion at *m/z* 420, representing the incorporation of a single ^{18}O-atom as expected. Thus the 12α-hydroxylation has features similar to those of the other microsomal monooxygenases involved in GA biosynthesis.

Incubations with the high speed supernatant system

The high speed (200000 *g*) supernatant fraction of the *C. maxima* system converts GA_{12}-aldehyde (**1**) via several intermediates to GA_{43} (**8**) and GA_4 (**10**) as end products, the latter in low yield [4]. It seemed probable, therefore, that 12α-hydroxy-GA_{12}-aldehyde (**4**) would also be converted by the soluble oxidases and thus be a precursor of the 12α-hydroxylated GAs present in the endosperm [6]. Consequently, the ^{14}C-labelled fraction B, prepared as described above, was incubated with the 200000 *g* supernatant supplemented with α-ketoglutarate, ferrous sulphate and ascorbate, the cofactors known to be required for the conversion of GA_{12}-aldehyde [5]. A low pH (6.5) was chosen for this incubation, since pH-values below 7 were best for the conversion of 12α-hydroxy-GA_{12}-aldehyde in trial incubations.

Separation by TLC of the products obtained in the incubation revealed five radioactive bands. The two most polar bands at R_f 0.1 (9% of the products recovered) and R_f 0.3 (14%) were analysed by GC/MS as the MeTMSi derivatives. However, no components containing ^{14}C-label could be detected and the identities of these products remain unknown. The remaining three bands at R_f 0.42 (46%), 0.48 (20%) and 0.67 (11%) were methylated and rechromatographed in a different solvent system. The band at R_f 0.42 gave a single new band, which was found by GC/MS to contain 12α-hydroxy-GA_{37} (**14**). This product contained ^{14}C-label as evidenced by an $[M+8]^+$ ion. However, the ratio of the peaks showed that it had been diluted 13 times by endogenous material. Rechromatography of the band at R_f 0.48 gave three bands in the ratio 12:7:3 in order of increasing mobility. The major component contained 12α-hydroxy-GA_{14} (**9**), which had been diluted three times with endogenous compound. The second band contained a single ^{14}C-labelled component, which had been diluted to the same extent as 12α-hydroxy-GA_{14}. It gave a weak molecular ion at *m/z* 478, but its identity could not be determined. The third component was not detected by GC/MS. The band at R_f 0.67 gave two new radioactive bands after rechromatography of the methyl esters. Although the major component could not be detected, the minor band contained a compound with a mass spectrum identical to that of 12α-hydroxy-GA_{15} (**15**), except for the ^{14}C-isotope peaks. It had the same specific radioactivity as the substrate and was thus undiluted by endogenous material.

The fact that fraction B was converted to endogenous GAs that have been shown to be 12α-hydroxylated [6] establishes the position of hydroxylation in fraction B as 12α. This taken together with the mass spectrum defines the structure of the radioactive component in fraction B as 12α-hydroxy-GA_{12}-aldehyde (**4**).

GA_{58} (**11**), the major endogenous C_{19}-GA in *C. maxima* endosperm [6, 12], was not detected in any of the fractions analysed and thus was not a product of the

incubation with 12α-hydroxy-GA_{12}-aldehyde. A possible reason for the failure to obtain GA_{58} in the cell-free system could be the presence of endogenous GAs, such as 12α-hydroxy-GA_{14} (**9**), that may be intermediates in the biosynthesis of GA_{58} and forming pools, preventing significant incorporation of radioactivity in further metabolites. A 200000*g* supernatant was therefore filtered through Sephadex G-25 to remove all endogenous GAs and then incubated with 12α-hydroxy-[^{14}C]GA_{12}-aldehyde. A higher pH (7.0) was chosen for this incubation although it was suboptimal for the conversion of 12α-hydroxy-GA_{12}-aldehyde, since it would cause less rapid closure of the δ-lactones in 12α-hydroxy-GA_{15} (**15**) and 12α-hydroxy-GA_{37} (**14**), which are likely intermediates in the pathway. Such lactone formation has been shown to prevent further oxidation at C-20 and thus the formation of other GAs [5,13,14].

The 200000*g* supernatant fraction after gel filtration was incubated at pH 7.0 with 12α-hydroxy-[^{14}C]GA_{12}-aldehyde, magnesium chloride, ferrous sulphate, α-ketoglutarate and ascorbate. After separation of the products by TLC, three radioactive bands were detected at R_f 0.40, 0.46 and 0.60 in the ratio 15:7:17. The bands at R_f 0.40 and 0.46 were found by GC/MS to contain 12α-hydroxy-GA_{37} (**14**) and 12α-hydroxy-GA_{14} (**9**), respectively, as sole ^{14}C-labelled components. There was no dilution with endogenous products this time. GC/MS analysis of the band at R_f 0.6 revealed a ^{14}C-labelled component with $[M]^+$ at *m/z* 506 (accompanied by an $[M+8]^+$ ion at *m/z* 514). The mass spectrum also showed losses of 15, 32 and 90 amu from the ion at *m/z* 506, supporting its assignment as the molecular ion. Although the spectrum was characteristic of a dihydroxylated C_{19}-GA, it was different from that of GA_{58} or any other known GA. Since it was produced from an incubation of 12α-hydroxy-GA_{12}-aldehyde, it can be assigned the structure 12α,X-dihydroxy-GA_9, where X is not the 3-position.

The pH-dependence for the oxidation of 12α-hydroxy-GA_{12}-aldehyde by the 200000*g* supernatant is shown in Fig. 2A. The overall conversion of substrate and incorporation into the main products, 12α-hydroxy-GA_{14} (**9**) and 12α-hydroxy-GA_{37} (**14**), are both optimal between pH 6.0 and 6.5. This contrasts the conversion of GA_{12}-aldehyde to GA_{43}, which is optimal between pH 7.0 and 7.5 (Fig. 2B).

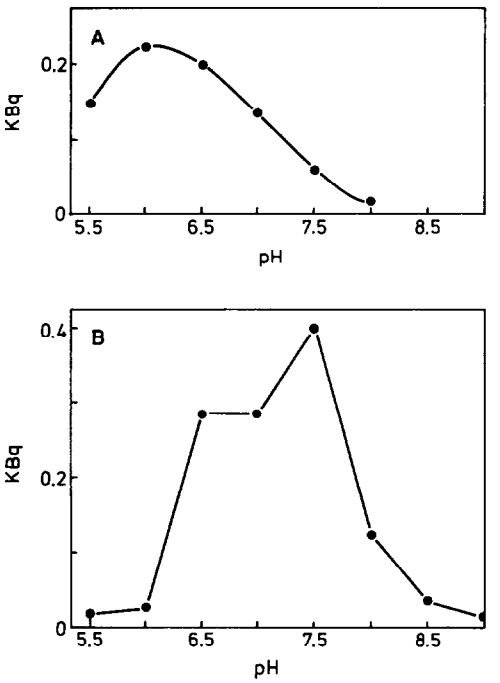


Fig. 2. Influence of pH on the conversions catalysed by the high speed supernatant fraction. A, Conversion of 12α-hydroxy-GA_{12}-aldehyde to 12α-hydroxy-GA_{14} and 12α-hydroxy-GA_{37} as the main products. B, Conversion of GA_{12}-aldehyde to GA_{13} and GA_{43} as the main products.

Incubation with [^{2}H]GA_9

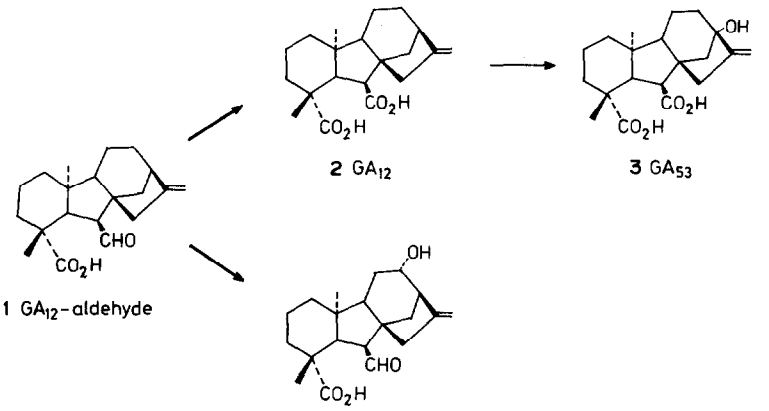
When [^{2}H]GA_9 (**12**) with a deuterium content of 0.92 atoms per molecule was incubated with dialysed 10000*g* supernatant preparation, [^{2}H]GA_4 (**10**) and [^{2}H]GA_{58} (**11**) were formed with a deuterium content of 0.86 atoms per mol. Gibberellins A_{13}, A_{43} and A_{49}, 12α-hydroxy-GA_{14} (**9**), 12α-hydroxy-GA_{37} (**14**), 7β,12α-dihydroxykaurenolide and *ent*-6α,7α-dihydroxykaurenoic acid were also detected by GC/MS, but all these compounds did not contain deuterium. The structures are shown in the preceding paper [6].

DISCUSSION

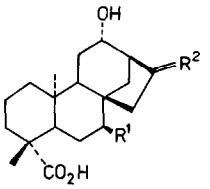
The conversion of GA_{12}-aldehyde (**1**) by microsomal preparations from *C. maxima* endosperm is both qualitatively and quantitatively pH-dependent. At low pH (6.0–6.5), hydroxylation at the 12α-position is favoured whereas above pH 6.5, oxidation in the 13-position predominates (Scheme 1). Above pH 7.0, 12α-hydroxylation becomes insignificant. Most probably, GA_{12}-aldehyde is the substrate for 12α-hydroxylation *in vivo*. Earlier intermediates cannot be considered for this role, since ring contraction does not take place when *ent*-12β-hydroxykaurenoic acid (**5**) is used as a substrate. Also GA_{12} (**2**) is not a substrate for 12α-hydroxylation, being instead 13-hydroxylated to GA_{53} (**3**). Thus GA_{12}-aldehyde lies at a branch-point in the GA pathway (Scheme 1). Its immediate products, GA_{12} and 12α-hydroxy-GA_{12}-aldehyde are oxidized further by soluble oxygenases giving rise to two pathways. 12α-Hydroxy-GA_{12}-aldehyde is not oxidized to 12α-hydroxy-GA_{12} by the microsomal system, which thus is specific for GA_{12}-aldehyde. The identification of GA_{53} (**3**) as a product was a surprise, since 13-hydroxylated GAs are not found in *C. maxima* endosperm, although small amounts of 13-hydroxylated *ent*-kaurenoic acids are [6]. GA_{12} is rapidly oxidized by the soluble oxidases and may never reach sufficiently high concentrations in the microsomal compartment for 13-hydroxylation to be of any importance *in vivo*.

The conversion of 12α-hydroxy-GA_{12}-aldehyde (**4**) to 12α-hydroxy-GA_{15} (**15**), -GA_{14} (**9**), -GA_{37} (**14**) and unidentified products by the 200000*g* supernatant proceeds best at low pH (6.0–7.0). This is in contrast to the conversion of GA_{12}-aldehyde (**1**) to GA_{43} (**8**), which is optimal above pH 7.0 (Fig. 1).

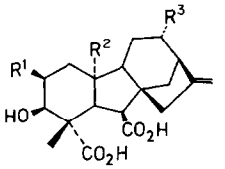
The failure to obtain GA_{58} (**11**), the major C_{19}-GA in *C. maxima* endosperm is noticeable. Since 12α-hydroxy-GA_{14} and 12α-hydroxy-GA_{37} accumulate both *in vivo*



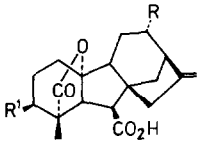
Scheme 1. Conversions of GA_{12}-aldehyde by the microsomal fraction from *C. maxima* endosperm.



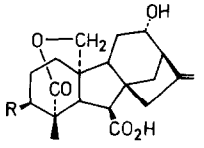
	R^1	R^2
5	H	CH_2
6	OH	CH_2
7	H	O



	R^1	R^2	R^3
8	OH	CO_2H	H
9	H	Me	OH



	R^1	R^2
10	OH	H
11	OH	OH
12	H	H
13	H	OH



	R
14	OH
15	H

and *in vitro*, they may not be intermediates on the pathway to GA_{58}. Instead GA_{58} could arise analogous to GA_4 *via* the open lactone form of 12α-hydroxy-GA_{15} (**15**) and 12α-hydroxy-GA_{36} [5, 15] (cf. Scheme 2 in ref. [6]), or it could be formed *via* 12α-hydroxy-GA_9 (**13**). In the latter case, 3β-hydroxylation would occur after the conversion to C_{19}-GAs. However, we have obtained no evidence for this; the only C_{19}-GA that was produced from 12α-hydroxy-GA_{12}-aldehyde was hydroxylated other than at the 3β-position. A third alternative would be the formation of GA_{58} via GA_9 (**12**) as suggested by the incorporation of [^{2}H]GA_9 into GA_4 (**10**) and GA_{58} by a preparation containing both microsomal and soluble components. In this case both the 3β- and the 12α-hydroxylations must have occurred at the C_{19}-stage. However, GA_9 has never been found among the products of the cell-free system, nor has it been identified as an endogenous GA in *C. maxima*. It is therefore possible that the conversion of GA_9 is due to nonspecific enzyme activity, not truly reflecting the metabolism *in vivo*. The route of formation of GA_{58} in the endosperm of *C. maxima* is therefore still unknown.

EXPERIMENTAL

Cell-free extracts. Fruits of field grown *C. maxima*, var.

'Riesenmelone, gelb vernetzt' were harvested when they had reached full size but the seeds were still immature. The endosperm was removed as described in ref. [16], homogenized lightly, centrifuged at 2000 *g* 5 min (pellet discarded) and dialysed (× 3) against K-Pi (0,05 M, pH 8) with $MgCl_2$ (2.5 mM). The preparations were frozen on solid CO_2 as pellets and stored in liquid N_2. The defrosted preparations are referred to as the 2000 *g* supernatant.

Substrates. RS-[2-^{14}C]Mevalonate (0.96 GBq/mmol) was prepared from the lactone by KOH hydrolysis. [^{14}C]GA_{12}-aldehyde was obtained by incubation of 2000 *g* supernatant fraction (7 ml) with 0.5 mM [2-^{14}C]MVA, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ATP, 5 mM PEP and 0.5 mM NADPH in a total vol. 10 ml for 2 hr at 30°. The products were extracted and separated by TLC in solvent system 1. Purity and sp. act. were determined by GC/MS. The sp. act. was lowered by the addition of non-radioactive GA_{12}-aldehyde whenever the experiments allowed this.

ent-[17-^{13}C,15,17-^{3}H]12*β-Hydroxykaur*-19-*en*-16-*oic acid. ent*-12β-Hydroxykaur-16-en-19-oic acid (**5**) (65 mg), isolated from *Helianthus decapetalus* [17], in THF–H_2O (1:1, 12 ml) was treated with OsO_4 (1 mg) and $NaIO_4$ (100 mg) at room-temp. overnight. The soln was concd in *in vacuo*, diluted with H_2O, acidified to pH 3 and extracted with EtOAc. The gum obtained by evaporation of the organic layer was eluted through a short column of silica gel in EtOAc–petrol (7:3) to give the norketone 7 (53 mg). This norketone in pyridine (0.5 ml) was treated with TMSiCl (150 μl) and HMDS (150 μl) at room temp. for 2 hr. The soln was then evaporated in a stream of N_2 and the residue taken up in EtOAc, filtered under N_2 through a small plug of celite and evaporated. The resulting silylated norketone was treated with supernatant ylide soln (2.5 ml) prepared from $P^+Ph_3{}^{13}C^3H_3Br^-$ (2.46 g), NaH (638 mg of 50% oil dispersion, washed with petrol) and THF (20 ml) in the normal way [18]. After 1 hr, Me_2CO was added and the reaction mixture poured into dil. HCl and extracted with EtOAc. The wet EtOAc extract was allowed to stand for 2 days until hydrolysis of the TMSi protecting group was complete (TLC monitoring). The gum obtained by evaporation was eluted through a short column of silica gel in EtOAc–petrol (1:1). The recovered product was then partitioned between 2 M KOH and EtOAc. Acidification of the KOH-layer and recovery in EtOAc gave 12α-hydroxy-[17-^{13}C, 15,17-^{3}H]kaur-16-en-19-oic acid (25.5 mg, sp. act. 8.18 GBq, 90.7 atom % ^{13}C). [3-^{2}H]GA_9 (0.92 ^{2}H-atoms/mol) was prepared as described in ref. [18].

GC/MS. Methylated and trimethylsilylated samples were injected (260°) into a fused SiO_2 capillary column (WCOT, OV-101, 25 m × 0.25 mm) by the Grob splitless method. The column was maintained at 50° for 1 min, then programmed at 15°/min to 200° and at 4°/min to 260°. The He flow rate was 2 ml/min and the column effluent was lead directly into the source (290°). The electron energy was 70 eV, the emission current 0.21 mA. For the experiment with [3-^{2}H]GA_9, a 2% SE-33 packed column was used under conditions as described in ref. [19].

Extraction and TLC of products. After incubation, the pH of the reaction mixture was adjusted to 2.5, Me_2CO was added and the products were extracted (× 3) with EtOAc. The organic extract was washed with a little H_2O and evaporated to dryness. TLC was on silica gel in solvent systems (1) $CHCl_3$–EtOAC–HOAc, in (1a) (70:30:1), in (1b) (30:70:1), in (1c) (20:80:1), in (1d) (10:90:1) and (2) petrol (40–60°)–EtOAc (1:1). For GC/MS, the eluted products were methylated with ethereal CH_2N_2 and trimethylsilylated with MSTFA (90°, 30 min). When quantitative data were obtained by liquid scintillation counting of the product on silica gel, the counts were corrected for adsorption and quenching.

Incubation with microsomes at pH 6.3. A microsomal fraction was prepared by centrifugation of the 2000 *g* supernatant (28 ml) at 200 000 *g* for 1 hr. The pellet was resuspended in 50 mM K-Pi (pH 7.5, 28 ml) with 5 mM $MgCl_2$ and sedimented again. The resulting pellet was resuspended in 50 mM K-Pi (pH 6.2, 15 ml), giving a final pH of 6.3. [^{14}C]GA_{12}-aldehyde (**1**) (1.2 GBq, sp. act. 5.0 GBq/mmol) in Me_2CO (25 μl) was added to the microsomal suspension, containing 4.5 mM $MgCl_2$, 0.9 mM NADPH and 1.0 μM ancymidol in a total vol of 16 ml. After incubation for 2 hr at 30°, the products were extracted, separated in solvent system 1a and identified by GC/MS of the MeTMSi-derivatives as follows: Product A at R_f 0.1—GA_{53} (**3**); Product B at R_f 0.25—a monohydroxy-GA_{12}-aldehyde; Product C at R_f 0.4—GA_{12} (**2**) and product D at R_f 0.7—GA_{12}-aldehyde (**1**) (substrate).

Products B and C (0.25 nmol) were reincubated with the microsomal suspension (125 μl) and cofactors as before. Only product B was further converted to a single product, migrating like A on TLC. To confirm the identity of this product, [^{14}C]GA_{12} (3.5 nmol) was incubated with the microsomal suspension (2.0 ml) at pH 6.3. The product was identified as GA_{53} by GC/MS.

Incubations with microsomes at different pH-values. Six aliquots (0.5 ml) of 2000 *g* supernatant were centrifuged at 200 000 *g* for 1.5 hr. The pellets were resuspended in 50 mM K-Pi (0.25 ml) at different pH-values, either between pH 5.5 and 8.0 in steps of 0.5 units or between pH 6.0 and 7.0 in steps of 0.2 units. The suspensions were incubated for 2 hr at 30° with [^{14}C]GA_{12}-aldehyde (0.3 nmol, sp. act. *ca* 1.48 GBq/mmol), 5 mM $MgCl_2$ and 1 mM NADPH (total vol. 3 ml). The products were separated by TLC in solvent system 1a and counted by liquid scintillation on the silica gel (Fig. 1).

Incubations in the presence of $^{18}O_2$. A microsomal pellet prepared as above from 2000 *g* supernatant (4 ml) was resuspended in 50 mM K-Pi (pH 6.2, 2 ml), containing $MgCl_2$ (10 μmol), NADPH (2 μmol) and ancymidol (4 nmol), and freeze-dried. After an atmos. of N_2–$^{18}O_2$ (4:1) had been introduced, degassed H_2O (1 ml) and [^{14}C]GA_{12}-aldehyde (**1**) (15.8 nmol, 170 MBq/mmol) in Me_2CO (5 μl) were injected through a rubber septum and the mixture was incubated for 3 hr at 30° in the sealed system. The products were extracted, separated and analysed by GC/MS of the MeTMSi-derivatives.

Incubation with ent-12*β-hydroxy*-[17-^{13}C,16,17-^{3}H]*kaur*-16-*en*-19-*oic acid.* This substrate (**5**) (20 nmol) was added in Me_2CO (20 μl) to a 2000 *g* supernatant fraction (5 ml) containing 7.5 mM $MgCl_2$, 1.0 mM $MnCl_2$, 5.0 mM ATP, 5.0 mM PEP, 0.5 mM NADPH and 50 mM K-Pi (pH 7.6) in a total vol. of 6 ml. The mixture was incubated for 2 hr at 30°. GC/MS of the extracted and derivatized products revealed *ent*-7α,12β-dihydroxy-kaur-16-en-19-oic acid (**6**) as the only ^{13}C-labelled component.

Incubation of 12α-*hydroxy*-[^{14}C]*GA*$_{12}$-*aldehyde with the high speed supernatant.* (a) *Without gel filtration.* 12α-Hydroxy-[^{14}C]GA_{12}-aldehyde (**4**) (15.8 nmol, 4.96 GBq/mmol) was added in MeOH (30 μl) to the 200 000 *g* supernatant fraction (7.0 ml), which had been adjusted to pH 6.5 and supplemented with 5 mM α-ketoglutarate, 5 mM ascorbate and 0.5 mM $FeSO_4$ [5]. After incubation for 2 hr at 30°, the products were extracted and separated in solvent system 1b. Five radioactive bands were detected and eluted. The material from the two bands with the lowest R_f-values was derivatized and analysed by GC/MS directly, whereas the material from the other three bands was first methylated and rechromatographed in solvent system 2. In order of increasing R_f-values, they were resolved into one, three and two radioactive bands, respectively, which were eluted, derivatized and analysed by GC/MS. (b) *With gel filtration.* The 200 000 *g* supernatant fraction (2.2 ml) was filtered through a Sephadex G-25 column, re-equilibrated with 50 mM K-Pi

(pH 7.0), containing 2.5 mM $MgCl_2$, and eluted with the same buffer. Fractions absorbing at 280 and 260 nm were pooled (9 ml) and stored in liquid N_2. 12α-Hydroxy-[^{14}C]GA_{12}-aldehyde (5.0 nmol, 4.96 GBq/mmol) was added in MeOH (30 μl) to the gel filtered preparation (5.0 ml), containing cofactors as before. After incubation for 2 hr at 30°, the products were extracted with EtOAc and BuOH. Chromatography of the combined extracts in solvent system 1b gave three radioactive bands, which were eluted, derivatized and analysed by GC/MS.

(c) *Incubation at different pH-values.* Six 200 000 *g* supernatant aliquots (300 μl) were supplemented with α-ketoglutarate, ascorbate and $FeSO_4$ to give the final concns described above in 330 μl. The pH was adjusted to different values between 5.5 and 8.0 in intervals of 0.5 units and 200 μl of each was incubated for 1 hr at 30° with 12α-hydroxy-[^{14}C]GA_{12}-aldehyde (**4**) (0.2 nmol, 4.96 GBq/mmol), added in MeOH (4 μl). Extraction and separation of the products in solvent system 1c yielded four radioactive bands from each sample, which were counted by liquid scintillation. From the large scale incubations, it was known that the most polar band contained predominantly 12α-hydroxy-GA_{14} (**9**) and 12α-hydroxy-GA_{37} (**14**), whereas the least polar band contained starting material (**4**).

Incubations of [^{14}C]GA_{12}*-aldehyde with the high-speed supernatant at different* pH*-values.* Eight 200 000 *g* supernatant aliquots (100 μl) were diluted 1:1 with 50 mM K-Pi at different pH values between 5.5 and 9.0 at intervals of 0.5 units. The pH of each aliquot was adjusted to the required value when necessary. Each aliquot was incubated for 15 min at 30° with [^{14}C]GA_{12}-aldehyde (**1**) (0.5 nmol, 1.3 GBq/mmol) and 0.5 mM $FeSO_4$. The products were separated first with solvent system 1a to 15 cm, then with solvent system 1d to 8 cm. The major, most polar products, which are known to be GA_{13} and GA_{43} [4], were counted by liquid scintillation.

Incubation with [3-$^2H_1$]GA_9. A 10 000 *g* supernatant fraction (pH 7.6, 20 ml) was incubated with [3-$^2H_1$]GA_9 (**12**) (4.5 μmol, 0.92 ^{2}H-atoms/mol), 5 mM $MgCl_2$, 5 mM ATP, 10 mM PEP, 0.5 mM NAD and 0.1 mM $FeSO_4$ in a total vol. of 30 ml for 2 hr at 30°. The products were extracted and dissolved in 0.05 M K-Pi (0.8 ml, pH 8.0). Neutral lipids were extracted with petrol and discarded. Acidic components were extracted into EtOAc at pH 3. Gibberellins A_4, A_{13}, A_{43}, A_{49} and A_{58}, 7β,12α-dihydroxykaurenolide, 12α-hydroxy-GA_{14}, 12α-hydroxy-GA_{37} and *ent*-6α,7α-dihydroxykaurenoic acid were identified by GC/MS of the MeTMSi-derivatives. Only GA_4 (**10**) and GA_{58} (**11**) contained deuterium (0.86 atoms/mol).

Reproducibility. The formation of 12α-hydroxy-GA_{12}-aldehyde at low pH and GA_{12} at higher pH is now routinely used in our laboratory for the preparation of these compounds to be used as substrates. The orderly pH-curve was done twice with the results shown in Fig. 1. The conversion of 12α-hydroxy-GA_{12}-aldehyde was also found in numerous experiments to proceed better at low pH; the optimum was determined in two experiments, one of which is shown in Fig. 2. The pH-optimum for the conversion of GA_{12}-aldehyde has been determined several times with identical results. All enzymes conversions were shown to be real by the use of heat denatured controls.

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