PERKIN 1

Probing the mechanism of loss of carbon-20 in gibberellin biosynthesis. Synthesis of gibberellin 3α,20-hemiacetal and 19,20-lactol analogues and their metabolism by a recombinant GA 20-oxidase

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Received (in Cambridge, UK) 1st August 2001, Accepted 20th November 2001
First published as an Advance Article on the web 21st December 2001

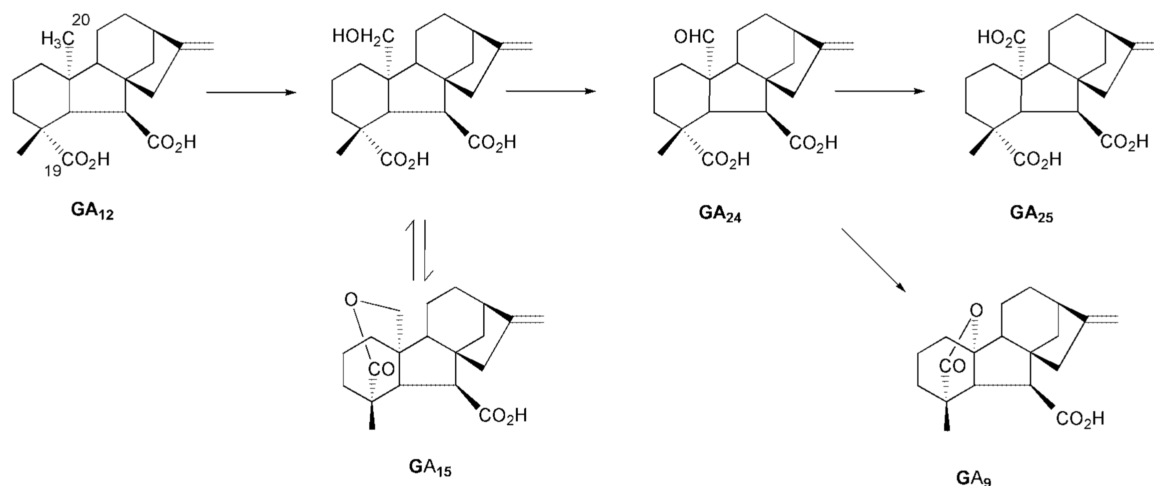
A gibberellin 3α,20-hemiacetal, **1** (equivalent to 3-*epi*-gibberellin A_{36}), has been synthesised from gibberellin A_{13}. Turnover of this hemiacetal by recombinant gibberellin 20-oxidase occurred with loss of the 20-carbon atom to give the 20-nor-19,10-lactone (3-*epi*-gibberellin A_4). In addition, two other enzyme products were detected and identified by synthesis as 3-*epi*-20-norgibberellin A_{13} and 3-*epi*-1,10-didehydro-20-norgibberellin A_{13}. These by-products indicate that the enzymatic reaction proceeds *via* a C-10 radical intermediate. Carbon radicals at C-10 and acyl radicals at C-20 were generated chemically and their decomposition products were studied with reference to the biological mechanism. The corresponding 19-nor-3α,20-hemiacetal **3** and 19-methylene analogue **4** were synthesised but were not oxidised at C-20 by the enzyme. These results indicate that the 19-carboxylic acid is an essential component of the enzyme reaction.

Introduction

The gibberellins (GAs) are well-known regulatory molecules important in plant growth and development.[1] They are tetracyclic diterpenoids that are biosynthesised from geranylgeranyl diphosphate by the action of two terpene cyclases followed by a series of oxidative modifications.[2] One of the most important sequences in the several parallel pathways, leading to hormonally active C_{19}-GAs, is the successive oxidation of the C-20 methyl group, as shown in Scheme 1 for the basic non-3β and 13-hydroxy pathway. Recently, genes encoding GA 20-oxidases have been cloned from a number of plant species including pumpkin,[3] *Arabidopsis thaliana*,[4] spinach[5] and pea.[6] These enzymes are 2-oxoglutarate-dependent dioxygenases, an important class of soluble, Fe^{II}-containing, oxygenases.[7] The availability of recombinant enzyme from expression in *E. coli* has allowed us to establish that the conversion of GA_{12} to GA_9 and GA_{25} (Scheme 1) is the result of action of a single protein with multifunctional enzyme activity.[3] We have also demonstrated[8] that the 20-*pro-R* hydrogen is stereospecifically abstracted in the conversion of the 20-alcohol GA_{15}, in its open lactone form, to the aldehyde GA_{24}. In this paper we report on mechanistic investigations into the next step of the sequence: the conversion of the 20-aldehyde into the 20-nor-19,10-γ-lactone function, a characteristic structural feature of the biologically active C_{19}-GAs.

Results and discussion

The enzymic loss of the 20-carbon atom is known to occur at the 20-aldehyde stage, as the 20-carboxylic acid, itself a product of the 20-oxidase activity, is not an intermediate.[9] The mechan-



Scheme 1 Reactions catalysed by GA 20-oxidases.

 DOI: 10.1039/b106990j

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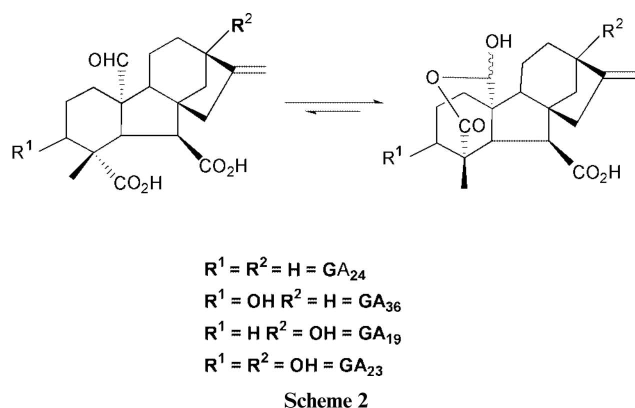
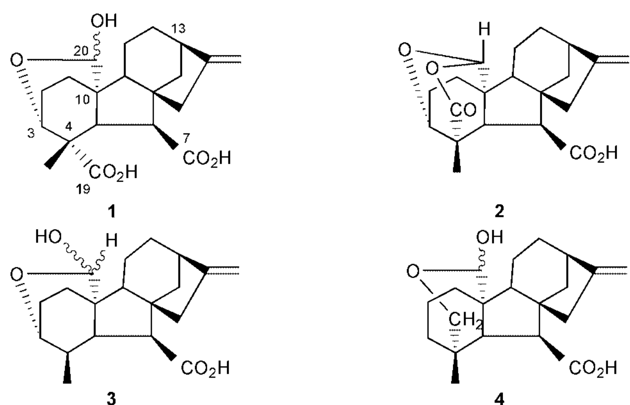
ism for the conversion of the 20-aldehyde to the 19,10-γ-lactone has not been determined. However, the various possibilities have been analysed by MacMillan.[2] Loss of an angular carbon at the aldehyde oxidation level occurs in the biosynthesis of oestrogen and other steroids.[10] In these cases the reaction involves vicinal elimination of formate. In C_{19}-GA biosynthesis, however, it is apparent, from studies with the fungus *Gibberella fujikuroi*, that the vicinal hydrogens at C-1, -5 and -9 are retained in the C-10/-20 bond cleavage reaction.[11] It is also known that, in the fungus, both oxygen atoms of the lactone arise from the 19-carboxylate group.[12] Carbon-20 is ultimately lost as CO_2 both in the fungus[13] and in cell-free systems from plants.[14]

In solution, the GA 20-aldehydes, such as GA_{24}, GA_{19}, GA_{36} and GA_{23} exist in a dynamic equilibrium between the 19,20-lactol form and the open 20-aldehyde, 19-carboxylate, with the lactol form predominating at room temperature in organic solvents[15,16] (Scheme 2). Presumably, one of these forms preferentially binds to the 20-oxidase active site and undergoes the carbon–carbon cleavage reaction, leading to the 19,10-lactone. To probe the lactol–aldehyde equilibrium, and to gain some insight into the conformation of the aldehydic substrate accepted by the enzyme, we have investigated the metabolism of synthetic analogues **1** and **2** containing a 3α-hydroxy group in the aldehyde structure.



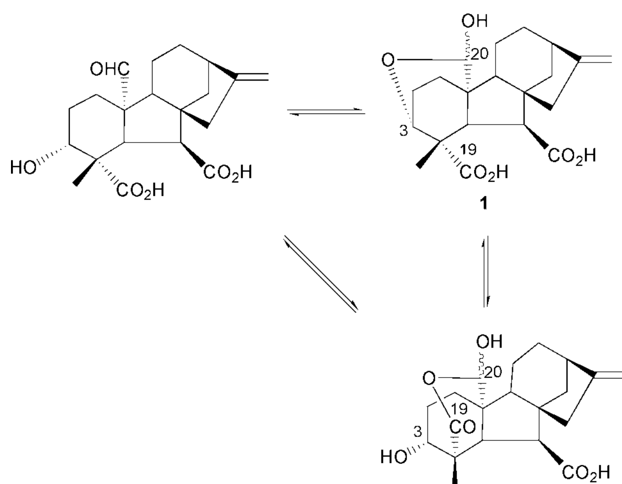
Scheme 2



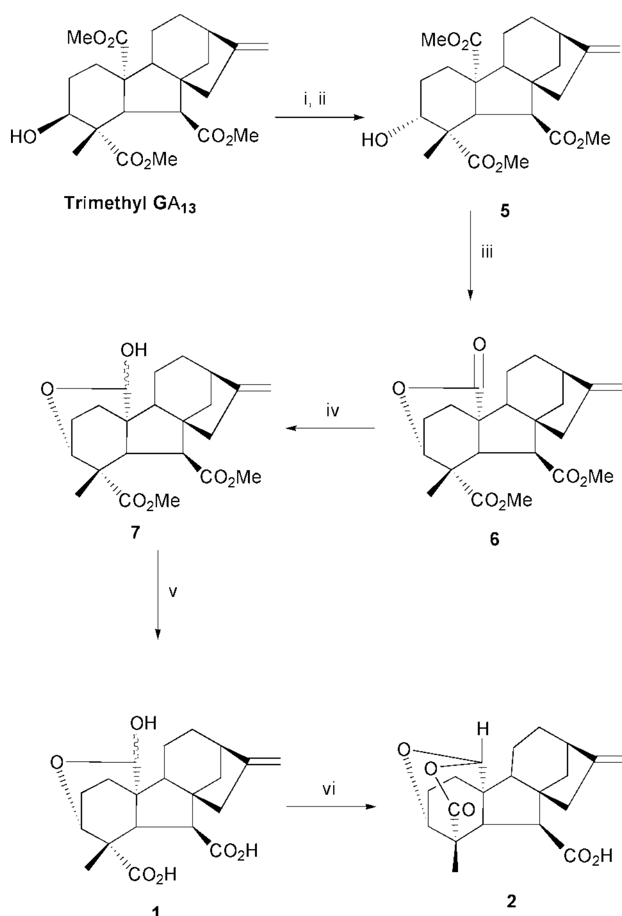
We anticipated that in the target compound **1** new equilibria would be set up as shown in Scheme 3. In addition we have probed the role of the 19-carbonyl function in the enzyme reaction by use of the 19-nor analogue **3** and the 19,20-hemiacetal analogue **4**.

Synthesis and conformation of substrate analogues

3α,20-Hemiacetals 1 and 2. The synthesis of **1** and **2** is outlined in Scheme 4, and represents a relatively short route to a GA C-20 aldehyde. These hindered aldehydes have previously proven very difficult to access. The only published route is that of Dawe *et al.*[17] who describe the conversion of GA_3 (a C_{19}-GA) into GA_{19}, C-20 being added *via* an intramolecular addition of a carbene generated from a C-19-diazomethyl ketone. As an alternative we have examined routes starting from the trimethyl ester of the C_{20}-GA, GA_{13}, which is available from methylation and subsequent separation of a mixture of GA_{13} and GA_3. Transformation of the C-20 carboxylate in GA_{13} to an aldehyde requires methods to distinguish between this group and the less hindered carboxylates at C-7 and C-19. One solution, based on exhaustive reduction to a 7,19,20-triol and selective protection of the 7- and 19-positions with bulky silyl ethers has already been described by us.[8] The route in Scheme 4 represents an additional solution and retains C-7 and C-19 at the carboxy oxidation level throughout.



Scheme 3

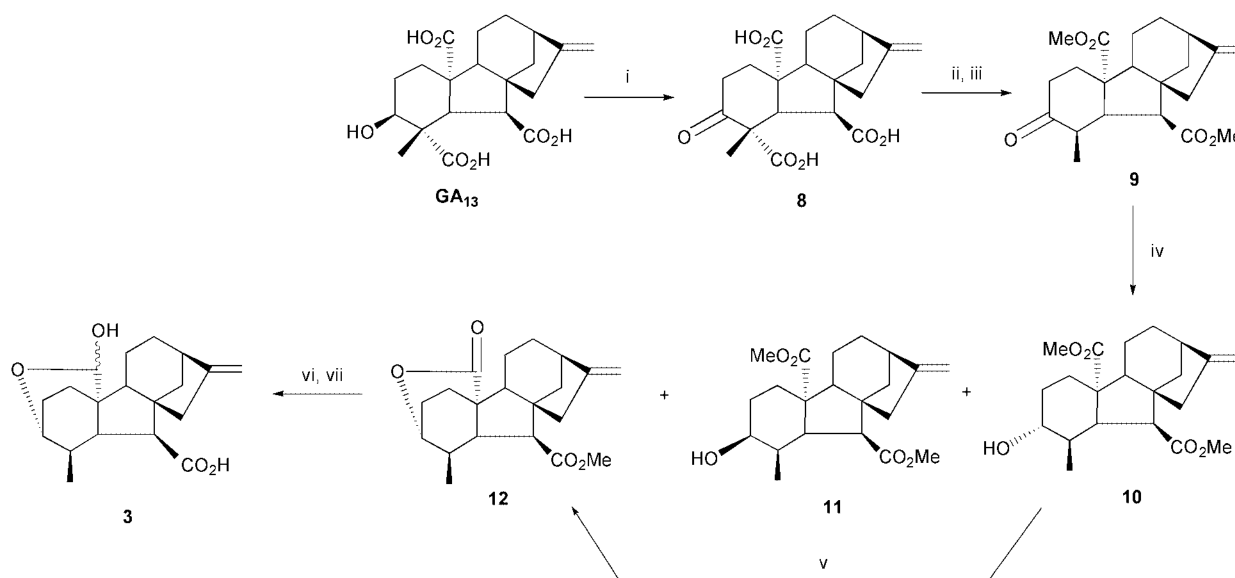


Scheme 4 *Reagents and conditions*: i, Jones reagent, 0 °C; ii, $NaBH_4$–MeOH; iii, KH–THF; iv, DIBAL-H, −78 °C; v, KOH–aq. MeOH; vi, PPTS, C_6H_6.

GA_{13} trimethyl ester was oxidised with Jones reagent to give the known 3-ketone trimethyl ester. Treatment of this ketone with sodium borohydride in methanol gave 3-*epi*-GA_{13} trimethyl ester **5** in high yield by addition of hydride from the least hindered β-face. In the ^{1}H NMR spectrum, the 3β-hydrogen appeared as a double triplet at δ 3.24, with couplings of 12 and 5 Hz, in agreement with its axial conformation. Treatment of **5** with potassium hydride and 18-crown-6 in tetrahydrofuran afforded the 20,3α-lactone **6**, in high yield. In the ^{1}H NMR spectrum the conversion was accompanied by the loss of one methoxy signal and the appearance of a broad singlet at δ 4.9 corresponding to the 3β-hydrogen, which in **6** occupies a quasi-equatorial position as ring A is now held in a boat conformation, forced on the closure of the lactone ring. This compound had previously been noted[18] as a minor product on refluxing of trimethyl GA_{13}-3-toluene-*p*-sulfonate in collidine. The corresponding 7-carboxylic acid has been prepared[19] in poor yield by pyrolysis of 3-*epi*-GA_{13}. Diisobutylaluminium hydride (DIBAL-H) is known to be an effective reagent for the partial reduction of lactones.[20] Reduction of **6** with DIBAL-H at −78 °C was selective for the lactone function over the remaining two methoxycarbonyl groups and gave the GA 3α,20-hemiacetal 7,19-dimethyl ester **7** as a mixture of epimers at C-20 in the ratio 2 : 1. In the ^{1}H NMR spectrum of **7** the 20-hydrogen appeared at δ 4.99 (major isomer) and δ 5.12 (minor isomer), chemical shifts that are characteristic of such anomeric hydrogens. In addition, the 3β-hydrogen had moved upfield, and now appeared as a broad singlet at δ 4.08 (major isomer) and δ 4.18 (minor isomer). Examination of the chemical shifts of H-6α revealed that in the major isomer (δ 3.85) this signal resonated well downfield of that in the minor isomer (δ 3.25). This indicates that the deshielding effect of the 20-hydroxy function on H-6α is greater in the major isomer. On this basis the major isomer was assigned the (*S*)-configuration at the anomeric 20-carbon. Demethylation of **7** with potassium hydroxide in refluxing aqueous methanol afforded the required 3α,20-hemiacetal-7,19-diacid **1**. ^{1}H NMR analysis confirmed the formation of the 7,19-dicarboxylic acid since both methoxy groups had disappeared. It also appeared that the 3α,20-hemiacetal arrangement was unchanged since the broad singlets, assigned to 20-H, at δ 5.08 and δ 5.22 were only slightly shifted from their positions in the starting dimethyl ester. If the alternative 19,20-lactol arrangement were present (Scheme 3), it would be expected that the chemical shift of the 20-H would be similar to that in GA_{24} and GA_{23} (*ca.* δ 5.7).[15,21] A key piece of confirmatory information was the 3β-H signal which had the same chemical shift in the dimethyl ester and the free acid. Thus, it appears that, on the NMR timescale, of the three possible equilibrium forms of **1** (Scheme 3) the 3α,20-hemiacetal predominates at room temperature in chloroform. The structure of **1** was also examined by GC-MS as the methyl ester trimethylsilyl ether. A single GC peak possessing a molecular ion at *m*/*z* 462 was observed, confirming the presence of two methoxy groups and one –OTMS group. The mass spectrum contained an ion at *m*/*z* 344 which corresponds to M^+ − TMSOCHO and thus indicates that the 3α–20 bridge is intact in the silylated derivative. ^{1}H NMR analysis of the methylated trimethylsilylated derivative also confirmed this.

The 3α,20-hemiacetal **1** was treated with pyridinium toluene-*p*-sulfonate in refluxing benzene to provide the (3α,20)(19,20)-acetal **2**. Changes in the ^{1}H NMR were quite dramatic. The 20-H signal now appeared downfield at δ 5.51, reflecting the increased deshielding of the 19,20 bridge. H-6α resonates at δ 2.76, significantly shielded relative to its position in **1** [δ 3.97 (major 20-epimer)] but similar to its position in GA_{24} (δ 2.67)[15] and GA_{23} (δ 2.72),[21] and thus is a feature of the 19,20-lactol ring structure. GC-MS analysis of the methyl ester, produced by treatment with ethereal diazomethane, gave a single peak with a molecular ion of *m*/*z* 358, consistent with simple monomethylation at the 7-carboxy group. In this respect, the 19,20-lactol ring in **6** differs from that in GA_{24} and its relatives[15,21] which on treatment with diazomethane opens to give the 7,19-dimethyl esters. Similarly **2** formed a single trimethylsilyl derivative (M^+ 416), corresponding to the 7-OTMS ester, whereas GA_{24} forms a bistrimethylsilyl derivative.[16]

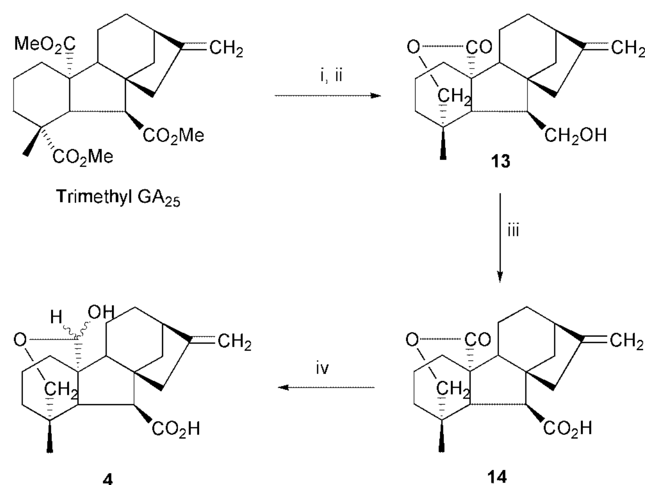
The 19-nor-3α,20-hemiacetal 3. This compound was designed to investigate the requirement for a C-19 carboxylic acid function in the enzymatic oxidation at the C-20 aldehydic function. Oxidation of a mixture (3 : 2) of GA_{13} and GA_3 with Jones reagent yielded the known 3-keto-GA_{13} **8** (Scheme 5), which was readily separable from the intractable products arising from GA_3 oxidation. As described by Galt,[18] the ketone **8** was decarboxylated at C-19 by heating and the resultant 7,20-diacid esterified to give the 19-nor-3-ketone **9**. The decarboxylation was stereospecific and returned the 4β-methyl isomer **9** with only small amounts of the 4α-methyl isomer (3% of total isolated product), which was separable by column chromatography. The ^{1}H NMR spectrum of **9** contained a doublet methyl signal at δ 0.96 (J = 7 Hz) and a multiplet at δ 2.73 identified by COSY-45 and single point irradiation experiments as H-4 with $J_{4–5}$ = 12 Hz confirming the axial orientation of the 4α-H atom.



Scheme 5 *Reagents and conditions*: i, Jones reagent, 0 °C; ii, heat; iii, CsF, MeI; iv, $NaBH_4$–MeOH; v, KH–THF; vi, DIBAL-H, −78 °C; vii, KOH–aq. MeOH.

Reduction of **9** with sodium borohydride gave mainly the 3α-alcohol **10** (68% of recovered product, H-3β = δ 3.27, ddd, J = 10, 10 and 4.5 Hz) accompanied by the corresponding 3β-alcohol **11** (14%, H-3α = δ 3.77, broad singlet) and the 20,3-lactone **12** (18%). Conversion of **10** to **12** was completed by treatment with potassium hydride. As for corresponding 19-methoxycarbonyl compound **6**, the 19-nor-20,3-lactone **12** has a boat conformation for ring A, with H-3β now quasi-equatorial and resonating as a broad singlet at δ 4.32 in the ^1H NMR. The synthesis was completed by reduction of the 20,3-lactone with DIBAL-H followed by demethylation to give the target 19-nor-3α,20-hemiacetal **3**, as a mixture (6 : 1) of epimers at the anomeric carbon-20. By analogy to **6**, the major isomer of **3** (20-H = δ 5.07, s; 6α-H = δ 3.49, d, J = 12 Hz) was assigned the (*S*)-configuration by virtue of the large downfield shift of H-6 relative to its position in the minor isomer (20-H = δ 5.22, s; 6α-H = δ 2.85, d, J = 12 Hz). Analysis of **3** by GC-MS, as the methyl ester, trimethylsilyl ether derivative, gave a single peak with a weak molecular ion at *m/z* 404. The presence of a major fragment at *m/z* 286 (M − TMSOCHO) as above indicates that this compound also trimethylsilylates in the closed form.

19,20-Hemiacetal 4. This compound, in which position 19 is a methylene, was designed to probe the enzyme's requirement for a carbonyl at this carbon. It was synthesised (Scheme 6)



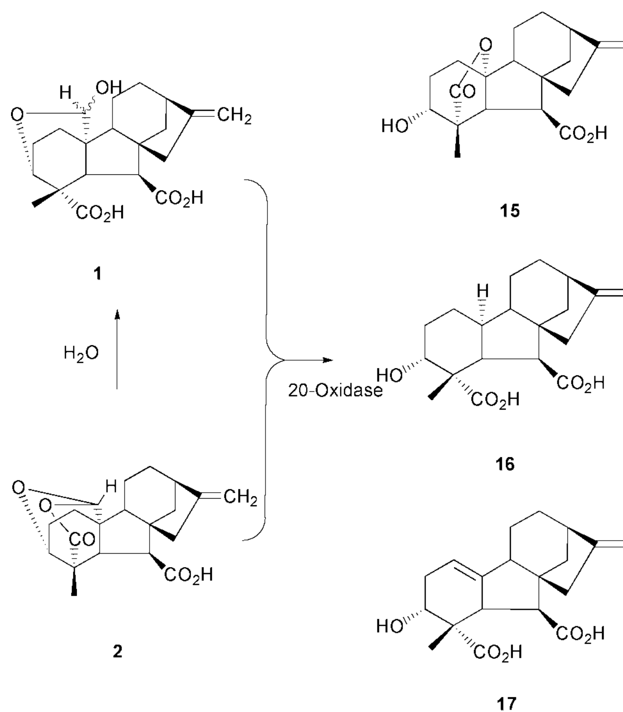
Scheme 6 *Reagents and conditions*: i, $LiAlH_4$–diethyl ether, Δ; ii, 2 M KOH, MeOH, Δ; iii, Jones reagent, acetone, 0 °C; iv, DIBAL-H, toluene, −70 °C.

from trimethyl GA_{25}, prepared from GA_{13} as previously described.[8] Partial reduction of trimethyl GA_{25} with lithium aluminium hydride in ether, as described by Ali *et al.*,[22] gave the 7,19-diol which was cyclised with base to yield 7-hydroxy-20,19-δ-lactone **13**. This was oxidised to the 7-acid **14** with Jones reagent. DIBAL-H reduction of the lactone function returned the required 19,20-lactol **4** as a mixture (3 : 2) of stereoisomers at C-20 as indicated by ^1H NMR and GC-MS. The 20-hydrogen resonated as a broad singlet at δ 4.94 and 4.87 in the two isomers. GC-MS analysis as the 7-methyl ester, 20-OTMS derivative showed a molecular ion at *m/z* 418.

Metabolism of substrate analogues by recombinant 20-oxidase

The analogues were incubated with soluble proteins extracted from *E. coli* cultures expressing recombinant Arabidopsis GA 20-oxidase clone AtGA20ox2 (At2353)[4] in the presence of 2-oxoglutarate, Fe^{2+} and ascorbate. The recovered products were examined by GC-MS.

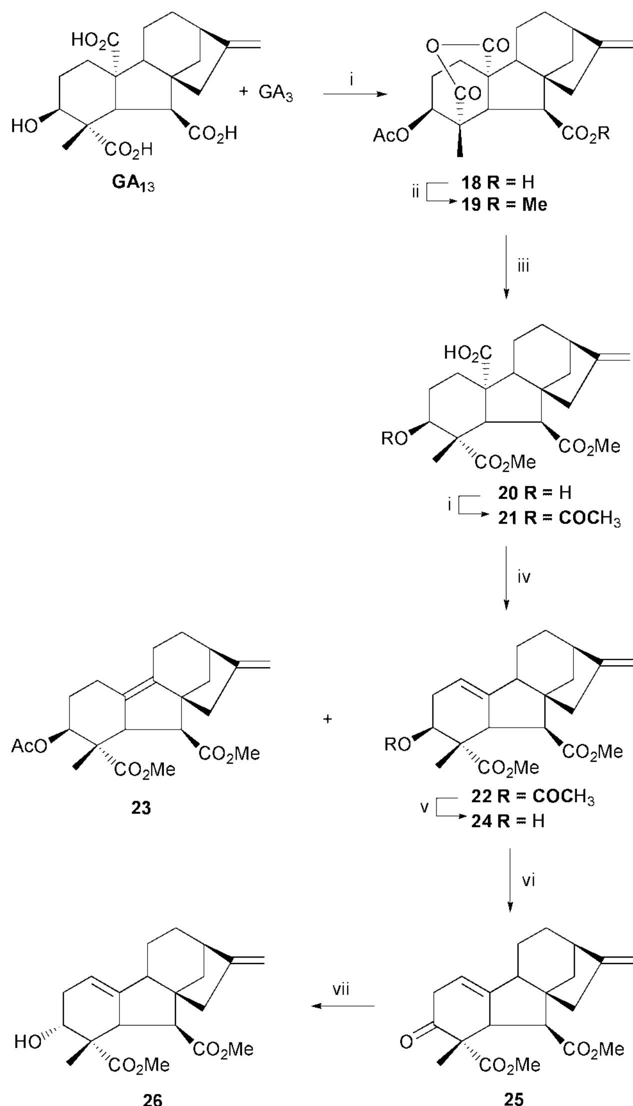
Metabolism of analogues 1 and 2. Both **1** and **2** were metabolised with loss of carbon-20 to give the same products, as shown in Scheme 7. The major product in both cases (75%) was identified as the C_{19}-GA, 3-*epi*-GA_4 **15**. Two other products were also



Scheme 7 Products from the incubation of **1** and **2** with the GA 20-oxidase enzyme.

detected. Based on the mass spectra the structures were assigned as the 20-nor-derivative **16** (18%) and the 1,10-didehydro derivative **17** (7%). These structures were confirmed by comparison with synthetic samples (see later). In control experiments we observed non-enzymic hydrolysis of the tricyclic compound **2** to the 3α,20-hemiacetal **1**. Thus, it was likely that in the enzyme incubation **2** is converted initially to **1**, which is then metabolised. The results demonstrate that the GA 20-oxidase is able to operate at carbon-20 in these compounds despite the strong transannular interaction with the 3α-hydroxy group. These experiments did not give conclusive data on the form of the substrate accepted by the enzyme. We know from NMR data in organic solvents that **1** exists in the 3α,20-hemiacetal form while GA_{24} and other natural aldehydes exist in the 19,20-lactol form. However, the situation within the enzyme active site may be quite different with preferential binding of open conformers a distinct possibility. The 19-methyl ester of GA_{12} was not metabolised by the enzyme (data not shown) indicating that for the first step at least a 19-carboxylic acid is required. Natural substrates with 20-CH_3 or 20-CH_2OH are converted by the Arabidopsis 20-oxidase to the 19,10-lactone with little build-up of 20-aldehydes (or 19,20-lactols). However, this is not a universal phenomenon and there are examples of GA 20-oxidases from other plants for which the aldehyde does build up during the multi-step reaction. The presence of the minor products **16** and **17** indicates that a C-10 radical intermediate is formed by the enzyme. This apparently reacts intramolecularly with the 19-COOH to form the 19,10-lactone as the major product, but can also abstract hydrogen to give the 20-nor compound **16** or eliminate giving rise to the 1,10-unsaturated derivative **17**.

Structure confirmation of 17. In order to confirm the structure assigned to **17** and also to examine the consequences of chemically induced radical formation at C-10, the protected 7,19,20-triacid 7,19-dimethyl ester **21** was synthesised from GA_{13} as shown in Scheme 8. A mixture of GA_{13} and GA_3 was treated with acetic anhydride and pyridine to afford a separable mixture of 3-acetyl-GA_3 and 3-acetyl-GA_{13} 19,20-anhydride **18**. In the ^1H NMR spectrum of **18**, the signal for the C-17 olefinic hydrogens appears as a broad singlet at δ 4.96. This effect is

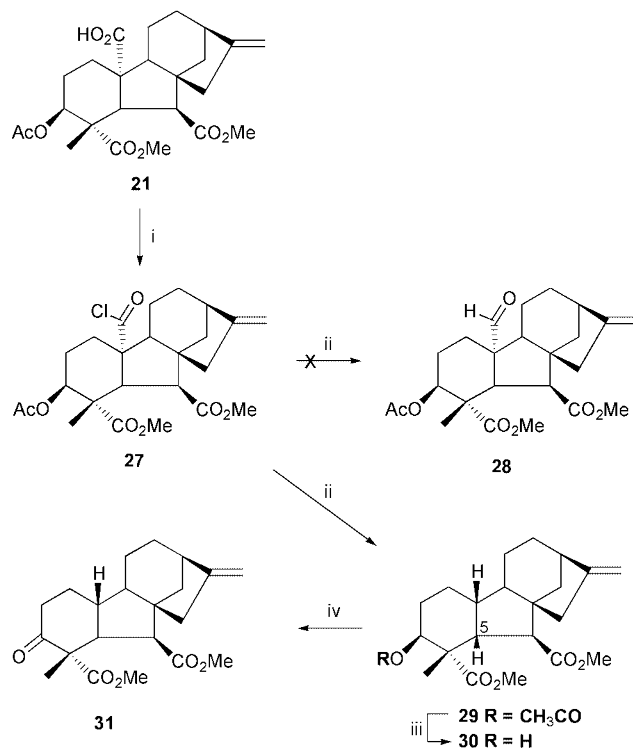


Scheme 8 *Reagents and conditions*: i, acetic anhydride–pyridine; ii, CH_2N_2; iii, NaOMe–MeOH; iv, $Pb(OAc)_4$, $Cu(OAc)_2$; v, K_2CO_3–MeOH; vi, Jones reagent; vii, $NaBH_4$–MeOH.

unusual in gibberellin spectra since the C-17 hydrogens normally appear as a pair of broad singlets at *ca.* δ 4.8–5.0, and can be associated with the A-ring anhydride structure. Treatment of the anhydride with ethereal diazomethane gave the 7-methyl ester derivative, **19**, which retained the anhydride ring. Treatment of **19** with sodium methoxide in methanol resulted in anhydride opening and hydrolysis of the 3-acetyl group to give GA_{13}-7,19-dimethyl ester **20**. Re-acetylation of the 3β-hydroxy group gave **21**.

Oxidative elimination of the 20-COOH group in **21** using lead tetraacetate and cupric acetate, afforded two olefinic products in a 3 : 2 ratio which were inseparable by flash chromatography. The presence of a quartet at δ 5.30, integrating to 0.4H, in the ^{1}H NMR spectrum indicated that the minor olefin was the 1,10-didehydro analogue **22**. The remaining 60% of the mixture appeared to be the 9,10-didehydro GA analogue **23**. Deprotection of the acetate group in the mixture with potassium carbonate in methanol afforded the 3β-hydroxy analogue **24** mixed with the corresponding 9,10-double-bond isomer. In order to confirm the structure of the enzyme product, **17**, it was necessary to invert the stereochemistry at C-3. This was easily achieved by oxidation of the 3β-hydroxy group to the ketone **25** which was then treated with sodium borohydride in methanol to afford the 3α-hydroxy analogue **26**, which was separated from the 9,10-double-bond isomer by preparative TLC. Direct GC-MS comparison of the synthetic 3α-hydroxy-1,10-didehydro-GA **26** (= dimethyl-**17**), as the TMS derivative (M^+ 432, KRI (Kovat's retention index) value 2481) with the methylated, trimethylsilylated products formed from incubation of **1** or **2** with the 20-oxidase, confirmed that the olefinic product formed by the enzyme was **17**.

Biomimetic acyl radical generation at C-20 and the structure of 16. To further probe the possible provenance of the C-10 carbon radicals and the products arising from them the corresponding acyl chloride, **27** was prepared from the acid **21** as shown in Scheme 9. The aim here was to examine the fate of chemically generated C-20 acyl radicals in an analogue where intramolecular reaction with C-19 carboxylic acids was blocked by use of the methyl ester. It is known that tri-*n*-butyltin hydride can reduce acyl halides to aldehydes *via* a radical mechanism.[23] However, when the acyl chloride **27** was treated with tri-*n*-butylstannane the observed product was not the 20-aldehyde **28** (MW 432) but was a product of decarbonylation with MW 404. The compound structure was assigned as 3β-acetoxy-20-nor-GA_{13} 7,19-dimethyl ester **29** by analysis of its ^{1}H and ^{13}C NMR spectra. 2-D experiments (COSY 45 and C–H shift correlation spectroscopy) allowed a full assignment of the protons in this molecule. The ^{1}H NMR spectrum contained a multiplet at δ 2.25, due to the hydrogen atom at C-10. Extra coupling on the signal for H-5β (δ 2.52, dd, J = 6 and 13 Hz) which would normally appear as a simple doublet confirmed the presence of a hydrogen at C-10. Analysis of other couplings around ring A demonstrated that the acetoxy group at C-3β was now in a quasi-equatorial position (δ 5.40, dd, J = 12 and 4 Hz), indicating a change in conformation from the normal gibberellin A ring. The stereochemistry at C-10 was determined by difference NOE experiments. Irradiation of the 10-hydrogen caused a signal enhancement to the 5β-hydrogen and the 18-methyl signal. This confirmed that the 10-hydrogen was on the same side of the molecule as these hydrogens and therefore the 10-hydrogen was assigned the 10β-configuration. Thus, the 'biomimetic' generation of acyl radicals at C-20 resulted in an unexpected decarbonylation presumably to a C-10 carbon radical. Interestingly, in the absence of intramolecular reaction



Scheme 9 *Reagents and conditions*: i, $(COCl)_2$–DMF; ii, $(n\text{-Bu})_3SnH$; iii, K_2CO_3–MeOH; iv, Jones reagent.

with C-19, this radical abstracts hydrogen from tributylstannane. The stereochemistry observed is reasonable, as delivery of hydrogen from the bulky tri-*n*-butylstannane would be expected to occur from the less hindered β-face. However, to produce this outcome, the carbon radical, which is generated on the α-face, and is presumably trigonal in structure, must adopt a more planar structure despite the restraints imposed by its ring junction location. The reaction did not give rise to any of the olefins observed in the enzyme reaction and in the lead tetraacetate reaction of the 20-carboxylic acid described above. The formation of 1,10- or 9,10-olefins involves the elimination of axial hydrogens at 1β and 9β and supports the trigonal structure of the C-10 radical in the enzyme and lead tetraacetate reactions. The observed differences in the reaction path in the three cases are presumably due to the influence of the environment on the lifetime, stability and structure of the radical.

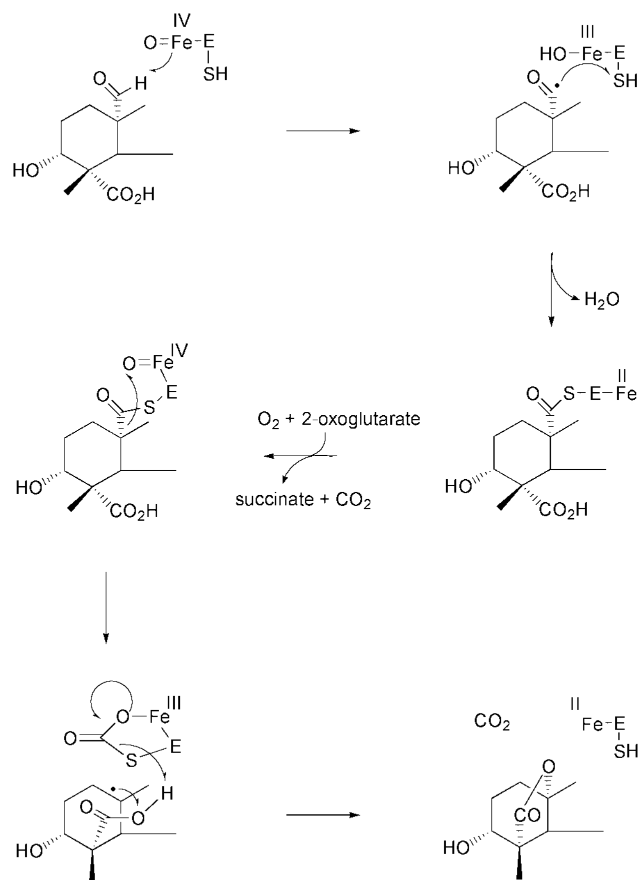
In relation to the enzyme mechanism, the tributylstannane reaction would be a more informative experiment if the equivalent reaction could be carried out on an analogue containing the 20-acyl chloride and 19-carboxylic acid. However, this type of compound was not synthetically accessible because of the need to select the more hindered 20-acid over the 19-acid in acyl chloride generation and also the propensity of the two groups to react intramolecularly to form the cyclic anhydride. An experiment that is related, but not involving 20-acyl radicals, has been previously been reported by Bearder and MacMillan,[24] who treated gibberellin A_{13} (*i.e.* a 19,20-dicarboxylic acid) with lead tetraacetate. This reaction gave a mixture of γ-lactones containing 60% of the 19,10-lactone. This product arises from decarboxylation at C-20 and intramolecular capture of the C-10 radical by the C-19 acid group, in line with the possible biochemical reaction path.

Having prepared a synthetic 20-nor-derivative with determined 10β-H stereochemistry we were able to make a direct comparison with the 20-nor enzyme product **16** observed in the metabolism of **1** and **2**. The analogue **29** has the opposite stereochemistry at C-3 to the metabolite **16**. Thus, for direct comparison it was necessary to equalise the functionality at this carbon. Deprotection of **29**, with potassium carbonate in methanol (Scheme 9) afforded the 3β-hydroxy derivative **30**. Oxidation of this alcohol with Jones reagent afforded the 3-keto derivative **31**. An aliquot of the products of incubation of the lactol **1** with enzyme, containing **16**, was also treated with Jones reagent to produce 3-ketone analogues. This sample, after methylation with diazomethane, was then compared with **31** by GC-MS. The two compounds were shown to be isomeric (M^+ 360) but had different retention indices and mass spectra that were very similar but with different relative intensities. The enzyme derived product (KRI 2471) had prominent ions at *m*/*z* 360, 328, 300 and 241(base), while the 10β-H compound **31** (KRI 2483) gave a weak molecular ion at *m*/*z* 360 with a base peak at 328. From these data it was concluded that the 20-nor enzyme product possessed a 10α-hydrogen and had been correctly assigned as structure **16**.

Metabolism of hemiacetals 3 and 4. Both **3** or **4** were recovered unchanged after incubation with the enzyme. These compounds contain the same C-20 functionality as **1** and the natural substrate GA_{24}. Thus, the 19-carboxylic acid group, which is absent from **3**, plays a key role in substrate binding and/or oxidation at C-20. Hemiacetal **4** is a direct analogue of the lactol form of the GA_{24}. Lack of turnover of this substrate again points to a requirement for a carboxy group at C-19.

Conclusions

The group of hemiacetal/lactol analogues prepared have revealed important clues to the structural requirements for oxidative cleavage of the carbon-20 in gibberellin biosynthesis. It would appear that a free C-19 carboxylic acid is required for successful oxidation of CH_3, CH_2OH and CHO functionality at C-20 and for the capture of the C-10 radical. The product profile from the 3α-hydroxy analogues **1** and **2** indicates the involvement of a carbon radical at C-10. The fate of this radical is very dependent on the environment. When generated by the enzyme, the major reaction is lactone formation from intramolecular reaction with the proximal 19-carboxy function. In the presence of the hindering 3α-axial hydroxy group this reaction is slower than with GA_{24}, the natural substrate, and other radical derived products are observed. Elimination reaction of the C-10 radical was specific in the enzyme active site and gave only the 1,10-olefin, whereas generation of this same radical with lead tetraacetate gave rise to a mixture of olefins. Interestingly when the same C-10 radical was generated by the spontaneous decarbonylation of the 20-acyl radical, in the presence of tributylstannane, no olefins were observed and the reaction was terminated by hydrogen abstraction onto the least hindered β face. This hydrogen abstraction reaction was also observed in the enzymic reaction, but with opposite stereochemistry, reflecting the constraints within the active site. These stereochemical differences between the enzyme and chemical reactions of the same radical further emphasise the degree of control imposed by the enzyme on this reaction. The results obtained clarify the role of the C-19 carboxylate. The exact mechanism of loss of C-20 remains unclear. Involvement of a C-19 carboxy radical reacting intramolecularly with a C-20 percarboxyiron function cannot be ruled out. However, the mechanism that we favour is shown in Scheme 10 and involves an enzyme-bound C-20



Scheme 10 Possible mechanism for the formation of C_{19}-GAs *via* an enzyme-bound intermediate.

thioester intermediate which undergoes a second round of oxidation to yield a C-10 radical, which can lactonise to produce **15**, along with CO_2 as shown. The elimination product **17** arises from the C-10 radical. However, the formation of **16** requires hydrogen abstraction, possibly from other peptide residues in the active site.

Experimental

Solvents were either analytical grade or HPLC grade and were redistilled, prior to use. Thin-layer chromatography was carried out on silica developed with ethyl acetate–hexane mixtures. Compounds were visualised under ultra-violet light (254 nm), with I_2 vapour or by spraying with methanol containing 5% conc. sulfuric acid and heating to *ca.* 100 °C. Flash chromatography, unless otherwise stated, was performed according to Still *et al.*[25] and carried out on columns of Merck Kieselgel 60 (40–63 mm), pre-washed with ethyl acetate–hexane (1 : 9) containing 0.5% v/v acetic acid. Compounds were pre-adsorbed onto silica and the columns eluted stepwise with 10% increments of ethyl acetate in hexane containing 0.5% v/v acetic acid (for acid derivatives). Relevant fractions were pooled, and the solvent removed *in vacuo*.

High resolution MS analyses were performed on a Kratos MS80RFA mass spectrometer by direct insertion probe. GC-MS analysis was carried out on a VG7070 HS mass spectrometer using a BP-1 capillary column (25 m × 0.2 mm, 0.1 µm film thickness) run with a temperature gradient of 35 °C for 2 min, then raised at 20 °C min^{-1} to 150 °C, followed by 4 °C min^{-1} to 300 °C. Samples, unless otherwise stated, were run as the methyl ester, trimethylsilyl ether (MeTMS) derivatives, prepared by treatment of the sample with ethereal diazomethane followed by treatment with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide.

Trimethyl *ent*-3β-hydroxygibberell-16-ene-7,19,20-trioate 5

Trimethyl GA_{13} (2.1 g) in acetone (25 cm^3) at 0 °C was treated with Jones reagent until the supernatant remained orange. The solution was stirred at this temperature. After 1 hour, water was added and the product extracted into ethyl acetate. Recovery of the organic layer afforded trimethyl GA_{13} 3-ketone (2.14 g) as a colourless gum which was treated with sodium borohydride (1.03 g) in methanol (110 cm^3) and the reaction allowed to stir for 2 hours. Acetone (10 cm^3) was slowly added and the methanol removed *in vacuo*. The product was partitioned between dilute HCl and ethyl acetate. Recovery of the organic layer afforded trimethyl 3-*epi*-GA_{13} **5** (2.3 g) as a colourless gum. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.28 (s, 18-H_3), 2.67 (d, J = 12 Hz, 5-H), 3.24 (m, 3-H), 3.59, 3.67 and 3.69 (3s, 3 × -OMe), 3.82 (d, J = 12 Hz, 6-H), 4.78 and 4.86 (2s, 17-H_2).

Dimethyl *ent*-3β-hydroxy-20-carboxygibberell-16-ene-7,19-dioate 20,3-lactone 6

To a suspension of potassium hydride (1.0 g of 35% oil dispersion, washed with hexane) in tetrahydrofuran (THF, 40 cm^3) with 18-crown-6 (20 mg) was added 3-*epi*-GA_{13} trimethyl ester **5** (2.3 g) in THF (8 cm^3) dropwise. After 2 hours, ethanol (2 cm^3) was added cautiously followed by water. The solution was acidified with conc. HCl to pH 3 and the product extracted into ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* afforded the 20,3-lactone derivative **6** (1.95 g) as a colourless gum which solidified upon standing. The material was used without further purification. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.46 (s, 18-H_3), 2.35 (d, J = 13 Hz, 5-H), 2.99 (d, J = 13 Hz, 6-H), 3.67 and 3.73 (2s, 7-OMe and 19-OMe), 4.79 and 4.84 (2s, 17-H_2), 4.93 (s, 3-H); *m/z* (EI) 388.1869 (M^+; $C_{22}H_{28}O_6$ requires 388.1886), 356 (100%), 328 (10), 296 (72), 283 (54), 269 (13), 251 (16), 225 (24), 199 (9), 181 (9), 143 (10), 105 (9), 91 (12).

Dimethyl *ent*-3β-hydroxy-20-oxogibberell-16-ene-7,19-dioate 20,3-hemiacetal 7

Gibberellin 20,3-lactone dimethyl ester **6** (1.9 g) in THF (80 cm^3) at −78 °C was treated with diisobutylaluminium hydride (DIBAL-H) (1 mol dm^{-3} in hexanes, 12 cm^3) and the mixture stirred at this temperature for 1 hour. Sodium fluoride (900 mg) was added and the solution stirred for 10 minutes. Water was added and the solution allowed to slowly warm to room temperature. The solution was acidified with conc. HCl to pH 3 and the product extracted into ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* followed by flash chromatography (hexane–ethyl acetate) afforded the 20,3-hemiacetal derivative **7** (1.0 g) as a mixture of two inseparable isomers. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.36 and 1.38 (2s, 18-H_3), 3.26 and 3.84 (both d, J = 12.5 Hz, 6-H), 3.64, 3.67, 3.72 and 3.73 (4 × s, 7-OMe and 19-OMe), 4.08 and 4.18 (2s, 3-H), 4.80 and 4.93 (2s, 17-H_2), 4.99 and 5.12 (2s, 20-H); *m/z* (EI) 372.1945 [(M^+ − 18), $C_{22}H_{28}O_5$ (M − 18) requires 372.1937], 356 (100%), 340 (29), 312 (28), 296 (24), 284 (48), 216 (21), 171 (14), 129 (14), 105 (12), 91 (27).

ent-3β-Hydroxy-20-oxogibberell-16-ene-7,19-dioic acid 20,3-hemiacetal 1

The 20,3-hemiacetal dimethyl ester **7** (70 mg) was dissolved in (1 : 1) methanol–aqueous potassium hydroxide (2 mol dm^{-3}, 5 cm^3) and heated under reflux for 12 hours. The solution was partitioned between ethyl acetate–water. The aqueous layer was acidified to pH 3 with conc. HCl and the product extracted into ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* afforded the GA 20,3-hemiacetals 7,19-diacid **1** (68 mg) as a colourless gum. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.41 (s, 18-H_3), 3.38 and 3.97 (both d, J = 12.5 Hz, 6-H), 4.08 and 4.15 (both s, 3-H), 4.80 and 4.96 (2s, 17-H_2), 5.08 and 5.22 (both s, 20-H); *m/z* (EI) 344.1637 [(M^+ − 18), $C_{20}H_{24}O_5$ (M − 18) requires 344.1624], 326 (36%), 298 (18), 284 (37), 270 (100), 225 (23), 202 (92), 184 (15), 129 (19), 91 (32).

ent-3β-Hydroxy-20-oxogibberell-16-ene-7,19-dioic acid 20,3-hemiacetal 19,20-hemiacetal 2

The GA 20,3-hemiacetal **1** (32 mg) in benzene (5 cm^3) was heated under reflux (Dean–Stark) for 10 minutes. Pyridinium toluene-*p*-sulfonate (50 mg) was added and refluxing continued for a further 8 hours. The solvent was evaporated *in vacuo* and flash chromatography (hexane–ethyl acetate) afforded the tricyclic derivative **2** (12 mg) as a colourless gum. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.23 (s, 18-H_3), 3.85 (d, J = 4 Hz, 3-H), 4.87 and 4.98 (2s, 17-H_2), 5.50 (s, 20-H); *m/z* (EI) 344.1618 (M^+; $C_{20}H_{24}O_5$ requires 344.1624), 326 (42%), 298 (18), 281 (7), 270 (100), 225 (27), 202 (94), 184 (20), 157 (23), 129 (19), 105 (18), 91 (32), 77 (21).

Dimethyl *ent*-3-oxo-19-norgibberell-16-ene-7,20-dioate 9

A mixture of GA_3 and GA_{13} (1.2 g) was dissolved in acetone (25cm^3) and the solution cooled to 0 °C in an ice bath. Jones reagent was added until the supernatant remained orange. Stirring was continued at 0 °C for 30 minutes. The solution was poured into water and extracted into ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* afforded the crude 3-keto-GA_{13} **8** (582 mg) which was purified by flash chromatography. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.46 (s, 18-H_3), 2.56 (d, J = 12.5 Hz, 6-H), 2.66 (br, 13-H), 2.72 (d, J = 12.5 Hz, 5-H), 4.86 and 4.97 (both s, 17-H_2). Dry 3-keto-GA_{13} **8** (512 mg) was moistened with a few drops of water and heated on a water bath at 100 °C for 30 minutes. The product was recovered into ethyl acetate, dried ($MgSO_4$) and the solvent removed *in vacuo* to afford the 3-keto-19-nor-GA_{13} (410 mg). This material was then treated with ethereal diazomethane and purified by flash chromatography to give the 3-keto-19-nor-GA_{13} dimethyl ester **9** (343 mg). δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.96 (d, J = 7 Hz, 18-H_3), 2.28 (d, J = 12.5 Hz, 5-H), 2.56 (br, 13-H), 2.72 (m, 4-H), 3.52 (d, J = 12.5 Hz, 6-H), 3.73 (s, OMe), 3.82 (s, OMe) 4.88 and 4.95 (both s, 17-H_2); δ_C(100 MHz, $CDCl_3$, Me_4Si) 12.28 (q, 18-C), 19.03 (t, 11-C), 31.72 (t), 34.23 (t), 37.76 (t), 38.78 (t), 39.53 (d), 45.67 (t), 46.40 (d), 51.65 and 51.73 (2 × q,

2 × OMe), 52.49 (d), 52.55 (s), 54.77 (d), 55.42 (d), 57.80 (s), 106.66 (t, 17-C), 155.63 (s, 16-C), 174.77 (s), 174.91 (s), 211.03 (s, 3-C); *m/z* (EI) 360.19373 (M^+; $C_{21}H_{28}O_5$ requires 360.19368), 328(36%), 310 (14), 300 (73), 269 (54), 241 (31), 192 (12), 165 (100), 133 (28), 91 (64), 59 (33).

Dimethyl *ent*-3β-hydroxy-19-norgibberell-16-ene-7,20-dioate 10

The 3-keto-19-nor-GA_{13} **9** (300 mg) in methanol (15 cm^3) was stirred at room temperature. Sodium borohydride (167 mg) was added and stirring was continued for 2 hours. The solution was poured into water, and extracted into ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* afforded the crude product which was purified by flash chromatography to afford the 3α-hydroxy-19-nor derivative **10** (150 mg), the 3β hydroxy isomer **11** (30 mg) and the GA 19-nor-20,3-lactone **12** (40 mg).

Dimethyl *ent*-3β-hydroxy-19-norgibberell-16-ene-7,20-dioate 10. δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.91 (d, J = 7 Hz, 18-H_3), 1.89 (d, J = 12 Hz, 5-H), 2.40 (m, 4-H), 2.56 (m, 13-H), 3.27 (ddd, J = 10, 10 and 4.5 Hz, 3-H), 3.37 (d, J = 12 Hz, 6-H), 3.68 (s, OMe), 3.69 (s, OMe) 4.82 and 4.88 (both s, 17-H_2); δ_C(100 MHz, $CDCl_3$, Me_4Si) 15.57, 19.03, 31.89, 33.32, 34.17, 38.01, 39.80, 40.47, 45.86, 51.35, 51.61, 51.89, 52.08, 54.07, 55.71, 58.18, 76.55, 106.36, 156.12, 175.51, 175.70; *m/z* (EI) 330.18085 [$M^+ - 32$; $C_{20}H_{26}O_4$ ($M - 32$) requires 330.18311], 298 (100%), 270 (77), 231 (58), 171 (29), 129 (36), 91 (55), 55 (19).

Dimethyl *ent*-3α-hydroxy-19-norgibberell-16-ene-7,20-dioate 11. δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.88 (d, J = 7 Hz, 18-H_3), 2.41 (t, J = 12 Hz, 5-H), 3.38 (d, J = 12 Hz, 6-H), 3.67 (s, OMe), 3.68 (s, OMe), 3.77 (s, 3-H), 4.82 and 4.89 (both s, 17-H_2); *m/z* (EI) 344.19988 ($M^+ - 18$); $C_{21}H_{28}O_4$ ($M - 18$) requires 344.19876), 312 (100%), 284 (31), 253 (49), 225 (84), 183 (10), 129 (14), 91 (31).

Methyl *ent*-3β-hydroxy-20-carboxy-19-norgibberell-16-en-7-oate 20,3-lactone 12. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.11 (d, J = 7 Hz, 18-H_3), 3.70 (s, OMe), 4.32 (s, 3-H), 4.80 and 4.92 (both s, 17-H_2); *m/z* (EI) 330.18408 (M^+; $C_{20}H_{26}O_4$ requires 330.18311), 298 (100%), 270 (63), 231 (55), 171 (31), 129 (43), 91 (57).

Methyl *ent*-3β-hydroxy-20-carboxy-19-norgibberell-16-en-7-oate 20,3-lactone 12

Potassium hydride (35% wt dispersion, 76 mg) was suspended in dry THF (3 cm^3) under nitrogen. 18-Crown-6 (10 mg) was added followed by 3α-hydroxy-19-nor-GA_{13} methyl ester **10** (150 mg) as a solution in THF (1 cm^3). The reaction was stirred at room temperature under a nitrogen atmosphere for 2 hours. Ethanol (2–3 drops) was added followed by water. The product was extracted with ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* afforded the crude product which was purified by flash chromatography to afford *methyl ent-3β-hydroxy-20-carboxy-19-norgibberell-16-en-7-oate 20,3-lactone* **12** (75 mg). δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.11 (d, J = 7 Hz, 18-H_3), 3.70 (s, OMe), 4.32 (s, 3-H), 4.80 and 4.92 (both s, 17-H_2); *m/z* (EI) 330.18408 (M^+; $C_{20}H_{26}O_4$ requires 330.18311), 298 (100%), 270 (63), 231 (55), 171 (31), 129 (43), 91 (57).

***ent*-3β-Hydroxy-20-oxogibberell-16-ene-7,20-dioic acid 20,3-hemiacetal 3**

19-Nor-GA 20,3-lactone **12** (73 mg) in dry THF (5 cm^3) was cooled to −78 °C. DIBAL-H (1 mol dm^{-3} in hexane, 266 mm^3) was added and the reaction was stirred at this temperature for one hour. Water was added and the reaction was allowed to warm to room temperature. The product was extracted into ethyl acetate and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded the crude product which was purified by flash chromatography to yield the 19-nor-20,3-hemiacetal (46 mg). This material was then treated with (1 : 1) methanol–aqueous potassium hydroxide (2 mol dm^{-3}, 2 cm^3) to afford the *ent-3β-hydroxy-20-oxogibberell-16-ene-7,20-dioic acid 20,3-hemiacetal* **3** (28 mg) as a mixture of C-20 epimers (6 : 1). Major epimer: δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.98 (d, J = 7 Hz, 18-H_3), 3.49 (d, J = 12 Hz, 6-H), 3.65 (s, 3-H), 4.81 and 4.93 (both s, 17-H_2), 5.07 (s, 20-H); minor epimer: δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.98 (d, J = 7 Hz, 18-H_3), 2.85 (d, J = 12 Hz, 6-H), 3.61 (s, 3-H), 4.81 and 4.93 (both s, 17-H_2), 5.22 (s, 20-H).

***ent*-7,19-Dihydroxygibberell-16-en-20-oic acid 20,19-lactone 13**

Trimethyl GA_{25} (3.04 g) in diethyl ether (200 cm^3) was treated with lithium aluminium hydride (400 mg) and the reaction mixture was heated at reflux temperature for two hours. Water was added slowly. The solution was acidified with conc. HCl to pH 3 and the organic layer recovered and evaporated *in vacuo*. Flash chromatography afforded the 7,19-dihydroxy 20-methyl ester (2.23 g) as a pale yellow gum. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.21 (s, 18-H_3), 2.5–4.0 (6H, m), 3.60 (s, 20-OMe), 4.70 and 4.83 (both br s, 17-H_2). The 20-methoxy-7,19-diol (610 mg) in methanol–2 mol dm^{-3} aqueous potassium hydroxide (1 : 1, 70 cm^3) was heated at reflux temperature for two hours. After cooling, the solvent was evaporated *in vacuo* and the product partitioned between ethyl acetate and dilute HCl to afford the 7-hydroxy-20,19-lactone **13** (460 mg) as a pale yellow gum which solidified upon standing. Mp 159–163 °C; lit.[22] mp 160–162 °C. δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.95 (s, 18-H_3), 3.6 and 3.78 (both dd, J = 2 and 11 Hz, 7-H_2), 4.15 and 4.35 (both d, J = 11 Hz, 19-H_2), 4.88 (br s, 17-H_2); *m/z* (EI) 316.20362 (M^+; $C_{20}H_{28}O_3$ requires 316.20385), 257 (54%), 204 (46), 173 (32), 129 (67), 91 (100).

***ent*-19-Hydroxygibberell-16-ene-7,20-dioic acid 20,19-lactone 14**

The 7-hydroxy-20,19-lactone **13** (100 mg) in acetone (2 cm^3) was treated with excess Jones reagent at 0 °C until a more polar spot was observed by TLC. Water was added and the product was extracted into ethyl acetate and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded the crude product which was purified by flash chromatography to afford the 7-carboxy-20,19-lactone **14** (68 mg) as a white solid (mp 183–184 °C; lit.[22] mp 182–184 °C). δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.88 (s, 18-H_3), 2.62 (d, J = 10 Hz, 6-H), 4.14 and 4.32 (both d, J = 11 Hz, 19-H_2), 4.91 (br s, 17-H_2); δ_C(100 MHz, $CDCl_3$, Me_4Si) 17.35, 21.25, 23.47, 30.96, 33.26, 37.49, 40.51, 41.85, 42.88, 43.24, 50.35, 51.38, 51.55, 54.64, 54.89, 75.80, 106.31, 153.26, 175.59, 180.34; *m/z* (EI) 330.1825 (M^+, $C_{20}H_{26}O_4$ requires 330.1831), 312 (10%), 284 (11), 271 (10), 257 (13), 241 (11), 230 (12), 218 (14), 197 (6), 173 (7), 155 (8), 143 (11), 129 (11), 117 (10), 105 (9), 91 (18).

***ent*-19-Hydroxy-20-oxogibberell-16-en-7-oic acid 20,19-hemiacetal 4**

The 19-hydroxy-20,19-lactone **14** (100 g) in toluene (3 cm^3) under nitrogen was cooled to −70 °C. A solution of DIBAL-H in toluene (1.5 mol dm^{-3}, 230 mm^3) was added and the reaction mixture was stirred at this temperature for a further hour. Sodium fluoride (50 mg) was added followed by water (10 cm^3). The product was extracted into ethyl acetate and the solvent removed *in vacuo*. Flash chromatography afforded the 20,19-hemiacetal **4** (44 mg) as a 3 : 2 mixture of inseparable isomers. δ_H(400 MHz, $CDCl_3$, Me_4Si) 0.72 and 0.74 (both s, 18-H_3), 3.02 (d, J = 13 Hz, H-5), 3.25 (d, J = 11 Hz, H-5), 3.4–3.95 (2H, m, 19-H_2), 3.64 (d, J = 13 Hz, H-6), 3.52 (d, J = 13 Hz, H-6), 4.79 and 4.91 (both br s, 17-H_2), 4.88 and 4.94 (both s, 20-H); *m/z* (EI) 314.1884 [$M^+ - 18$, $C_{20}H_{26}O_3$ ($M - 18$) requires 314.1881], 286 (72%), 271 (21), 257 (28), 241 (27), 217 (29), 149 (54), 91 (52).

ent-3α-Acetoxygibberell-16-ene-7,19,20-trioic acid 19,20-anhydride 18

A mixture of GA_3 and GA_{13} (5 g) was treated with acetic anhydride (24 cm^3) and pyridine (48 cm^3) and stirred at room temperature overnight. The solution was poured into water, acidified to pH 3 with c.HCl and extracted into ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* afforded a mixture of GA_3 3-acetate and 3-acetoxy GA_{13}-19,20-anhydride **18** (4.32 g) as a colourless gum which solidified upon standing (mp 268–270 °C; lit.[18] mp 264–267 °C); δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.22 (s, 18-H_3), 2.07 (s, -$OCOCH_3$), 2.50 (d, J = 11 Hz, 6-H), 2.68 (br, 13-H), 2.75 (d, J = 11 Hz, 5-H), 4.96 (s, 17-H_2), 5.12 (s, 3-H); m/z (EI) 402.1673 (M^+, $C_{22}H_{26}O_7$ requires 402.1679), 388 (2%), 356 (56), 314 (52), 270 (100), 241 (18), 225 (32), 155 (19), 105 (23), 91 (42), 79 (22).

ent-3α-Acetoxygibberell-16-ene-7,19,20-trioic acid 7-methyl ester 19,20-anhydride 19

The GA_{13} anhydride derivative **18** (3.0 g) was treated with ethereal diazomethane until a yellow colour persisted. The solvent was removed *in vacuo* to afford the 7-methyl ester **19** (2.8 g) as a colourless gum. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.15 (s, 18-H_3), 2.12 (s, -$OCOCH_3$), 2.45 (d, J = 11 Hz, 6-H), 2.66 (br, 13-H), 2.77 (d, J = 11 Hz, 5-H), 3.72 (s, -OMe), 4.94 (s, 3-H), 4.94 and 5.07 (both s, 17-H_2); m/z (EI) 416.1849 (M^+ $C_{23}H_{28}O_7$ requires 416.1834), 403 (2%), 385 (11), 370 (49), 356 (62), 328 (88), 284 (100), 224 (78), 181 (18), 129 (19), 91 (32).

ent-3α-Acetoxygibberell-16-ene-7,19,20-trioic acid 7,19-dimethyl ester 21

The GA_{13} anhydride 7-methyl ester **19** (2.8 g) in methanol (90 cm^3) was treated with sodium (930 mg) and heated under reflux for 2 hours. The solvent was removed *in vacuo* and the product partitioned between dilute HCl and ethyl acetate. Recovery of the organic layer and removal of solvent *in vacuo* afforded the dimethyl ester **20** (3.04 g) as a colourless gum, which was used without further purification. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.24 (s, 18-H_3), 2.60 (5-H), 3.63 (s, 19-OMe), 3.70 (d, 6-H), 3.72 (s, 7-OMe), 4.01 (s, 3-H), 4.82 and 4.90 (both s, 17-H_2). The GA_{13} dimethyl ester **20** (3 g) was treated with acetic anhydride (14 cm^3) and pyridine (28 cm^3) and stirred for 48 hours at room temperature. The solution was poured into water, acidified to pH 3 with conc. HCl, extracted into ethyl acetate and then washed (H_2O). The solvent was removed *in vacuo* to afford the 3-acetoxy derivative **21** (2.91 g) as a pale yellow gum which solidified on standing. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.24 (s, 18-H_3), 2.09 (s, -$OCOCH_3$), 2.57 (d, J = 11 Hz, 5-H), 3.62 (s, 19-OMe), 3.70 (s, 7-OMe), 3.78 (d, J = 11 Hz, 6-H), 4.82 and 4.90 (both s, 17-H_2), 5.25 (s, 3-H); m/z (EI) 448.2081 (M^+, $C_{24}H_{32}O_8$ requires 448.2098), 416 (19%), 388 (5), 370 (45), 356 (86), 328 (84), 296 (100), 268 (60), 223 (51), 129 (27), 91 (48).

Dimethyl ent-3β-hydroxygibberella-1,16-diene-7,19-dioate 26

The 3-acetoxy 7,19-dimethyl ester derivative **21** (50 mg) in benzene (5 cm^3) was treated with lead tetraacetate (87 mg), cupric acetate (5 mg) and pyridine (5 mm^3). The mixture was heated under reflux for one hour during which time the solution changed from blue to green. After cooling the solvent was removed *in vacuo* and the residue diluted with water. The solution was adjusted to pH 3 with conc. HCl and the product was extracted with ethyl acetate. The organic layer was washed with water, dried ($MgSO_4$) and the solvent removed *in vacuo* to afford the 1,10-didehydro derivative **22** and a 9,10-didehydro isomer **23** as a 2 : 3 mixture of compounds (31 mg). δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.12 and 1.21 (s, 18-H_3), 2.07 and 2.09 (s, $OCOCH_3$), 3.64 and 3.65 (s, 19-OMe), 3.70 and 3.72 (s, 7-OMe), 4.87 (br s, 17-H_2), 5.23 and 5.26 (s, 3-H), 5.30 (q, J = 2 Hz, 1-H). The 1,10-didehydro analogue **22** (29 mg) in methanol (1 cm^3) was treated with potassium carbonate (100 mg). The solution was allowed to stir at room temperature for 12 hours. Aqueous citric acid (5% w/v) was added and the product extracted into ethyl acetate. The organic layer was recovered, washed with water, dried ($MgSO_4$) and the solvent removed *in vacuo* to afford the 3β-hydroxy-1,10-didehydro derivative **24** and the corresponding 9,10-didehydro isomer as a 2 : 3 mixture of compounds (20 mg). δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.21 and 1.31 (s, 18-H_3), 3.64 and 3.65 (s, 19-OMe), 3.70 and 3.72 (s, 7-OMe), 4.04 (s, 3-H), 4.89 (br s, 17-H_2), 5.25 (q, J = 2 Hz, 1-H). The 1,10-didehydro analogue **24** (19 mg) in acetone (1 cm^3) was treated with Jones reagent until the supernatant remained orange. The solution was allowed to stir at room temperature for 1 hour. Water was added and the product extracted into ethyl acetate. The organic layer was recovered, washed with water, dried ($MgSO_4$) and the solvent removed *in vacuo* to afford the 3-keto-1,10-didehydro derivative **25** and the corresponding 9,10-didehydro isomer as a 2 : 3 mixture of compounds (10.7 mg). δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.26 and 1.33 (s, 18-H_3), 3.67 (s, 19-OMe), 3.71 and 3.72 (s, 7-OMe), 4.92 (br s, 17-H_2), 5.49 (q, J = 2 Hz, 1-H). The 1,10-didehydro analogue **25** (10 mg) in methanol (500 mm^3) was treated with sodium borohydride. The solution was allowed to stir at room temperature for 20 minutes. Acetone (1 cm^3) was added and the methanol removed *in vacuo*. Water was added to the residue and the product was extracted into ethyl acetate. The organic layer was recovered, washed with water, dried ($MgSO_4$) and the solvent removed *in vacuo* to afford the 3β-hydroxy-1,10-didehydro derivative **26** and the corresponding 9,10-didehydro isomer as a 2 : 3 mixture of compounds (7.1 mg). The two isomers were separated by preparative TLC and a pure sample of **26** was isolated for characterisation by ^1H NMR and GC-MS. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.36 (s, 18-H_3), 3.67 (s, 19-OMe), 3.71 (s, 7-OMe), 4.89 (br s, 17-H_2), 5.31 (q, J = 2 Hz, 1-H).

Dimethyl ent-3α-acetoxy-20-norgibberell-16-en-7,19-dioate 29

The 3-acetoxy 7,19-dimethyl ester derivative **21** (56 mg) in benzene (2 cm^3) was treated with oxalyl chloride (60 mm^3) and dimethylformamide (DMF, 1 drop). The resultant solution was stirred under nitrogen for 2.5 hours during which time a white precipitate formed. The solvent was removed *in vacuo* and the product taken up in benzene (×2) and evaporated *in vacuo* to remove any excess oxalyl chloride. The acid chloride **27** (62 mg) was used immediately. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.18 (s, 18-H_3), 2.10 (s, -$OCOCH_3$), 2.63 (d, J = 13 Hz, 5-H), 3.59 (d, J = 13 Hz, 6-H), 3.70 and 3.72 (both s, 19 and 7-OMe), 4.85 and 4.92 (both s, 17-H_2), 5.32 (s, 3-H). A solution of tri-*n*-butyltin hydride (100 mm^3) in benzene (10 cm^3) was heated under reflux (Dean–Stark) whilst a solution of the acid chloride **27** (62 mg) in benzene (1 cm^3) containing AIBN (10 mg) was added dropwise. Refluxing was continued for a further hour. The solvent was removed *in vacuo* to give the crude product which was identified as the 10β-hydrogen derivative **29** (31 mg). δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.43 (s, 18-H_3), 1.96 (s, -$OCOCH_3$), 2.55 (dd, J = 6, 13 Hz, 5-H), 3.16 (d, J = 13 Hz, 6-H), 3.57 and 3.63 (both s, 19 and 7-OMe), 4.76 and 4.91 (both s, 17-H_2), 5.40 (dd, J = 4, 12 Hz, 3-H); δ_C(100 MHz, $CDCl_3$, Me_4Si) 18.03 (q, 18-C), 19.03 (t, 11-C), 21.78 (q, -$OCOCH_3$), 25.82 (d), 26.38 (t), 32.49 (t), 36.92 (t), 39.42 (d, 10-C), 39.83 (d, 13-C), 46.21 (t, 15-C), 48.37 (s, 8-C), 48.60 (d, 9-C), 50.45 (s, 4-C), 50.58 (d, 6-C), 50.80 (t, 1-C), 51.93 and 52.43 (2q, 2 × -OMe), 73.71 (d, 3-C), 106.85 (t, 17-C), 158.37 (s, 16-C), 170.86 (s, -$OCOCH_3$), 174.44 (s, 7-C), 175.87 (s, 19-C); m/z (EI) 404.2144 (M^+ − $C_{23}H_{32}O_6$ requires 404.2141), 372 (91%), 344 (6), 326 (12), 312 (48), 284 (100), 252 (30), 225 (40), 197 (12), 171 (21), 91 (21).

Dimethyl *ent*-3α-hydroxy-20-norgibberell-16-ene-7,19-dioate 30

The 20-nor acetate derivative **29** (25 mg) in methanol (3 cm^3) was treated with potassium carbonate (100 mg) and the suspension stirred at room temperature for 2.5 hours. The product was partitioned between 5% w/v aqueous citric acid and ethyl acetate. Recovery of the organic layer followed by removal of solvent *in vacuo* afforded the 3-hydroxy derivative **30** (19 mg) as a colourless oil. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.35 (s, 18-H_3), 2.53 (dd, J = 6, 13 Hz, 5-H), 3.08 (d, J = 13 Hz, 6-H), 3.60 and 3.62 (both s, 7- and 19-OMe), 4.33 (dd, J = 4, 12 Hz, H-3), 4.75 and 4.88 (both br s, 17-H_2); GC-MS (OTMS) m/z 434 (M^+, 1%), 419 (59), 402 (97), 374 (100), 359 (8), 342 (9), 314 (12), 284 (54), 252 (15), 225 (48), 129 (76), 73 (91).

Dimethyl *ent*-3-oxo-20-norgibberell-16-ene-7,19-dioate 31

The 20-nor-3-hydroxy derivative **30** (19 mg) in acetone (1 cm^3) at 0 °C was treated with Jones reagent until the supernatant remained orange. The solution was stirred at this temperature for a further 10 minutes. The solution was poured into water, acidified to pH 3 with conc. HCl and extracted into ethyl acetate. Recovery of the organic layer and removal of the solvent *in vacuo* afforded the 3-ketone **31** (9 mg) as a colourless oil. δ_H(400 MHz, $CDCl_3$, Me_4Si) 1.49 (s,18-H_3), 2.68 (dd, J = 5, 12 Hz, 5-H), 3.07 (d, J = 12.5 Hz, 6-H), 3.53 and 3.58 (both s, 7- and 19-OMe), 4.73 and 4.87 (both br s, 17-H_2).

***E. coli* expression and protein extraction**

Electro-competent *E. coli* BL21 cells were transformed with Arabidopsis 20-oxidase cDNA At2353 inserted into the pET-9d vector. Kanamycin resistant colonies were selected, grown up and plasmids isolated and checked for insert by restriction analysis. For preparation of 20-oxidase enzyme, selected cell lines were cultured in 2 × YT medium at 37 °C to OD_{600} equals 0.6 and enzyme expression induced with IPTG (1 mM) for 3 h at 30 °C. The cultures were centrifuged at 2000 *g* for 20 min and the cells resuspended in Tris buffer (100 mM, pH 7.5), containing lysozyme and DNAse. The cells were ruptured by freeze–thawing and sonication. Cell debris was separated by centrifugation at 15000 *g* for 5 min. Aliquots of this cell-free enzyme preparation were used directly in empirical qualitative gibberellin turnover experiments.

Incubations of GA analogues with a recombinant GA 20-oxidase

Control incubations to confirm activity for the conversion of GA_{12} to GA_9 were carried out for each enzyme prepared as above. Incubations were carried out in a total volume of 100 mm^3 made up as follows. Enzyme preparation (70 mm^3), cofactor mix [10 mm^3, containing 2-oxoglutarate (4 mM), ascorbic acid (4 mM), ferrous sulfate (0.5 M), dithiothreitol (2 mM), BSA (bovine serum albumen) (2 mg cm^{-3}) and catalase (1 mg cm^{-3}) made up in Tris HCl (10 mM)], substrate (0.005 mg in 50% aqueous methanol, 10 mm^3) and Tris buffer (100 mM, pH 7.5, 10 mm^3). Reactions were incubated at 30 °C overnight. Following this, acetic acid (10 mm^3) and distilled water (140 mm^3) were added and the sample centrifuged at 13000 rpm for 5 min. The supernatant was loaded onto a 1 cm^3 C_{18} (octadecylsilyl) SPE column which had been previously washed with methanol (3 cm^3) and then acidified water (pH 3, 3 cm^3). After application of the GA sample a further 3 cm^3 of acidified water were washed through the column. The GA was eluted with methanol (3 cm^3) and dried down. To remove the lipid, the sample was redissolved in 50% aqueous methanol (1 cm^3) and partitioned against an equal volume of *n*-hexane. The lower aqueous phase was collected and dried down. After redissolving in methanol the GA was methylated by the addition of diazomethane and dried down. The sample was dissolved in ethyl acetate (1cm^3) and partitioned against an equal volume of doubly distilled water (pH 3). The upper organic layer was removed and applied to a 200 mm^3 aminopropyl SPE column that had been pre-equilibrated with ethyl acetate (3 cm^3). The eluate was collected and further ethyl acetate (3 cm^3) was washed through the column and collected. The combined ethyl acetate fractions were then dried down and analysed by GC-MS as TMS derivatives.

Acknowledgements

IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. We also thank the BBSRC for a linked research group grant (CLW, PH and MHB) and the Royal Society for a returning fellowship (RGSB)

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