Stereochemistry of the oxidation of gibberellin 20-alcohols, GA_{15} and GA_{44}, to 20-aldehydes by gibberellin 20-oxidases

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(20*R*)- and (20*S*)-[20-$^2H_1$]-gibberellins A_{15} and A_{44} have been used to determine the stereochemistry of the conversion to the 20-aldehyde catalysed by gibberellin 20-oxidases.

The stepwise oxidation and subsequent removal of carbon-20 from C_{20}-precursors is one of the most important sequences of reactions in the biosynthesis of biologically active C_{19} gibberellin (GA) plant hormones (Scheme 1). This conversion is unusual in that carbon-20 is lost at the aldehyde oxidation level in a reaction which also involves concerted formation of the 19,10-lactone function, a characteristic structural feature of the C_{19}-GAs, such as GA_9 and GA_{20}. The 20-carboxylic acid is also a biosynthetic product, but is not an intermediate in C_{19}-GA formation. Genes encoding GA 20-oxidases have been cloned from several plants.[1–5] These enzymes are 2-oxoglutarate-dependant dioxygenases, an important class of soluble, Fe^{II}-containing oxygenases .[6] Functional expression of GA 20-oxidase clones in *E. coli* has provided the means for a detailed study of the mechanism. Although studies in cell-free systems of spinach[7] indicated that more than one enzyme may be involved in the conversion of 20-methyl compounds *via* the 20-alcohol and 20-aldehyde to the C_{19}-lactone, all GA 20-oxidases so far cloned are able to convert substrates with 20-methyl functions through to C_{19}-lactones and/or 20-carboxylic acids.

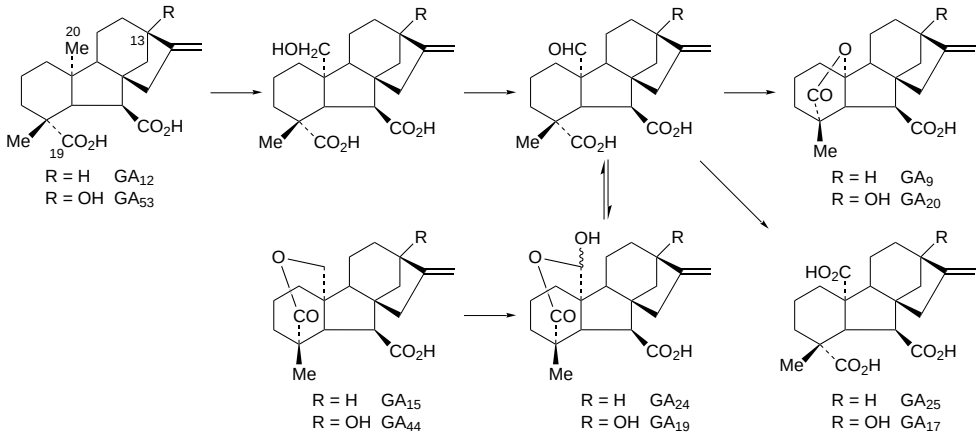
Here we report studies on the stereochemistry of the conversion of the 20-alcohol to the 20-aldehyde. Results are compared in cell-free systems from spinach with those obtained using recombinant enzyme from high-level expression of the *Arabidopsis thaliana* GA 20-oxidase clone At2353[2] in *E. coli* using the pET9d vector;† this enzyme converts GA_{12} to GA_9 in high yield.

The syntheses of (20*R*)- and (20*S*)-[20-^{2}H]-GA_{15} are summarised in Scheme 2. The route relies on the use of bulky *tert*-butyldiphenylsilyl (TBDPS) protecting groups to distinguish between the alcohol functions in the triol **1**, allowing manipulation of C-20, the most sterically hindered of the three hydroxymethyl groups. Triol **1** and its [$^2H_6$]-isotopomer **1a** were prepared by $LiAlH_4$ ($LiAlD_4$) reduction of GA_{25} trimethyl ester, itself prepared by deoxygenation of GA_{13} trimethyl ester.

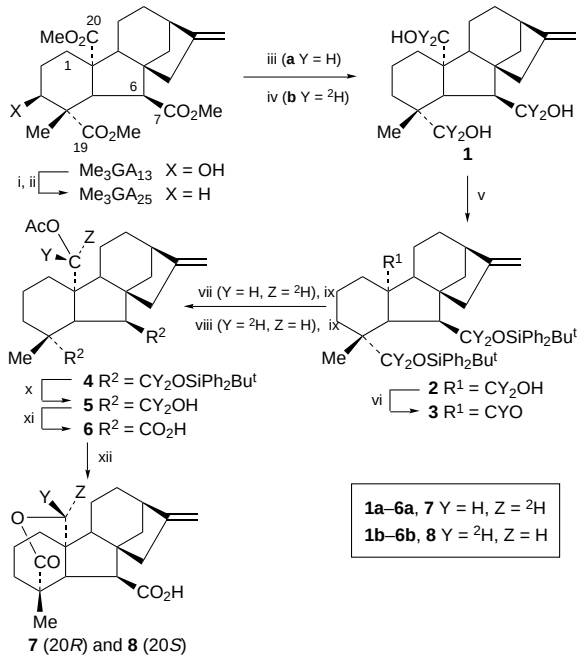
Treatment of **1** with TBDPSCl and imidazole gave the 7,19-*bis*-TBDPS derivative **2** in high yield. Swern oxidation of **2** gave the 20-aldehyde **3**. Reduction of the aldehyde with $NaBD_4$ was stereoselective (see later) and returned the [20-$^2H_1$]-alcohol **4**, which was protected as the acetate, before removal of the TBDPS groups with Bu_4NF to give 7,19-diol **5**. Oxidation to the 7,19-dicarboxylic acid **6** followed by hydrolysis yielded (20*R*)-[20-^{2}H]GA_{15} (d_1-65%, d_0-35%), in the lactone form **7**. The corresponding (20*S*) isomer **8** (d_1-99%, d_0-1%), was prepared in an analogous way from **1a,** reducing the 20-deuterio-aldehyde **3a** with $NaBH_4$ as shown in Scheme 2.

The configurations of **7** and **8** were assigned by ^{1}H NMR spectroscopy. In fully protio species, the 20-hydrogens resonate at δ 4.12 (d, *J* 12.5 Hz) and 4.48 (dd, *J* 12.5 and 2.5 Hz). The downfield signal was assigned to the 20-*proR* proton by virtue of the long range W-coupling (2.5 Hz) to 1β-H, as confirmed by a difference NOE experiment, which revealed a clear NOE from 6-H to the signal at δ 4.48 and the absence of any enhancement of the upfield doublet at δ 4.12, assigned to the 20-*proS* hydrogen. Both **7** and **8** were labelled stereospecifically.

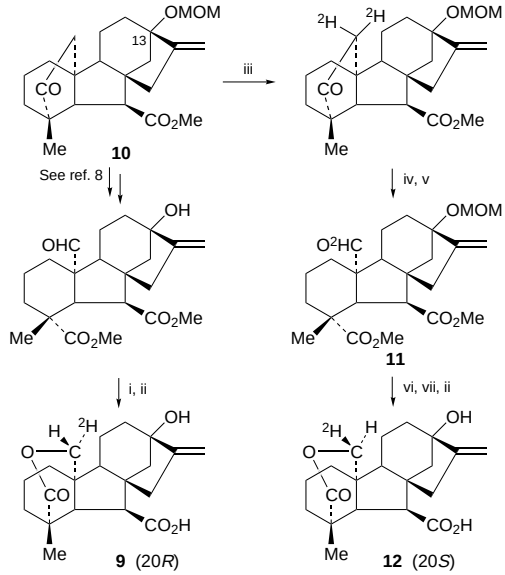
The corresponding [20-$^2H_1$] isotopomers of the 13-hydroxylated gibberellin, GA_{44} **9** and **12**, were synthesised as shown in



Scheme 1 Reactions catalysed by GA-20-oxidases



Scheme 2 Synthesis of (20*R*)- and (20*S*)-[20-$^2H_1$]-GA_{15}. *Reagents*: i, KH, CS_2, MeI; ii, Bu_3SnH; iii, $LiAlH_4$; iv, $LiAl^2H_4$; v, Bu^tPh_2SiCl, imidazole; vi, $(COCl)_2$, Me_2SO, Et_3N; vii, NaB^2H_4; viii, $NaBH_4$; ix, Ac_2O, pyridine; x, $Bu_4N^+F^-$; xi, Jones oxidn; xii, NaOH, aq. MeOH.



Scheme 3 Synthesis of (20*R*)- and (20*S*)-[20-$^2H_1$]-GA_{44} *via* GA_{19}. *Reagents*: i, NaB^2H_4; ii, NaOH, aq. MeOH; iii, ^2H_2O, K_2CO_3; iv, KH, DMF, THF, O_2; v, CH_2N_2; vi, Dowex 50W(H^+); vii, $NaBH_4$.

Scheme 3. (20*R*)-[20-$^2H_1$]-GA_{44} **9** (d_1-95%, d_0-5%) was produced by $NaBD_4$ reduction of GA_{19} dimethyl ester followed by hydrolysis of the esters (2 mol dm^{-3} aq. NaOH–MeOH, 1 : 1, reflux, 2 h). For the synthesis of (20*S*)-[20-$^2H_1$]-GA_{44}, the starting point was ketone **10**, an intermediate in the synthesis[8] of GA_{19}. Deuterium exchange at C-20, oxidative cleavage[8] and methylation gave [20-$^2H_1$]-GA_{19} dimethyl ester, 13-methoxymethyl ether **11**. Deprotection at C-13 and then reduction of the aldehyde followed by demethylation gave the required (20*S*)-[20-$^2H_1$]-GA_{44} **12** (d_1-61%, d_0-39%). The stereochemistry at C-20 was assigned by ^1H NMR spectroscopy as above.

Incubation of unlabelled GA_{15}, as the lactone or as the 20-hydroxy-19-carboxylic acid ('open lactone'),‡ with recombinant At2353 20-oxidase and analysis of the products by GC–MS revealed that only the open lactone was accepted as a substrate by the enzyme. A similar result has been obtained in cell-free systems from *C. maxima* endosperm[9] and pea cotyledons,[10] whereas homogenates of spinach leaves oxidise the closed-lactone form of GA_{44}.[7] Although the product of metabolism of GA_{15} open lactone was mainly GA_9, under short incubation times§ the intermediate 20-aldehyde GA_{24} was readily detectable by GC–MS.

Incubation§ of (20*R*)- and (20*S*)-[20-$^2H_1$]-GA_{15} **7** and **8,** and GA_{44} **9** and **12** in the open-lactone form‡ with recombinant enzyme and GC–MS analysis of the 20-aldehyde (GA_{24} and GA_{19}, respectively) products, revealed that, for both GA_{15} and GA_{44} substrate, there is stereospecific loss of the 20-*proR* hydrogen (Table 1). In contrast, incubation of **9** and **12,** in the closed-lactone form, with the spinach cell-free system[7] and analysis of the 20-aldehyde (GA_{19}) produced showed that, in this case, the 20-*proS* hydrogen is lost (Table 1). Thus, this cell-free system contains an enzyme that is capable of oxidising C-20, presumably in the lactone form, and it does so with the opposite stereochemistry to that observed with the recombinant *A. thaliana* protein, which only accepts the open-lactone form. In the lactone form, the 20-*proR* hydrogen is fixed in a severely sterically hindered position under ring B and, thus, it is not surprising that the lactone 20-oxidase, present in spinach, removes the 20-*proS* hydrogen. However, in the enzymes that accept only the 'open-lactone' form, the substrate presumably is held such that the 20-*proR* hydrogen is more exposed.

Table 1 Deuterium incorporations in enzymic products

Substrate	$d_1 : d_0$(%)	Enzyme	Product	$d_1 : d_0$(%)[a]
7 (20*R*) Open lactone	65 : 35	At2353	GA_{24}	0 : 100
8 (20*S*) Open lactone	99 : 1	At2353	GA_{24}	97 : 3
9 (20*R*) Open lactone	95 : 5	At2353	GA_{19}	0 : 100
12 (20*S*) Open lactone	61 : 39	At2353	GA_{19}	50 : 50
9 (20*R*) Lactone	95 : 5	Spinach	GA_{19}	85 : 15
12 (20*S*) Lactone	61 : 39	Spinach	GA_{19}	13 : 87

[a] GA_{24} and GA_{19} were identified by GC–MS comparison (KRI and spectra), as Me or Me, Me_3Si derivatives, with authetic samples. Due to weak molecular ions isotope enrichments were calculated on $M^+ - 32$ (*m*/*z* 342) for GA_{24} and $M^+ - 28$ (*m*/*z* 434) for GA_{19}.

Footnotes

† Full details of the functional expression and characterisation of GA 20-oxidases will be reported elsewhere.

‡ The lactone-opened form of 20-hydroxymethyl GAs was produced by treatment with 2 mol dm^{-3} NaOH.

§ Recombinant enzyme incubations were carried out in a total volume of 100 μl of TrisCl buffer (1 mol dm^{-3}, pH 7.5) containing 70 μl enzyme preparation [45% pure enzyme (estimated from SDS gel), 2.5 mg protein ml^{-1}]. GA substrate (5 μg), 2-oxoglutarate (8 mmol dm^{-3}), ascorbic acid (8 mmol dm^{-3}), dithiothreitol (8 mmol dm^{-3}), $FeSO_4$(1 mmol dm^{-3}), catalase (2 mg ml^{-1}) and bovine serum albumin (4 mg ml^{-1}).

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