Plant Physiol. (1998) 117: 559–563

Function and Substrate Specificity of the Gibberellin 3β-Hydroxylase Encoded by the Arabidopsis *GA4* Gene[1]

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cDNA corresponding to the *GA4* gene of *Arabidopsis thaliana* L. (Heynh.) was expressed in *Escherichia coli*, from which cell lysates converted [^{14}C]gibberellin (GA)$_9$ and [^{14}C]GA$_{20}$ to radiolabeled GA$_4$ and GA$_1$, respectively, thereby confirming that *GA4* encodes a GA 3β-hydroxylase. GA$_9$ was the preferred substrate, with a Michaelis value of 1 μM compared with 15 μM for GA$_{20}$. Hydroxylation of these GAs was regiospecific, with no indication of 2β-hydroxylation or 2,3-desaturation. The capacity of the recombinant enzyme to hydroxylate a range of other GA substrates was investigated. In general, the preferred substrates contained a polar bridge between C-4 and C-10, and 13-deoxy GAs were preferred to their 13-hydroxylated analogs. Therefore, no activity was detected using GA$_{12}$-aldehyde, GA$_{12}$, GA$_{19}$, GA$_{25}$, GA$_{53}$, or GA$_{44}$ as the open lactone (20-hydroxy-GA$_{53}$), whereas GA$_{15}$, GA$_{24}$, and GA$_{44}$ were hydroxylated to GA$_{37}$, GA$_{36}$, and GA$_{38}$, respectively. The open lactone of GA$_{15}$ (20-hydroxy-GA$_{12}$) was hydroxylated but less efficiently than GA$_{15}$. In contrast to the free acid, GA$_{25}$ 19,20-anhydride was 3β-hydroxylated to give GA$_{13}$. 2,3-Didehydro-GA$_9$ and GA$_5$ were converted by recombinant GA4 to the corresponding epoxides 2,3-oxido-GA$_9$ and GA$_6$.

Dwarf mutants with reduced biosynthesis of the GA plant hormones have been valuable tools in studies of the function of these compounds (Ross, 1994). In *Arabidopsis thaliana*, mutations at six loci (*GA1-GA6*) that result in reduced GA biosynthesis have been identified (Koorneef and van der Veen, 1980; Sponsel et al., 1997), and three of these loci have recently been cloned. The *GA1* locus was isolated by genomic subtraction (Sun et al., 1992) and shown by heterologous expression in *Escherichia coli* to encode the enzyme that cyclizes geranylgeranyl diphosphate to copalyl diphosphate (Sun and Kamiya, 1994). This enzyme was formerly referred to as *ent*-kaurene synthase A but has been renamed copalyl diphosphate synthase (Hedden and Kamiya, 1997; MacMillan, 1997). The *GA5* locus was shown to correspond to one of the GA 20-oxidase genes (Xu et al., 1995), the products of which catalyze the conversion of GA$_{12}$ to GA$_9$ and GA$_{53}$ to GA$_{20}$ (Phillips et al., 1995; Xu et al., 1995). GA 20-oxidases are 2-oxoglutarate-dependent dioxygenases that are encoded by small multigene families, members of which are differentially expressed in plant tissues (Phillips et al., 1995; Garcia-Martinez et al., 1997).

The *GA4* locus was isolated by T-DNA tagging and, on the basis of the derived amino acid sequence, was also shown to encode a dioxygenase (Chiang et al., 1995). Several lines of evidence indicate that the *GA4* gene encodes a GA 3β-hydroxylase. Shoots of a *ga4* mutant, all alleles of which are semidwarf, contained reduced concentrations of the 3β-hydroxy GAs GA$_1$, GA$_4$, and GA$_8$ compared with the Landsberg *erecta* wild type, whereas levels of immediate precursors to these GAs were elevated (Talon et al., 1990). Furthermore, metabolism of [^{13}C]GA$_{20}$ to [^{13}C]GA$_1$ was substantially less in the mutant than in the wild type (Kobayashi et al., 1994). In the present paper we confirm by functional expression of its cDNA in *E. coli* that *GA4* encodes a GA 3β-hydroxylase. In addition, we determine the substrate specificity of recombinant GA4 using a number of C$_{20}$- and C$_{19}$-GAs and show by kinetic analysis that the enzyme has a higher affinity for GA$_9$ than for GA$_{20}$, which is consistent with the non-13-hydroxylation pathway predominating in Arabidopsis (Talon et al., 1990).

MATERIALS AND METHODS

Expression of *GA4* in *Escherichia coli*

The coding region of *GA4* was amplified by reverse transcription-PCR from mRNA extracted from floral apices of the *ga1–2* mutant of *Arabidopsis thaliana* (L.) Heynh. as described previously (Phillips et al., 1995) using oligonucleotide primers with *Nco*I and *Bam*HI sites: forward primer, CAACCATGGCTGCTATGTTAACAGA; reverse primer, CAAGGATCCTCATTCTTCTCTGTGATTT.

The cloned PCR product was transferred into pET3d and pET9d *E. coli* expression vectors (Pharmacia). Although the expression products contained 3β-hydroxylase activity, sequencing of the PCR product identified a point mutation, resulting in a change of amino acid within the coding region. To prepare expression constructs with the correct sequence (Chiang et al., 1997), a *Sac*I-*Bam*HI fragment containing the mutation was replaced in both vectors with the corresponding fragment from the *GA4* cDNA clone pCD7 (Chiang et al., 1995). Cultures (50 mL) of *E. coli* BL21 transformed with the recombinant plasmids were grown

[1] IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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Abbreviation: IPTG, isopropyl-β-thiogalactoside.

with shaking at 37°C in 2× YT broth (1.6% [w/v] bactotryptone, 1% [w/v] yeast extract, and 0.5% [w/v] NaCl) containing 200 μg L^{-1} carbenicillin (pET3d) or 100 μg mL^{-1} kanamycin (pET9d). At the mid-logarithmic stage, cultures were transferred to 30°C and shaken for 30 min, after which expression was induced by the addition of IPTG (final concentration, 5 mM). At the same time, more antibiotics were added (400 μg mL^{-1} carbenicillin or 200 μg mL^{-1} kanamycin). Cultures were grown for another 3 h at 30°C and then placed on ice for 10 min, and cells were harvested by centrifugation at 5000 rpm at 4°C for 5 min. Pellets were resuspended in 25 mL of 100 mM Tris-HCl, pH 7.1, at 25°C containing 4 mM DTT, and recentrifuged as described above. Pellets were then resuspended in the same buffer (2 mL) containing lysozyme at 1 mg/mL, incubated at 30°C with shaking for 15 min, and, after cooling on ice, sonicated (three 5-s pulses). After centrifugation of the lysates at 12,000*g* for 15 min at 4°C, the supernatants were frozen in liquid N_2 and stored at −80°C.

Analysis of Expressed Protein by SDS-PAGE

Proteins from cultures induced with IPTG, described above, were analyzed by SDS-PAGE. For comparison, proteins were analyzed from cells grown under the same conditions but without the addition of IPTG. Induced cells contained typically 0.5 to 1.0 mg protein mL^{-1} culture, whereas noninduced cells contained approximately 30% of the protein concentration present in induced cells. Soluble and insoluble protein fractions were obtained after lysis of cells (1 mL) using lysozyme, as described above. After the sample was centrifuged, the supernatant and resuspended pellet, in 100 mM Tris-HCl buffer, pH 7.5 (0.1 mL), were diluted 1:1 with loading buffer. Equal quantities of protein for each sample were loaded onto the gel.

Enzyme Assays with Recombinant Protein

Provision of Substrates

[17-^{14}C]GA_9 (specific radioactivity 2.10 TBq mol^{-1}) and [17-^{13}C,^{3}H]GA_5 (1.51 TBq mol^{-1}) were gifts from Dr I. Yamaguchi (University of Tokyo) and Prof. J. MacMillan (Long Ashton Research Station), respectively. [17-^{14}C]GA_{24} (1.72 TBq mol^{-1}) and [17-^{14}C]GA_{19} (1.72 TBq mol^{-1}) were obtained from Prof. L.N. Mander (Australian National University, Canberra). 2,3-Didehydro[17-^{14}C]GA_9 (1.75 TBq mol^{-1}) and [17-^{14}C]GA_{20} (1.84 TBq mol^{-1}) were synthesized as described by MacMillan et al. (1997). [1,7,12,18-$^{14}C_4$]GA_{12} (5.74 TBq mol^{-1}), −GA_{12}-aldehyde (6.90 TBq mol^{-1}), and −GA_{15} (6.32 TBq mol^{-1}) were prepared from *R*-[2-^{14}C]mevalonic acid using a cell-free system from pumpkin endosperm, as described by Graebe et al. (1974). [1,7,12,18-$^{14}C_4$]GA_{53} (5.59 TBq mol^{-1}) and [17-^{14}C]GA_{44} (1.42 TBq mol^{-1}) were prepared from [$^{14}C_4$]GA_{12} and [$^{14}C_1$]GA_{12}, respectively, using a homogenate of developing pea cotyledons (Kamiya and Graebe, 1983). [$^{14}C_4$]GA_{25} (6.83 TBq mol^{-1}) was prepared from [$^{14}C_4$]GA_{12} using a partially purified GA 20-oxidase from pumpkin endosperm (Lange et al., 1994). The open-lactone forms of GA_{15} and GA_{44} were prepared by heating the lactones in 0.5 M KOH at 90°C for 1 h in a sealed vial. An appropriate volume (3–5 μL) of the hydrolysate was then added directly to the incubation mixture.

Enzyme Assays

For incubations with different substrates, cell lysates (5 or 50 μL) were incubated for 1 h at 30°C with the substrate, which was added in 5 μL of methanol in the presence of 100 mM Tris-HCl, pH 7.5, and a cofactor mixture (5 μL, containing 80 mM 2-oxoglutarate, 80 mM ascorbate, 80 mM DTT, 10 mM $FeSO_4$, 40 mg mL^{-1} BSA, and 20 mg mL^{-1} catalase in 100 mM Tris-HCl, pH 7.5) in a total volume of 0.1 mL. After the addition of acetic acid (10 μL) and water (140 μL), the incubation mixture was centrifuged at 3000 rpm for 10 min and then analyzed directly by HPLC with on-line radiomonitoring (MacMillan et al., 1997). Product identities were determined by full-scan GC-MS of methyl ester-trimethylsilyl ether derivatives by comparison with published data (Gaskin and MacMillan, 1991).

For kinetic studies [^{14}C]GA_9 added in 5 μL of methanol was incubated for 15 min at 30°C at different concentrations in the presence of cell lysate (equivalent to 0.22 μL containing 2 μg of protein) and 10 μL of the cofactor mixture in a total volume of 0.2 mL. [^{14}C]GA_{20} was incubated with cell lysate (equivalent to 0.55 μL containing 5 μg of protein) under the same conditions except that 2.5 μL of the cofactor mixture was used and the total volume was 50 μL. The dependence of the GA-hydroxylation rate (determined by HPLC with on-line radiomonitoring as described above) on GA concentration was established by nonlinear regression analysis using Enzfitter (Elsevier, Cambridge, UK).

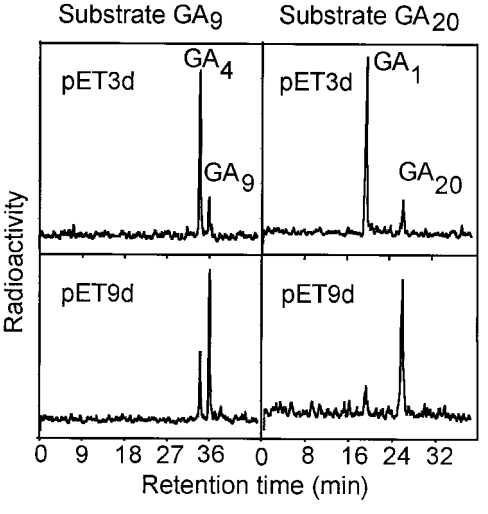

Figure 1. HPLC-radiochromatograms from incubations of [^{14}C]GA_9 and [^{14}C]GA_{20} with cell lysates of *E. coli* expressing *GA4* in pET3d or pET9d. The equivalent of 0.05 or 0.12 μL of lysate was incubated in 100 μL total volume with GA_9 or GA_{20}, respectively, plus the necessary cofactors.

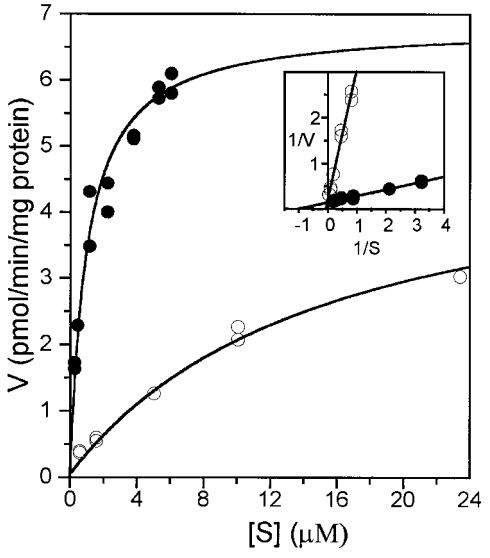

Figure 2. Michaelis-Menten and Lineweaver-Burk (inset) plots for the 3β-hydroxylation of GA_9 to GA_4 (●) and of GA_{20} to GA_1 (○) by cell lysates from recombinant *E. coli* expressing *GA4* in pET3d.

RESULTS AND DISCUSSION

Heterologous Expression of *GA4* in *E. coli*

GA4 cDNA was inserted into pET3d and pET9d vectors, and expression was induced in *E. coli* with 5 mM IPTG. Cell lysates from cultures of bacteria containing either expression construct metabolized [^{14}C]GA_9 to [^{14}C]GA_4, and [^{14}C]GA_{20} to [^{14}C]GA_1, with higher activity obtained with pET3d (Fig. 1). Lysates from bacteria containing vector with no insert did not metabolize either substrate (data not shown). Therefore, it is confirmed that *GA4* encodes a GA 3β-hydroxylase. The *LE* gene of pea has now also been cloned and, after expression in *E. coli*, shown to encode a GA 3β-hydroxylase with 54% amino acid identity to the corrected (Chiang et al., 1997) GA4 sequence (Lester et al., 1997; Martin et al., 1997). Separation of proteins from total cell extracts of both *E. coli* cultures by SDS-PAGE revealed a band of the anticipated size (M_r approximately 40,000). Although this band was more intense in extracts from cells containing the pET9d construct, virtually all of the protein was present in the insoluble fraction in this case and was presumably present in inclusion bodies. Therefore, the pET3d vector was used to produce active protein for characterization of enzyme activity.

Table I. *Efficiency of 3β-hydroxylation of potential GA substrates by cell lysates from E. coli expressing GA4*

Products were identified by comparison of their mass spectra with published data for unlabeled compounds (Gaskin and MacMillan, 1991).

Potential Substrate	Potential Product	Conversion in 1 h		Mass Spectrum of Product
		5 μL of Lysate	50 μL of Lysate	
		%		*m/z (% relative abundance)*
GA_{12}-aldehyde[a]	GA_{14}-aldehyde	–[b]	0	
GA_{12}[a]	GA_{14}	–	0	
GA_{53}[a]	GA_{18}	–	0	
GA_{15}[a]	GA_{37}	75	100	M^+ 440(33), 438(19), 432(25), 425(14), 408(25), 350(34), 348(12), 342(10), 318(100), 316(32), 310(50), 292(43), 290(21), 288(46),
GA_{44}[c]	GA_{38}	–	60	M^+ 528(43), 526(25), 520(22), 513(3), 497(3), 467(4), 438(11), 377(9), 282(13), 240(16), 209(100), 207(49)
GA_{15} open lactone[a]	GA_{37}	0	25	
GA_{44} open lactone[c]	GA_{38}	–	0	
GA_{24}[c]	GA_{36}	19	67	M^+ 464(5), 449(5), 432(39), 404(29), 376(15), 375(14), 342(26), 314(48), 286(100), 255(22), 227(41), 211(51), 173(48), 129(82)
GA_{19}[c]	GA_{23}	–	0	
GA_{25}[a]	GA_{13}	–	0	
GA_{25}-anhydride[d]	GA_{13}	–	40[e]	M^+ 492(0), 477(2), 436(7), 400(19), 342(8), 282(17), 251(8), 223(11), 160(13), 129(100)
GA_9[c]	GA_4	100	–	M^+ 420(19), 402(9), 392(12), 388(23), 360(12), 345(6), 330(22), 302(15), 298(22), 291(63), 286(100), 263(32), 234(46), 230(34), 227(90), 226(82), 203(37), 175(35), 129(63)
GA_{20}[c]	GA_1	73	–	M^+ 508(100), 493(9), 450(21), 378(19), 377(15), 359(7), 315(12), 237(11), 209(41), 195(18), 182(13)
2,3-Didehydro-GA_9[c]	2,3-Oxido-GA_9	43	100	M^+ 346(6), 314(100), 286(6), 267(8), 242(95), 225(43), 224(51), 214(18), 181(26), 155(57)
GA_5[f]	GA_6	–	1[e]	M^+ 433(100), 417(8), 374(15), 304(76), 236(44), 208(61)

[a] [1, 7, 11, 18-$^{14}C_4$]; [b] –, Not determined. [c] [17-^{14}C]. [d] Unlabeled. [e] Determined by GC-MS. [f] [17-^{13}C, $^3H_2$].

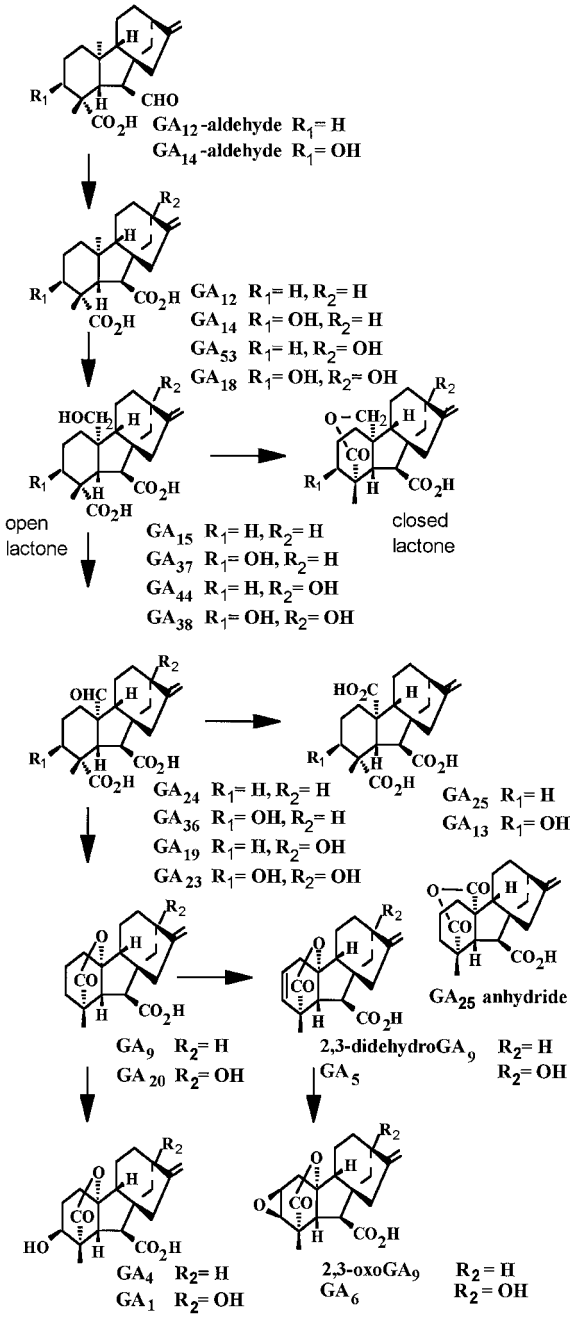

Figure 3. Structures of potential substrates and products arranged in their proposed biosynthetic relationship.

Characteristics of Recombinant GA4

[^{14}C]GA_9 and [^{14}C]GA_{20} were compared as the substrates for recombinant GA4 in cell lysates. Plots of reaction rate against substrate concentration, produced by nonlinear regression analysis (Fig. 2), yielded K_m values of 1.0 μM (V_{max} 6.8 pmol min^{-1} mg^{-1}) and 15 μM (V_{max} 2.8 pmol min^{-1} mg^{-1}) for GA_9 and GA_{20}, respectively. The preference for GA_9 as a substrate is consistent with the predominance of non-13-hydroxylated GAs in Arabidopsis; the major C_{19}-GA identified from entire shoots is GA_4 (Talon et al., 1990). Furthermore, the Arabidopsis GA 20-oxidases convert non-13-hydroxylated substrates, e.g. GA_{12}, more effectively than their 13-hydroxylated equivalents, e.g. GA_{53} (Phillips et al., 1995). Therefore, the preferred biosynthetic pathway in Arabidopsis appears to involve conversion of GA_{12} to GA_9, which is then 3β-hydroxylated to GA_4. The K_m values are very similar to those determined recently for the GA 3β-hydroxylase from pea, obtained by expression of the *LE* cDNA in *E. coli* (Martin et al., 1997). In pea shoots, which produce mainly 13-hydroxylated GAs, the preference of the enzyme for GA_9 was unexpected.

The substrate specificity of GA4 was further tested by incubating cell lysate with a number of nonhydroxylated and 13-hydroxylated C_{19}- and C_{20}-GAs at similar concentrations (1.8 μM; Table I); products, when formed, were identified by full-scan GC-MS. The structures of the tested potential substrates and products are shown in Figure 3, where they are arranged in their proposed biosynthetic relationship. In most cases the substrates were labeled with ^{14}C, enabling their degree of conversion to be quantified by radiomonitoring after HPLC. Therefore, their relative effectiveness as substrates could be readily compared. To compare the broad range of hydroxylation efficiencies encountered with these substrates, incubations were performed with both 5- and 50-μL cell lysate volumes, when appropriate. In general, C_{19}-GAs were much better substrates than C_{20}-GAs, and the presence of a 13-hydroxy group reduced the efficiency of conversion. No conversion was observed with GA_{12}-aldehyde, GA_{12}, GA_{53}, GA_{19}, or GA_{25}, whereas GA_{15}, GA_{44}, and GA_{24} were hydroxylated to GA_{37}, GA_{38}, and GA_{36}, respectively. GA_{15}, as the lactone, was hydroxylated almost as efficiently as GA_{20}. In contrast, the open-lactone forms of GA_{15} and GA_{44} were poor substrates.

It seems likely that these GAs in the lactone form mimic the C_{19}-GA substrates, which contain a γ-lactone. The low level of hydroxylation of the open-lactone form of GA_{15} may be due to some lactone formation during incubation. In solution, GA_{24} is likely to exist partially as a lactol between the C-20 aldehyde and the C-19 carboxylic acid group and would therefore also mimic the C_{19}-GA structure. Whereas the tricarboxylic acid GA_{25} was not metabolized by GA4, its 19–20 anhydride (Fig. 3) was hydroxylated to give GA_{13}. The incubation was conducted with unlabeled GA_{25} anhydride and, therefore, it was not possible to determine the conversion efficiency accurately. However, on the basis of GC-MS on the total extracted products, about 40% of the 1.4 nmol of substrate was converted by 120 μL of cell lysate (140 μL total incubation volume) in 1 h, indicating relatively efficient conversion. The expected product, GA_{13} anhydride, would be converted to GA_{13} on acidification of the incubation mixture prior to extraction.

These results indicate that a presumably polar bridge between C atoms 4 and 10 (lactone, lactol, or anhydride) is necessary for substrate binding to the 3β-hydroxylase. This requirement is in contrast to that of a GA 2β,3β-dihydroxylase, which was recently cloned from pumpkin endosperm and shown to utilize C_{20}-GAs, particularly GA_{25}, more effectively than C_{19}-GAs (Lange et al., 1997).

The high substrate specificity of the Arabidopsis 3β-hydroxylase for bridged, non-13-hydroxylated C_{20}-GAs may account for the occurrence of the 3β-hydroxylated C_{20}-GAs GA_{37}, GA_{36}, and GA_{13} in shoots of this species (Talon et al., 1990). The first two compounds would be formed by 3β-hydroxylation of the relatively abundant intermediates GA_{15} and GA_{24}, whereas GA_{13} may be a minor product of 20-oxidase activity on GA_{36}. The major product of this activity is likely to be GA_4.

The 2,3-didehydro-GAs GA_5 and 2,3-didehydroGA_9 were converted by GA4 to the corresponding epoxides, GA_6 and 2,3-oxidoGA_9, but these conversions were less efficient than those of their saturated analogs, GA_{20} and GA_9. Epoxidation of GA_5 has also been observed with a GA 3β-hydroxylase from immature seeds of *Phaseolus vulgaris* (Kobayashi et al., 1991) and contrasts the conversion of GA_5 to GA_3 in seeds of *Marah macrocarpus*, a reaction that is initiated by oxidation at C-1 (Albone et al., 1990). The 3β-hydroxylation of GA_9 and GA_{20} by GA4 is regiospecific, with no indication of 2,3-desaturation or 2β-hydroxylation, as was found for the enzyme from *P. vulgaris* (Smith et al., 1990).

ACKNOWLEDGMENTS

We thank Dr. I. Yamaguchi (University of Tokyo), Professor J. MacMillan (IACR-Long Ashton Research Station), and Professor L.N. Mander (Australian National University, Canberra), for gifts of radiolabeled GAs, and Dr. C.L. Willis (School of Chemistry, University of Bristol, UK) for providing the GA_{25} anhydride.

Received November 26, 1997; accepted March 11, 1998.

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