Phytochemistry, Vol. 26, No. 11, pp. 2895–2900, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

A RADIOIMMUNOASSAY FOR THE DIMETHYL ESTERS OF GA_{24} AND GA_{19}

S. KUROGOCHI, I. YAMAGUCHI,* M. FEYERABEND,† N. MUROFUSHI,* N. TAKAHASHI,* S. KUYAMA and E. W. WEILER†

Nihon Tokushu Noyaku Seizo K. K., Yuki Institute, Ibaraki 307, Japan; *Department of Agricultural Chemistry, University of Tokyo, Tokyo 113, Japan; †Pflanzenphysiologie, Universitaet Osnabrueck, D-4500 Osnabrueck, F.R.G.

(*Revised received* 31 *March* 1987)

Key Word Index—*Pharbitis nil;* Convolvulaceae; analysis of gibberellins; HPLC/RIA; GC/MS; radioimmunoassay for the dimethyl esters of GA_{19} and GA_{24}.

Abstract—GA_{24}-7,19-dimethyl ester was converted to dihydro GA_{24}-Me-17-chloroformate and dihydro GA_{24}-Me-17-cyanoacetate and coupled to bovine serum albumin. Antisera raised in rabbits against these immunogens were highly reactive with GA_{24}-7,19-dimethyl ester and were employed in a sensitive HPLC-radioimmunoassay (detection limit of RIA: 0.1–0.2 pmol) using GA_{24}-7-methyl ester-19-tritiated methyl ester as tracer. Some sera were also reactive with GA_{19}-Me, GA_{53}-Me and GA_{12}-Me. Levels of GA_{19} in immature seeds of *Pharbitis nil* obtained by RIA were compared to those obtained with an established GC/MS procedure and were found identical within the limits of experimental error. The synthetic procedures reported here should be applicable to a range of other gibberellins. The results exemplify the applicability of immunological assays to the analysis of C_{20}-gibberellins.

INTRODUCTION

The biosynthesis of bio-active C_{19}-gibberellins (GAs) proceeds via C_{20}-GAs. The immediate precursors of GA_9 and GA_{20} are GA_{24} and GA_{19}, respectively [1]. The transition from C_{20}- to C_{19}-GAs may be a key step regulating the biosynthesis of active GAs, and it has been shown that photoperiod influences the level of GA_{19} and GA_{20} in spinach [2], perhaps by regulating the conversion of GA_{19} to GA_{20}.

The quantitative analysis of C_{20}-GAs is exceedingly difficult because of their low levels, especially in vegetative tissues. From earlier studies, it became apparent that more selective sera were obtained for unsubstituted GAs such as GA_9 [3] while cross-reactions were more commonly observed for antisera raised against substituted GAs. As an example, GA_3 and GA_9 frequently cross-react with sera raised against GA_1 [3–5]. This can be explained on structural grounds in that, for example, replacement of a hydrogen by the more bulky hydroxyl-group may severely impair antibody fit of the whole GA structure while the reverse substitution (e.g. –H for –OH) may not give this effect. It appeared favourable, therefore, to raise group-selective antisera and combine them with efficient separation techniques such as HPLC. The feasibility of this approach was recently demonstrated for the HPLC/RIA of GA_4 and related GAs using a monoclonal antibody raised against GA_{13}-19,20-imide [6].

A similar approach was adopted for this study. The aim was to raise antibodies against GA_{19} starting from the more readily available GA_{24}, which differs from GA_{19} only in the absence of the 13-hydroxy function. In order to render the 13-position less accessible for the immune system (i.e. to allow for cross-reaction of GA_{19}), immunogen synthesis was carried out through the neighbouring exocyclic double bond, using different bridging structures. The GA_{24}-7,19-dimethyl ester (1, GA_{24}-Me) was used as starting material. This approach also allowed a facile synthesis of a ^{3}H-labelled GA_{24} derivatives of high specific radioactivity to be used as RIA tracers. It is shown here that by use of GA_{24} derivatives coupled to bovine serum albumin (BSA) through C-17, antibodies can be obtained which allow quantification of GA_{19}, by HPLC/RIA, in pg amounts. The immunoassay was validated using GC/MS as the reference technique and samples obtained from immature seeds of *Pharbitis nil*. These seeds are known to contain GA_{19}, at different levels, at various stages of their development.

RESULTS

Immunochemicals

To render GA_{24}-Me immunogenic, it was coupled to a protein. To this end, GA_{24}-Me (1) was hydroborated with bis-(3-methyl-2-butyl) borane in the presence of hydrogen peroxide to yield the dihydro GA_{24}-Me-17-ol (2) in 70% yield. From this starting material two conjugates with bovine serum albumin (BSA) were prepared (4, 6). Immunogen I (4) was obtained by reaction of dihydro GA_{24}-Me-17-chloroformate (3) with the protein in aqueous dioxan at pH 5.8. The conjugate contained 1 mol of GA per mol of protein. Immunogen II (6) was obtained via coupling of dihydro GA_{24}-Me-17-cyanoacetate (5) to diazotized *p*-aminohippurate substituted BSA. The conjugate contained 9 mol of GA per mol of protein. The use of GA_{24}-Me as hapten allowed a convenient preparation of tritiated tracers via alkylation, with ^{3}H-MeI, of the sodium salt of GA_{24}-7-monomethyl ester (7) in anhydrous conditions. The resultant GA_{24}-[^{3}H]Me (8) was isolated in 45% yield and high specific activity (9.6×10^{14} Bq/mol). The same approach has been used with similar results to prepare the 7-[^{3}H]methyl esters of GA_1,

GA_5 and GA_{20} (data not shown) and it should have a wide applicability in GA immunoanalysis.

Antiserum production

Significant binding of GA_{24}-[^{3}H]Me (**8**) was detected in sera from all immunized animals (immunogen I: two animals, immunogen II: three animals), after the first booster injection. Immunization was continued until after boost six according to the scheme in ref. [7] and the individual serum fractions were characterized for binding capacity. Serum fractions binding at least 30% of added GA_{24}-[^{3}H]Me (0.75 pmol, 721 Bq) at a final assay dilution of 1:900 were pooled, and the pooled sera characterized further. Both immunogens elicited similar antibody titers, but better sensitivity was obtained with immunogen I derived sera (Table 1). Whereas the reactivity of GA_{19}-7,19-dimethyl ester (**1**) with the group II sera was similar, immunogen I elicited a more variable response. From the initial screen, it could also be concluded that cross reactions of C_{19}-GAs are, apparently, negligible. Therefore, sera nos 422 and 423, both derived from the short-spacer immunogen I (**4**) were characterized in detail. For the analysis of GA_{19}, serum 423 was used.

Serum specificity

A detailed analysis of cross-reactions of antiserum 422 and 423 is given in Table 2. The structures of the relevant C_{20}-GAs are given in Fig.1. All compounds were analysed as methyl esters. If assessed by structural similarities, then neither of the γ-lactone GAs (C_{19}-GAs) tested were more than marginally reactive. Similarly, all δ-lactone GAs (C_{20}-GAs) tested (GA_{15}, GA_{27}, GA_{38}, GA_{44}) had low reactivities. Both sera clearly identify a very narrow group of C_{20}-GAs. Serum 422 was most selective in that even the difference of functionality of C-20 distinguishing GA_{12} (-Me) from GA_{24} (-CHO) was sharply discriminated. The presence of hydroxyl groups, e.g. in the 13-position (GA_{19}), 3-position (GA_{36}) or 3,13-position (GA_{23}) further reduce the reactivity to 4.3, 2.5 and 0.1% relative to GA_{24}.

In contrast, serum 423 did not discriminate, to the same extent, 13-non hydroxylated from 13-hydroxylated C_{20}-GAs. Consequently, GA_{19} was highly react-

Table 2. Cross-reactions of anti GA_{24}Me poolsera

	Cross-reactions (% molar)	
GA*	Serum 422	Serum 423
1	0	0
2	0.1	0
3	0	0
4	0	0
5	0	0
6	0.1	0
7	0	0.1
8	0.2	0.1
9	0.1	0.2
10	0	0.1
12	2.4	26
15	0.2	0.2
16	0	0.2
17	0	0
19	4.3	62
20	0	0
21	0.1	0.1
22	0	0.1
23	0.1	0.1
24	100	100
27	0	0.2
28	1.1	0.9
30	0	0.1
31	0.1	0.2
33	0	0
34	0	0
35	0	0.2
36	2.5	0.4
38	0.3	0
39	0.5	1.6
40	0.1	0.1
41	0	0.4
44	0.1	0
47	0	0
53	0.1	54

*All compounds tested as the methyl esters.

Table 1. Characterization of anti-GA_{24}-Me poolsera

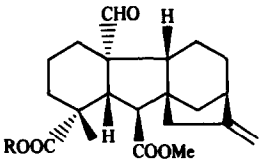
				Percent cross-reaction‡	
Immunogen	Serum	Titre*	Midrange† (pmol)	GA19Me	C-19 GAs
I	422	1:1080	0.75	4	n.d.
	423	1:1170	0.75	62	n.d.
II	420	1: 630	1.8	15	n.d.
	430	1:1710	1.4	20	n.d.
	431	1:1620	1.6	33	n.d.

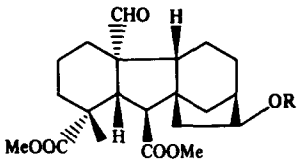
*Final dilution, standard RIA protocol, see Experimental.

†The amount of standard GA_{24}Me displacing 50% of tracer from antibody.

‡Included in this experiment were GA_1, GA_3, GA_4, GA_5, GA_9, GA_{20}.

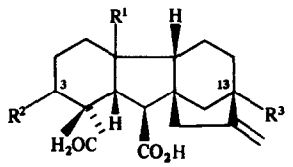
n.d. no detectable displacement of GA_{24}-[^{3}H]Me (**8**) by 100 pmol gibberellin added per assay tube.





1 R = Me
7 R = H
8 R = C^3H_3

2 R = H
3 R = COCl
4 R = CONH—BSA
5 R = $COCH_2CN$
6 R = CO—C(=N—NH—Ph—$CONHCH_2CONH$—BSA)—CN



R^1 (C-20)	$R^2 = R^3 = H$	$R^2 = OH$ $R^3 = H$	$R^2 = H$ $R^3 = OH$	$R^2 = R^3 = OH$
– Me	GA_{12}	GA_{14}	GA_{53}	GA_{18}
$-CH_2OH$	GA_{15} open lactone	GA_{37} open lactone	GA_{44} open lactone	GA_{38} open lactone
– CHO	GA_{24}	GA_{36}	GA_{19}	GA_{23}
$-CO_2H$	GA_{25}	GA_{13}	GA_{17}	GA_{28}

Fig. 1. Structures of C_{20}-gibberellins.

ive. Likewise, the functionality of C-20 was not discriminated against to the same extent as with serum 422 (compare GA_{12} with GA_{24} and GA_{53} with GA_{19}). However, GA_{17}, the C-20 carboxyl analogue of GA_{19}, was only marginally reactive with this serum and with serum 422. This suggests that this bulky group, present as the methyl ester under our experimental conditions, reduces immunoreactivity severely. The 3β-hydroxylation also resulted in near complete loss of immunoreactivity with serum 423, demonstrating the importance of the A-ring for antibody binding in both sera.

Radioimmunoassay

Antisera were used at a final dilution (Table 1) such that 30% of the GA_{24} tracer (**8**) (total added: 0.75 pmol, 721 Bq) was specifically bound under standard conditions (see Experimental). Under these conditions, between 7.5 and 14.5% (average 10%) of the total tracer was non-specifically precipitated in the separation step. However, an attempt to reduce this non-specific binding (NSB) by addition of detergents such as Triton X-100 or Tween 80, or organic solvents such as DMSO or DMF was unsuccessful. The aldehyde function of GA_{24} was considered not to be a major cause of non-specific binding (via Schiff's base formation with proteins), because the addition of excessive acetaldehyde to the incubation mixture failed to reduce NSB. The major factor contributing to the observed non-specific binding appeared to be the low polarity of the GA_{24} tracer used. Various sources of carrier protein were evaluated and found to give different NSB values as high as 25–35%. All experiments described herein were thus performed with a single batch of bovine serum diluted to 0.7 mg/ml (final protein concentration during incubation). NSB was also sensitive to changes in the composition of the assay mixture since it was observed that the addition of plant extract fractions (e.g. acidic ethyl acetate fractions of rice extracts) markedly increased NSB (from 9.9 to 28.7% in this example). Increasing NSB values were also observed in HPLC fractions of solvent blank runs using a Shodex gel filtration column and THF as eluant. The reason for this unique effect, not seen with other GA immunoassays, is unknown at present. To avoid misinterpretations, it is therefore necessary to monitor UB values carefully and run 'individual sample blanks' (i.e. assay tubes with sample, but without antiserum).

Under optimized conditions, the measuring ranges extended from 0.05–10 pmol of GA_{24}-Me (**1**) for both antisera, and from 0.1 to 10 pmol of GA_{19} Me for serum 423. For the analysis of GA_{19} in plant material, GA_{19} was used as an assay standard. Typical standard curves for both GAs (and serum 423) are given in Fig. 2.

Application to plant analysis and validation

Immature seeds of *Pharbitis nil* were chosen to validate the assay for GA_{19} because (a) the GAs of this tissue are well characterized and (b) their levels change considerably during embryogenesis. Samples of immature seeds were collected and extracted as described [8]. Extracts were split and either processed for GC/MS(SIM) (Tokyo) or HPLC/RIA (Osnabrück) as detailed in the Experimental section. All values were corrected for recovery using 15,17,17-trideuteroGA$_{19}$ (d_3-GA_{19}) internal standards for the GC/MS(SIM) procedure and GA_{19} (in split sample runs) for the HPLC/RIA procedure. The acidic ethyl acetate fraction of the crude *P. nil* seed extracts, after passage through a SepPak-C_{18} cartridge (to remove lipophilic material) and then subjected to HPLC on Nucleosil-N$(CH_3)_2$, yielded several distinctly separate areas of immunoreactivity, amongst them the completely resolved peak of GA_{19} (Fig. 3). This finding substantiates the necessity for sample separation prior to GA_{19}-RIA, but the finding was not surprising given the group-selectivity of the antibody 423 employed.

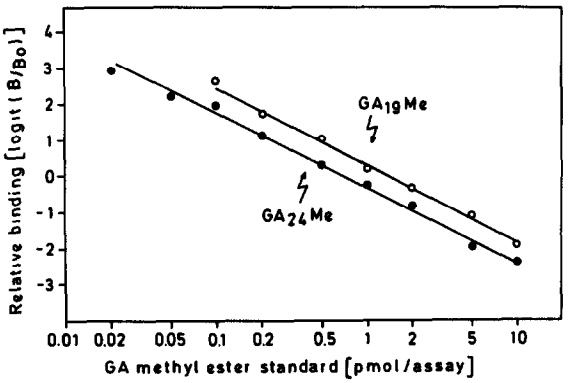


Fig. 2. Linearized standard curves for the RIA of GA_{24}-Me and GA_{19}-Me (antiserum: 423). For calculations, see [7].

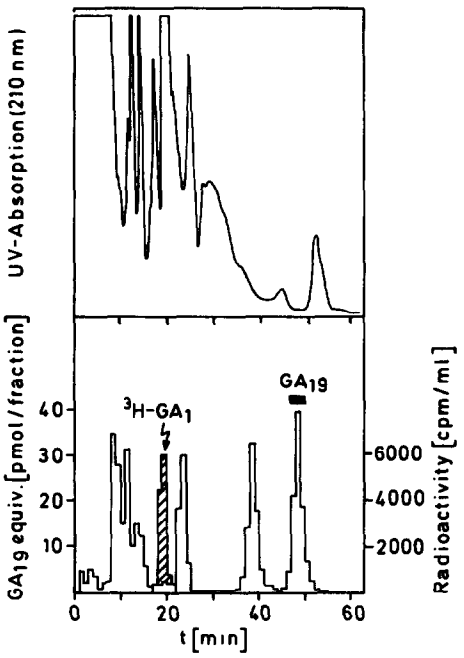


Fig. 3. Immunohistogram of HPLC separated *P. nil* (20–30 days post anthesis) seed extract. The shaded peak gives the location of the ^3H-GA_1 internal standard. The bar indicates the R_t of the internal standard of GA_{19} in the split sample (histogram not shown).

Following HPLC, RIA was used to quantitate GA_{19} in extracts of *P. nil* seeds collected at various stages of development. When compared to the quantitative data obtained by the GC/MS (SIM) technique, a close agreement of both methods became apparent, both in terms of precision and accuracy (Table 3).

Table 3. Comparison of GA_{19} levels in extracts of immature *Pharbitis nil* as determined by GC/MS(SIM) and RIA

Sample Days post anthesis	GA_{19}[ng/g fr. wt ± s.d. (n = 3)] GC/MS	RIA
2	7.9 ± 1.3	8.8 ± 1.0
4	19.7 ± 5.5	14.3 ± 3.2
8	15.5 ± 2.5	14.8 ± 1.6
16	69.5 ± 1.4	59.7 ± 1.7
20–30	290 ± 14.0	333.0 ± 24.7

DISCUSSION

Because of its importance as a key intermediate in the biosynthesis of C_{19}-GAs, a facile analysis of GA_{19} by RIA was attempted. The results presented here show that GA_{24}-Me coupled to protein via C-17 gives rise to high-affinity antibodies in rabbits. These antibodies react significantly with GA_{19} and some also react with related GAs such as GA_{12}. This, as well as another recent report [6], substantiate the usefulness of group selective antibodies in conjunction with fast and efficient HPLC separation techniques for the analysis of GAs in semi-purified samples. More specifically, for *P. nil* it is shown here that acidic ethyl acetate fractions of seed extracts, after HPLC on Nucleosil-$N(CH_3)_2$, yield sufficiently clean GA_{19} fractions to allow an accurate determination at the low ng/g tissue level. An analysis of the unfractionated extract (cf Fig. 3) is, however, not possible due to the presence of other immunoreactive constituents. GA_{12}, GA_{53} and GA_{24}, all cross-reactive with serum 423 employed for this experiment, chromatographed at R_t 9.8, 13 and 37.4 min, respectively, and thus they might contribute to the immunoreactive material found in the corresponding HPLC fractions. However, the present study was aimed at analysing GA_{19} and thus the identity of the other zones of immunoreactivity was not established. RIA offers a convenient alternative to the elaborate GC/MS techniques in that it apparently accepts samples of a considerably lower overall purity. This finding extends similar observations made for GA_9 analysis in Norway spruce [9]. On the other hand, the exact degree of purity to which sample processing must proceed needs careful assessment. General guidelines for the validation of immunoassays for plant hormones have recently been proposed [7, 10] and these were followed here. In addition to this, we observed that not only the antigen–antibody interaction can be subject to interference but also the unspecific binding of tracer can be influenced by sample composition.

With the reaction sequences detailed here, a range of synthetic procedures to GA immunogens is now available accessing most of the relevant structures of the GA molecule, i.e. C-3 [11], C-7 [3], C-17 ([11] and this study) and the α-plane via C-19 and C-20 [6]. With the experience now amassed about synthetic routes to GA-immunogens, the construction of a panel of monoclonal antibodies, suitable for the quantitation and isolation by immunoaffinity chromatography of both C_{20}- and C_{19}-GAs appears feasible, and studies in this direction have already met with success [6, 11].

EXPERIMENTAL

Immunization. Immunizations of outbred 12–16-week-old rabbits were carried out as described [7] but using 0.2 mg of

conjugate per injection (pre-immunization and booster injections). The development of serum titres was constantly monitored by RIA with GA_{24}-[^{3}H]Me (8) as tracer.

Plant materials and extraction procedures. Immature seeds of *Pharbitis nil* were harvested every two days from 2 to 18 days after anthesis and 20–30-day-old seeds were collected together. Among these harvests, 2,4,8,16 and 20–30-day-old seeds were used for analysis. The seeds were extracted (× 3) with 80% Me_2CO (15 ml of 80% Me_2CO/g seed/extraction) [8]. The Me_2CO extracts were used as common sources for radioimmunoassay and GC/MS.

Purification procedure of GA_{19} for GC/MS. In order to determine the amounts of int. standard to be added to the plant extracts, endogenous GA_{19} was estimated by GC/SIM without any int. standard. Deuterium labelled GA_{19} [15,17,17-$^2H_3$]GA_{19} prepared from deuterated steviol [12] fed to *Gibberella fujikuroi* in the presence of *N,N,N*-trimethyl (2',6',6'-trimethylcyclohex-2'-en-1'-yl)-2-propyl-ammonium iodide [13] was added to the extracts of *P. nil*. The amounts of the added int. standard were about 2–6 times in excess of the endogenous levels estimated by preliminary experiments d_3-GA_{19} added: 50 (2*d* sample), 90 (4*d*), 60 (8*d*), 190 (16*d*), and 1000 ng (20*d*). The dried extracts of *P. nil* (0.8–1.5 g fr.wt eq.) were suspended in a 0.1 M K-Pi buffer (pH 8.0, 10 ml) and the soln was partitioned with 20 ml CH_2Cl_2. The CH_2Cl_2 layer was extracted with another 10 ml of the buffer. The combined buffer was acidified with 3 ml 1 M HCl and extracted with EtOAc (2 × 15 ml). The EtOAc was dried over anhydrous Na_2SO_4 and concd.

HPLC of gibberellin for GC/MS analysis

Gel filtration. THF (freshly distilled from CaH_2) was used as eluant. The gibberellin fraction was dissolved in THF and passed through a filter (Shodex DT, E-D-13 CR). The filtrate was concd, redissolved with 500 μl THF, and subjected to GPC on a Shodex HF-2001 column (20 i.d. × 500 mm, exclusion limit = 1000) with a precolumn (Shodex GPC H = 2000 P). The effluent was monitored by UV_{254} nm and RI. The gibberellin eluting zone was collected. The R_t of GA_{19} was 22.8 min at the flow rate of 3.2 ml/min.

Nucleosil 5N(Me)$_2$. The gibberellin fraction after Shodex LC was dissolved in 100 μl MeOH for injection. The HPLC system was composed of a Jasco Tri-rotar SR-IV fitted with Nucleosil 5N(Me)$_2$ column (6 i.d. × 150 mm). the column was run under constant temp (40°). The elution solvent was MeOH–H_2O–HOAc (99:0.95:0.05). The flow rate was 1.5 ml/min. The R_t of GA_{19} was *ca* 37 min.

GC/MS. A Hitachi M-80A with data processing system M-003 fitted with a 2% OV-1 column (3 mm × 1 m) was used in the SIM mode. The following conditions were used: column temp. 210°, He 60 ml/min, ionization EI 22 eV. The R_t of GA_{19} methyl ester trimethylsilyl ether (GA_{19}-Me-TMSi) was 4.5 min. The samples were methylated with ethereal CH_2N and then trimethylsilylated with an aliquot of pyridine-bis (trimethylsilyl)acetamide—trimethylchlorosilane (10:5:1,). The ions ($[M-28]^+$ *m/z* 434/437:d_0/d_3) were used for quantitation by area-internal standard mode. A calibration curve was made using standards containing GA_{19} and d_3-GA_{19} in the ratios of 0–0.6 (d_0/d_3); the peak area ratios of *m/z* 434–*m/z* 437 were in the range of 0.14 ($d_0/d_3 = 0$) to 3.30 ($d_0/d_3 = 0.6$). The correlation coefficient of the standard curve was 0.9975. The peak area ratios of analysed samples were in the range of 0.17 (2 *d* sample) to 0.56 (16 *d* sample).

Sample processing and HPLC of gibberellins for RIA analysis. The dried residues of the Me_2CO extracts equivalent to 1.5–3.2 g fr. wt of seeds were redissolved in 7 ml MeOH. An int. standard of ^{3}H-GA_1 (1867 Bq, 0.37 × 10^{15} Bq/mol) was added followed by sufficient K-Pi, (50 mM, pH 8.0) to give a final MeOH concentration of 70%. Samples were then filtered through a glass fibre filter (Schleicher and Schüll, No. 6) and passed through a SepPak C_{18} (Waters) cartridge. The MeOH was removed on a rotary evaporator and the aq. fraction was acidified to pH 2,5 with 1 M HCl. The acidic aq. phase was partitioned (× 4) against EtOAc, the combined organic phases dried over anhydrous Na_2SO_4, followed by removal of the EtOAc. The residues were redissolved in MeOH, split in half. One half was spiked with an appropriate amount of GA_{19} to allow identification of its R_t on HPLC and assessment of its recovery. The fractions were then dried again, redissolved in a small vol of MeOH and filtered through a Gelman Acro LC 13 0.45 μm filter.

HPLC separation was carried out on a Nucleosil 5N(CH$_3$)$_2$ column as described above, but at 22°; R_t, GA_{19} int. standard was 49–41 min (decreasing with column usage), R_t, ^{3}H-GA_1 int. standard was 19–17.5 min.

The collected fractions of the 20 day sample were reduced to dryness, redissolved in 0.5 ml MeOH and methylated as described [14]. The dry, methylated fractions were redissolved in 10% MeOH in phosphate buffered saline, pH 7,4 and aliquots assayed two times in triplicate by RIA.

For the 2,4,8 and 16 day samples, HPLC separated fractions corresponding to the location of GA_{19} were pooled for each sample before further processing. Fractions adjacent to these were also processed identically to validate the effectiveness of the 'GA_{19}-cut'. In these fractions, no immunoreactive material could be detected. All samples were analysed in triplicate. Results were corrected for losses by using the internal standards included. Work-up losses were assessed by analysing the recovery of [^{3}H]-GA_1 added during extraction prior to the HPLC step. Recovery was from 64% to 77%. GA_{19} (1 μg) added to split extracts prior to HPLC was analysed by RIA in the final samples and recovered from 9.8 to 11.4%.

Radioimmunoassay. The RIA protocol used here followed the detailed description in [7].

Synthesis of dihydro GA_{24}Me-17-ol(2). GA_{24}-Me (5 mg, 0.013 mmol) containing ^{3}H-labelled GA_{24}-Me (7.7×10^4 Bq) was dissolved in 150 μl THF under dry N_2. To this sol, 250 μl bis-(3-methyl-2-butyl)borane (0.2 mmol) [prepared by adding 160 mg 2-methyl-2-butane (2.3 mmol) to 1 ml borane–THF complex (1 mmol) in a dry flask under N_2 and stirring for 1 hr at 0° and 0.5 hr at room temp.] was added, and the mixture stirred for 30 min at 0°. To the above mixture, 60 μl 3 M NaOH (0.18 mmol) was added, cooled on ice, and stirred for 1 hr at 0° after addition of 72 μl 30% H_2O_2 soln. The mixture was poured into EtOAC (*ca* 50 ml), washed with brine and aq. $Na_2S_2O_3$, dried over anhydrous Na_2SO_4, and concd. The concentrate was submitted to TLC (silica gel 0.2 mm thickness) developed with *n*-hexane–EtOAc (1:2), and the desired dihydro GA_{24}-Me-17-ol (R_f 0.48) was obtained, CIMS *m/z* (rel. int.): 393 $[M+1]^+$ (40), 361 $[M-31]^+$ (62); EIMS *m/z* (rel. int.): 392 ($[M]^+$ absent), 364 $[M-28]^+$ (6), 332 $[M-60]^+$ (58), 304 (29), 301 (15), 273 (17), 245 (42). The yield was 3.9 mg (5.8×10^4 Bq).

Synthesis of GA_{24} immunogen-1. Dihydro GA_{24}-Me-17-ol (250 μg, 0.64 μmol, 3440 Bq) in 100 μl dioxan and excess phosgene (100 mg, 1 mmol) in 1 ml Et_2O were mixed and left for 1 hr at room temp. The solvent was removed with a stream of N_2 and the crude dihydro GA_{24}-Me-17-chloroformate (3) was redissolved with 100 μl dioxan. This soln was added dropwise to a BSA (5 mg) soln (0.5 ml water, 7 μl 0.1 M sodium hydroxide and 275 μl dioxan) with stirring at 4°. A further 150 μl of dioxan was used to rinse the reaction vessel and this was also added dropwise to the BSA soln. An aliquot (7 μl) of a 0.1 M NaOH soln was added immediately and a further 10 μl was added after 30 min. The final

pH was 5.8. The mixture was stirred for 1 hr at 4°. The product was dialysed against distilled H_2O and lyophilized. The yield was 4.7 mg (408 Bq) and the coupling ratio (GA_{24}/BSA) was determined to be 1 based on the radioactivity of the [^{3}H]-GA_{24}-Me (8) incorporated into the conjugate.

Synthesis of GA_{24} immunogen-II. Dihydro GA_{24}-Me-17-ol (2) (374 μg, 0.96 μmol, 5145 Bq), 25 μl CH_2Cl_2 and 5 μl triethylamine were mixed. To this, four 10 μl aliquots of cyanoacetylchloride (10% in CH_2Cl_2) were added every 5 min. The mixture was stirred for 30 min at room temp. The crude product was dissolved in EtOAc (*ca* 50 ml) and washed with brine (*ca* 25 ml) and $NaHCO_3$ (*ca* 25 ml) soln and then dried over anhydrous Na_2SO_4. Dihydro GA_{24}-Me-17-cyanoacetate (5) was purified by TLC (*n*-hexane–EtOAc, 1:1). The yield was 74% (325 μg, 3790 Bq) based on radioactivity.

p-Aminohippuric acid-BSA conjugate [15] (5 mg) was dissolved in 0.5 ml H_2O and 36 μl 1 M HCl (pH 1.4). The mixture was cooled to 0° and $NaNO_2$ soln (828 μg/15 μl H_2O, 12 μmol) added. The mixture was stirred for 10 min at 0° until diazotization was complete. The soln consisting of dihydro GA_{24}-Me-17-cyanoacetate (5) (320 μg), H_2O (100 μl) and DMF (400 μl) was added to the diazotized protein soln dropwise with stirring. During this addition, Na_2CO_3 (3.8 mg/40 μl H_2O) was added (10 μl × 4) to keep the pH alkaline. The reaction mixture was stirred for 10–15 min at 0° and dialysed against distilled H_2O and lyophilized. The yield was 4.6 mg with a coupling ratio (GA_{24}/BSA) of 9.

Synthesis of GA_{24}-[^{3}H]Me (8). GA_{24}-Me (1) (615 μg, 1.6 μmol) was dissolved in MeOH (250 μl) and heated at 100° for 30 min with 10% aq. KOH (250 μl). H_2O (3 ml) was added and the resulting soln was extracted with EtOAc (5 ml × 3). After drying (Na_2SO_4) the extract was evaporated and purified by TLC (*n*-hexane–EtOAc–HOAc, 20:10:1). The main product (ca 500 μg) was recovered from R_f zone 0.40 and was tentatively assigned to be the GA_{24}-7-monomethyl ester (7) because the 7-methyl esters of C_{20}-GAs are hardly hydrolysed under the conditions employed.

The GA_{24}-7-monomethyl ester (7) (100 μg, 0.28 μmol) was dissolved in DMF (125 μl). NaH (50 μg of 55–60% in oil dispersion, 1 μmol) was added and mixed and the soln was then left for 30 min at room temp. with occasional mixing. ^{3}H-MeI (2.5 mCi/250 μl toluene, 3.14×10^{15} Bq/mol) was introduced and was left at room temp. overnight. After removal of the solvent under a stream of N_2 the crude product was purified by silica gel TLC (*n*-hexane–EtOAc, 2:1). The GA_{24}-Me (R_f 0.79) zone was eluted with EtOAc to give ^{3}H-labelled GA_{24}-Me. The yield was 45% based on radioactivity. Specific radioactivity was determined by the self displacement technique [16].

Acknowledgements—We wish to thank Dr K. Lürssen (Bayer AG) for organizing this project, Dr H. J. Riebel and Dr U. Priesnitz (Bayer AG) for the preparation of cyanoacetylchloride and physiochemical analysis of synthetic intermediates of immunogens, Mr J. Kwiatkowski for technical assistance, and Dr Nagin Lallu, Auckland, New Zealand, for checking the English. This work, was partly funded by the Fonds der Chemischen Industrie, Frankfurt, FRG. E. W. is also grateful to Dr A. Crozier, Glasgow, U.K., for providing some rare GAs.

REFERENCES

1. Sponsel, V. (1983) in *The Biochemistry and Physiology of Gibberellins* (Crozier, A., ed.) Vol. 1, p. 151. Praeger, New York.
2. Metzger, J. D. and Zeevaart, J. A. D. (1980) *Plant Physiol.* **66**, 679.
3. Atzorn, R. and Weiler, E. W. (1983) *Planta* **159**, 1.
4. Atzorn, R. and Weiler, E. W. (1983) *Planta* **159**, 7.
5. Yamaguchi, I., Nakagawa, R., Kurogochi, S., Murofushi, N., Weiler, E. W. and Takahashi, N. (1987) *Plant and Cell Physiol.* (in press).
6. Eberle, J., Yamaguchi, I., Nakagawa, N., Takahashi, N. and Weiler, E. W. (1986) *FEBS Letters* **202**, 27.
7. Weiler, E. W., Eberle, J., Mertens, R., Atzorn, R., Feyerabend, M., Jourdan, P. S., Arnscheidt, A. and Wieczorek, U. (1986) in *Immunology in Plant Science* (Wang, T. L., ed.) p. 27. Cambridge University Press, Cambridge.
8. Yamaguchi, I., Fujisawa, S. and Takahashi, N. (1982) *Phytochemistry* **21**, 2049.
9. Odèn, P. C., Weiler, E. W., Schwenen, L. and Graebe, J. E. (1985) *Physiol. Plant.* **62**, 21A.
10. Weiler, E. W. (1986) in *Modern Methods of Plant Analysis* (Linskens, H.-F. and Jackson, J. F., eds) Vol. 4. p. 1. Springer, Berlin.
11. Knox, J. P., Beale, M. H., Butcher, G. W. and MacMillan, J., (1987) *Planta* **179**. 86.
12. Gianfagna, T., Zeevaart, J. A. D. and Lusk, J. W. (1983) *Phytochemistry* **22**, 427.
13. Murofushi, N., Shigehiro, N. and Takahashi, N. (1979) *Agric. Biol. Chem.* **43**, 1159.
14. Weiler, E. W. and Wieczorek, U. (1981) *Planta* **152**, 159.
15. Weiler, E. W. (1980) *Planta* **148**, 262.
16. Chervu, L. R. and Murty, D. R. K. (1975) in *Radioimmunoassay* (Freeman, L. M. and Blaufox, M. D., eds) p. 33. Grune & Stratton, New York.