Preparation of Gibberellin Hapten–protein Conjugates. Part 1. Conjugation *via* Carboxymethylenation at C-17

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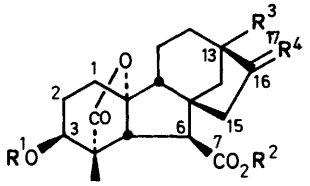
The preparation of C-17 carboxylated derivatives of gibberellin A_4 using anions of α-trimethylsilylethanoic acid derivatives is described. It is demonstrated that such derivatives are useful for the preparation of GA_4-17-linked hapten–protein conjugates for use as antigens.

The gibberellin (GA) family of plant hormones at present comprises 79 similar tetracyclic diterpenoid acids.[1] The problem of obtaining monoclonal antibodies which specifically recognise certain members of this group of small molecules reduces to the chemical synthesis of suitable hapten–protein conjugates for use as antigens. These conjugates must be prepared in such a way as to expose the required recognition sites on the GA to the mammalian immune system. As well as the required ligand binding specificities, another major consideration in the design of conjugates is the location, on available GA starting materials, of functional groups suitable for the construction of linkers to the carrier protein. The majority of GA-protein conjugates described in the literature have been prepared by amide bond formation at the C-7 carboxyl group either directly or *via* a spacer arm.[2] However, antibodies obtained using these antigens do not recognise free C-7 carboxylic acids. Although such antibodies are useful for the immunoassay of GA-methyl esters, they are not reasonable mimics of plant GA binding proteins and thus cannot be used in anti-idiotypic approaches to GA-receptors.



	R^1	R^2	R^3	R^4
(1) GA_4	H	H	H	CH_2
(2) GA_1	H	H	OH	CH_2
(3)	H	H	H	O
(4)	TMS	Me	H	O
(5)	TMS	TMS	H	O
(6)	H	H	H	$CHCO_2Et$
(7)	H	H	H	$CHCO_2H$
(8)	H	H	H	$CHCO_2CO_2Bu^t$
(9)	H	Me	H	$CHCO_2Me$
(10)	H	Me	H	$CHCONHCH_2CO_2Me$
(11)	H	$CONHCH_2CO_2Me$	H	$CHCO_2Me$
(12)	H	Me	H	O

In this paper, we describe a method for the functionalisation of C-17 of gibberellin A_4 (1) and its use in the preparation of protein conjugates *via* selective amide bond formation at C-17. Gibberellin A_4 (1) is the 13-deoxy analogue of GA_1 (2) which has been shown to be the major gibberellin involved in stem elongation in maize and pea.[3] The biosynthetic precursors of the active GA, and the inactive catabolites all differ in their ring A structure (Scheme 1). Thus a strategy for the generation of monoclonal antibodies which specifically recognise GA_1 in plant systems containing the GAs of Scheme 1 would be the use of a protein conjugate of GA_1 prepared *via* the C-16, 17 alkene function. This function is remote from ring A and thus it would be expected that the required A/B ring discrimination could be obtained. For synthetic convenience GA_4 (1) was used as starting material for the preparation of the conjugate. It was assumed that the absence of a 13-hydroxy group adjacent to the bridge to the carrier would be unlikely to affect antibody specificity to a large extent.

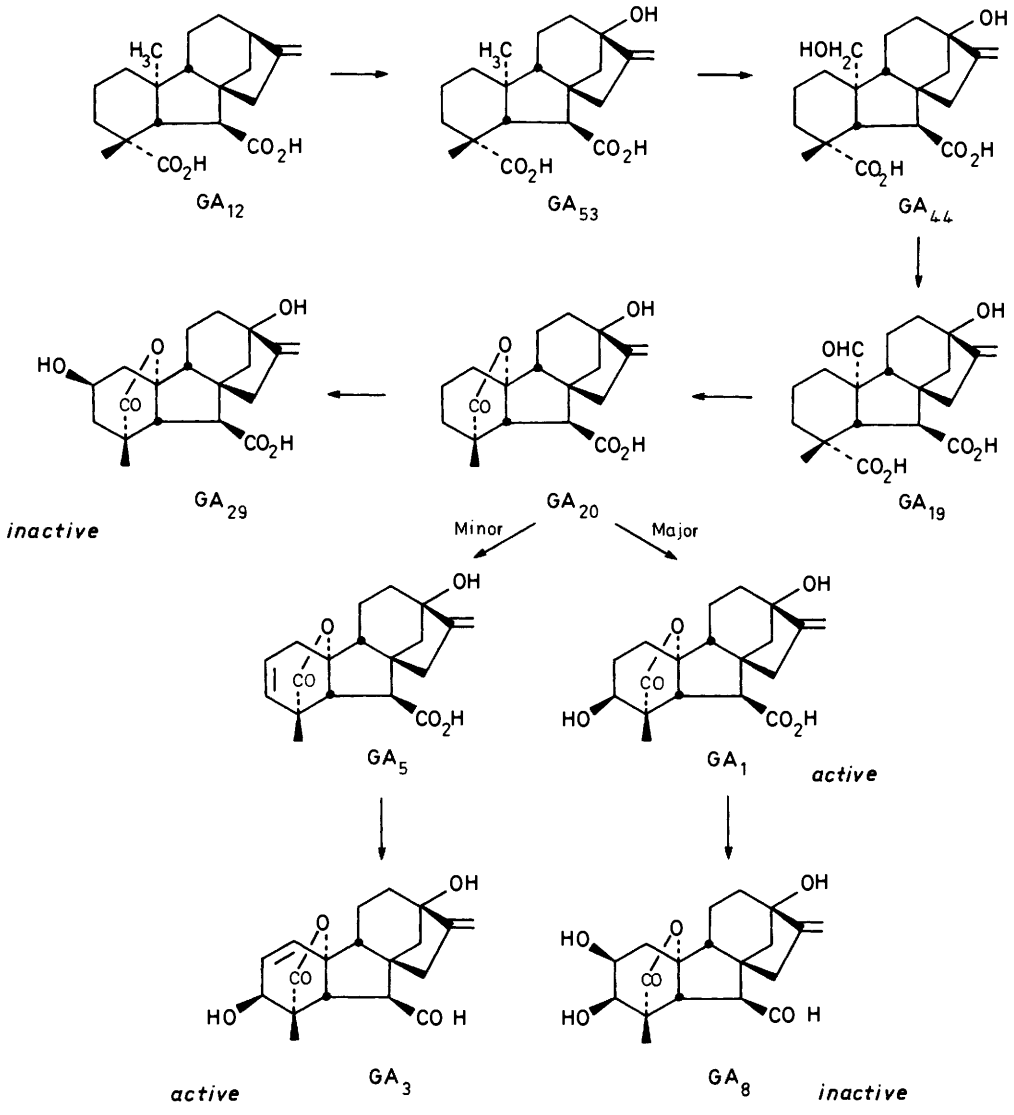
Results and Discussion

The Wittig reaction of gibberellin 17-nor-16-ketones such as (3) with methylene(triphenyl)phosphorane is a well-known method for the regeneration of the 16,17-alkene function.[4] Our initial attempts to add a functional group at C-17 were with ylides generated from phosphonium salts such as $Ph_3P^+(CH_2)_n$ $X\,Br^-$ where $n = 2$ or 3 and X = CO_2R, NH_2, or phthalimide and also from the phosphonate $(EtO)_2P(O)CH_2CO_2Et$. However in all cases no reaction could be achieved with the norketone (3) or its 7-methyl ester–3-trimethylsilyl ether derivative (4). The failure of this approach was shown to be due to the low reactivity of the ketone rather than the presence of the terminal group, X, as the ylide generated from Ph_3P^+-$(CH_2)_5CH_3Br^-$ also failed to react with these ketones even at reflux.

We then turned our attention to the Peterson alkenation reaction[5] using anions generated from α-trimethylsilylethanoic acid derivatives [*e.g.* (15)] with lithium di-isopropyl amide. Initial experiments were carried out with anions of ethyl trimethylsilylethanoate (15) generated at −70 °C in tetrahydrofuran (THF) and the bis-trimethylsilyl norketone (5) (−70 to 20 °C). These reactions smoothly yielded the required α,β-unsaturated ester (6) after acidic work-up to remove the Me_3Si protecting groups. The product (6) was an inseparable mixture of *E* and *Z* isomers (1:2) as revealed by the ^1H NMR spectrum (Figure) which when run in pyridine showed 13-H for the major *Z*-isomer at δ 3.65 while for the minor *E*-isomer it resonated at δ 2.63. This was interpreted as the deshielding effect of a (*Z*)-CO_2Et group on 13-H in the major isomer. Similarly, in the minor *E*-isomer, the germinally coupled 15-H_α and 15-H_β protons (δ 3.35 and 2.90, respectively) were well downfield of their positions in the major *Z*-isomer (δ 2.45 and 2.62).

To obtain the α,β-unsaturated acid (7) directly, the protected norketone (5) was condensed with the dianion of trimethylsilylethanoic acid (16) prepared at 0 °C as described by Grieco *et al.*[6] After acidic hydrolysis of protecting groups the diacid (7) was obtained in good yield. Analysis of the ^1H NMR spectrum run in pyridine as before revealed the *E*/*Z* ratio to be 2:1 in this case (major isomer: 15-H_α and 15-H_β at δ 3.51 and 3.01; minor isomer: 13-H at δ 3.90).

Before attempting to couple the diacid (7) to a protein it was



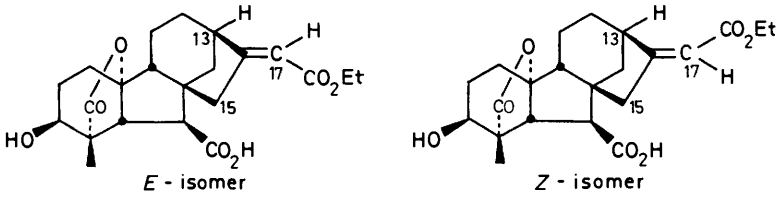
Scheme 1.

necessary to show that selective amide bond formation could be achieved at the C-17 carboxy group rather than at the more sterically hindered C-7 acid. This was done by modelling the coupling reaction using glycine as the amine (Scheme 2). Activation of the diacid was by treatment with isobutyl chloroformate (1 equiv.) and tributylamine to form the mixed anhydride (**8**). Reaction of (**8**) with glycine yielded, after treatment with diazomethane, a mixture (*ca.* 2:1) of two compounds. The major component was identified as the (*E*/*Z*) dimethyl ester (**9**) of the starting diacid, presumably formed by hydrolysis of the mixed anhydride (**8**). The other more polar compound was shown by mass spectrometry and ^{1}H NMR spectroscopy to be a mono-amide. This compound was shown to have structure (**10**) (*E*/*Z*, 2:1) rather than the alternative structure (**11**) by treatment with osmium tetraoxide/sodium metaperiodate which yielded the methyl ester of GA_4-17-nor-16-ketone (**12**) identical with a sample prepared by treatment of (**3**) with diazomethane.

For the synthesis of the conjugate the diacid (**7**) was prepared radio-labelled with tritium using [1,2-$^3H_2$]GA_4 (*ca.* 1 μCi) which was converted to the [^{3}H]-17-nor-16-ketone (**3**) by osmium tetraoxide/sodium periodate treatment and then reaction of its bis-trimethylsilyl derivative (**5**) with (**16**). In this instance, as well as the required [^{3}H]-diacid (**7**), considerable amounts of a separable isomer were obtained. This compound was assigned the *endo* structure (**13**) on the basis of its lack of UV absorbance at 225 nm [*cf.* λ_{max} 222 nm, ε = 10 090 for (**7**)] and its ^{1}H NMR spectrum which contained doublets (*J* 11 Hz) at δ 2.25 and 1.96, assigned to the geminally coupled 17-H_2. These signals were obscured in hexadeuterioacetone and were best observed in the spectrum of the dimethyl ester (**14**) run in deuteriochloroform. The formation of this isomer is thought to be due to over-exposure of the reaction mixture to acid during the removal of the trimethylsilyl protecting groups. Analysis of the [^{3}H]diacid (**7**) obtained from this reaction by ^{1}H NMR spectroscopy revealed the *E*/*Z* ratio to be 1:4. The predominance of *Z* isomer observed here may be due to a faster rate of endomerisation of the *E* isomer to give (**13**). The [^{3}H] diacid (**7**) was treated with isobutyl chloroformate/tributylamine to give the activated anhydride (**8**) and then with bovine serum albumin (BSA) at pH 8.1. The resulting GA_4-BSA conjugate, purified by dialysis, contained 8.6 molecules of GA_4 to each BSA as determined by [^{3}H] counting of an aliquot of the lyophilised protein product.

Experimental

General details are described in ref. 7. Gibberellin A_4 norketone



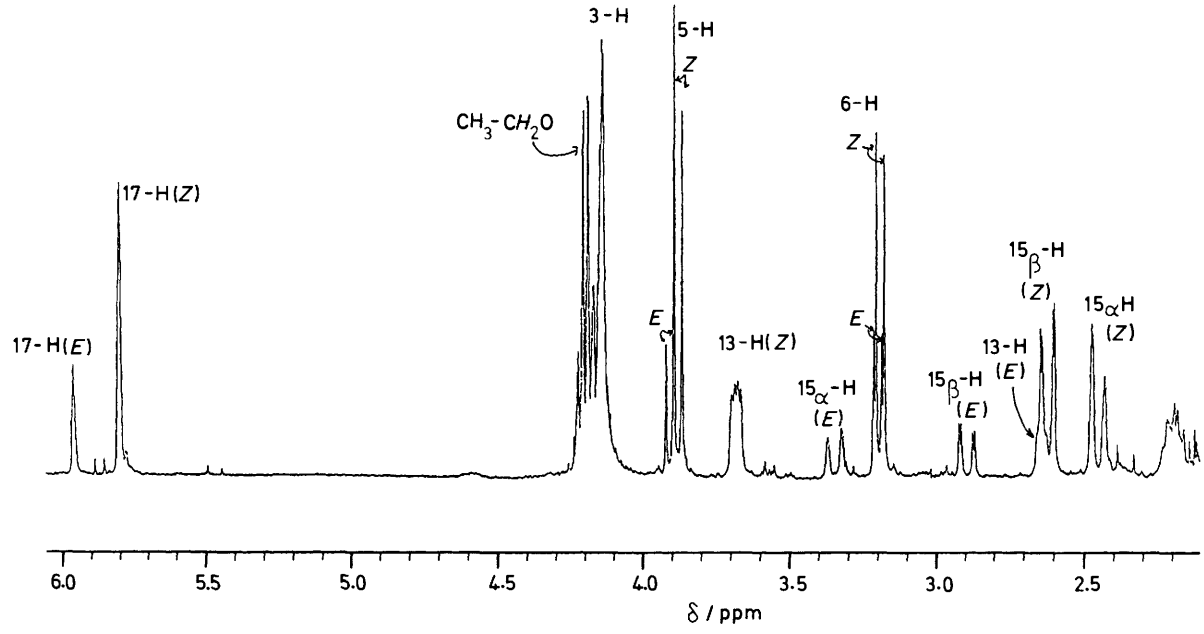
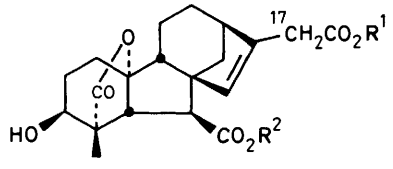


Figure. ^{1}H NMR (C_5D_5N) spectra of compound (**6**) (*E*:*Z*, 1:2).



(**13**) $R^1 = R^2 = H$
(**14**) $R^1 = R^2 = Me$

$Me_3SiCH_2CO_2Et$ (**15**)

$Me_3Si-\bar{C}H-CO_2^- 2Li^+$ (**16**)

(3) was prepared by osmium tetraoxide/sodium periodate oxidation of gibberellin A_4, m.p. 122–135 °C (lit.,[8] 120–140 °C).

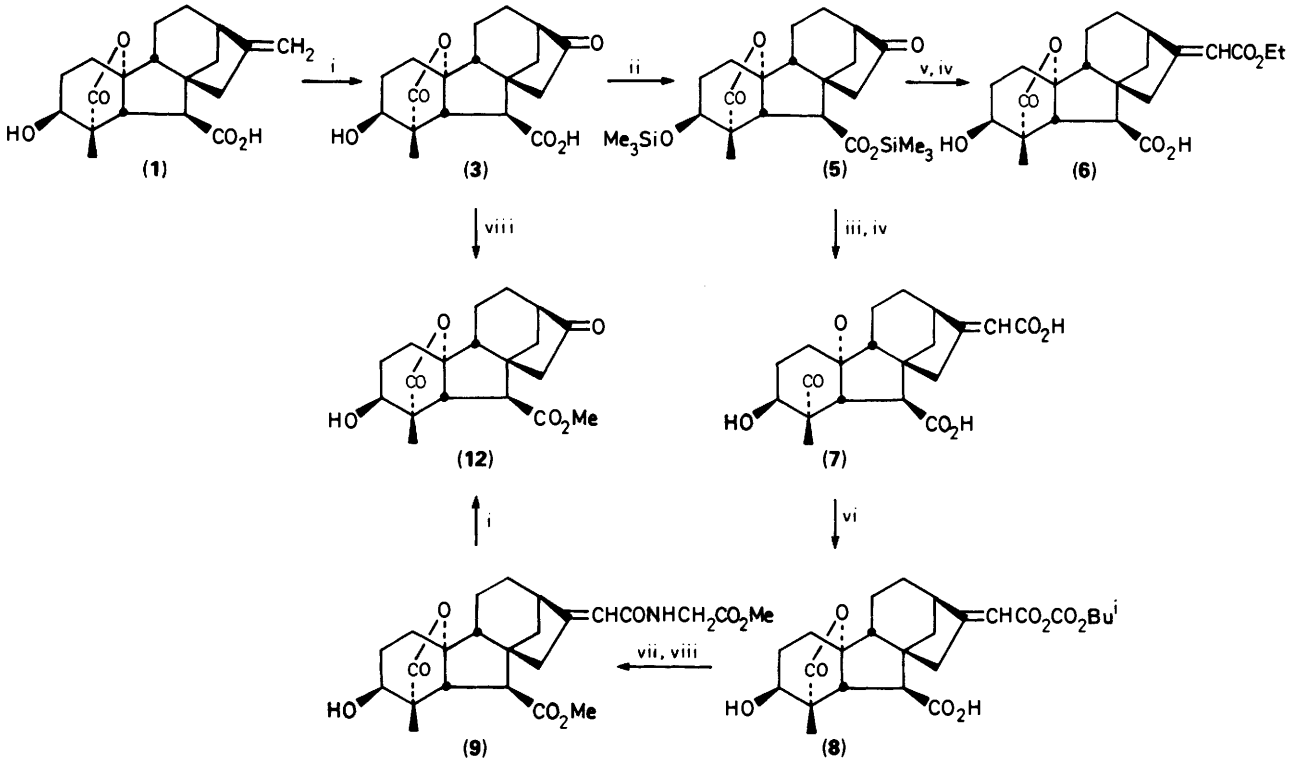
ent-17-*Ethoxycarbonyl*-3α,10-*dihydroxy*-20-*norgibberell*-16-*ene*-7,19-*dioic Acid* 19,10-*Lactone*. (17-*Ethoxycarbonylgibberellin* A_4) (**6**).—(a) *Preparation of* GA_4 *norketone trimethylsilyl ester trimethylsilyl ether* (**5**). To gibberellin A_4 norketone (**3**) (800 mg, 2.4 mmol) in dry pyridine (1 ml) was added hexamethyldisilazane (1.5 ml) and chlorotrimethylsilane (1.5 ml). After 1 h at room temperature the solution was evaporated with a stream of N_2 gas. Dry dichloromethane (2 × 5 ml) was added and similarly evaporated. Finally, the product was dissolved in dichloromethane, filtered under N_2, evaporated and stored under vacuum until use.

(b) *Preparation of ethyl trimethylsilylethanoate anion*. To dry THF (20 ml) containing butyl-lithium (3.7 ml; 1.3M solution in hexane) at 0 °C was added di-isopropylamine (675 μl, 4.82 mmol). The solution was cooled to −70 °C and ethyltrimethylsilylethanoate (**15**) (882 μl, 4.82 mmol) was added and the solution was stirred for 10 min.

(c) 17-*Ethoxycarbonyl*-GA_4 (**6**). To the solution of ethyl trimethylsilylethanoate anion (prepared above), at −70 °C was added the GA_4-norketone trimethylsilyl ether ester (**5**) (2.41 mmol) in THF (10 ml). The reaction mixture was then allowed to warm to room temperature over 3 h. Water was added and the stirred mixture was acidified to pH 3 with concentrated hydrochloric acid. The organic material, recovered in ethyl acetate was dissolved in acetone (50 ml) and 2M hydrochloric acid (20 ml) and stirred for 30 min after which time TLC indicated that hydrolysis of the trimethylsilyl protecting groups was complete. The acetone was evaporated and the product recovered in ethyl acetate. The product was purified by flash chromatography using ethyl acetate–light petroleum–acetic acid mixtures as follows: 20:80:0.5 (100 ml), 50:50:0.5 (300 ml) and 100:0:0.5 (300 ml) collected in 30 fractions. Fractions 21 and 22 contained 17-ethoxycarbonyl-GA_4 (**6**) (375 mg) as a 1:2 mixture of *E*:*Z* isomers.

^{1}H NMR (C_5D_5N): *E* isomer, δ 5.96 (s, 17-H), 3.92(d, *J* 10 Hz, 5-H), 3.35 (d, *J* 17 Hz, 15α-H), 3.19 (d, *J* 10 Hz, 6-H), 2.90 (dd, *J* 17, 2 Hz, 15β-H), and 1.15 (t, *J* 6 Hz, 17-$CO_2CH_2CH_3$); *Z* isomer, δ 5.79 (s, 17-H), 3.88 (d, *J* 10 Hz, 5-H), 3.58 (m, 13-H), 3.18 (d, *J* 10 Hz, 6-H), 2.62 (dd, *J* 17 and 2 Hz, 15β-H), 2.45 (d, *J* 17 Hz, 15α-H), and 1.20 (t, *J* 6 Hz, 17-$CO_2CH_2CH_3$). Signals common to both isomers: δ 4.20 (q, *J* 6 Hz, 17-$CO_2CH_2CH_3$), 4.15 (br, s, 3-H), and 1.63 (s, 18-CH_3). *m*/*z* (%) 404 (M^+, 90%), 386 (23), 359 (78), 358 (72), 342 (34), 296 (100), and 268 (37).

ent-17-*Carboxy*-3α,10-*dihydroxy*-20-*norgibberell*-16-*ene*-7,19-*dioic Acid*, 19,10-*Lactone*. (17-*Carboxygibberellin* A_4) (**7**).—(a) GA_4 norketone $TMSi_2$ (**5**) was prepared as above from the norketone (**3**) (807 mg, 2.40 mmol), hexamethyldisilazane and chlorotrimethylsilane in pyridine.



Scheme 2. *Reagents:* i, $OsO_4/NaIO_4$; ii, TMSiCl/HMDS/py; iii, $Me_3SiCHCO_2^- 2Li^+$; iv, H^+; v, $Me_3SiCHCO_2Et\ Li^+$; vi, ClCOOBu^i/Bu_3N; vii, $H_2NCH_2CO_2H$, pH 8.1; viii, CH_2N_2.

(b) *Preparation of trimethylsilylacetic acid dianion* (**16**).[6]—To dry THF (40 ml) containing butyl-lithium (1.6M, solution in hexane 15 ml; 24 mmol, 10 equiv.) at 0 °C under nitrogen was added di-isopropylamine (3.38 ml, 24 mmol) dropwise over 10 min. A solution of trimethylsilylethanoic acid (1.59 g, 12 mmol, 5 equiv.) in tetrahydrofuran (5 ml) was then added dropwise over 5 min to give a clear solution of the dianion $TMSiCHCO_2\ Li^+Li^+$ (**16**).

(c) *17-Carboxy-*GA_4 (**7**). To the dianion solution prepared above, at 0 °C, was added the GA_4 norketone $TMSi_2$ (2.12 mmol) in THF (10 ml). The solution was then allowed to warm to room temperature over 4 h. Acetone (10 ml) was then added. The solution was acidified to pH 3 with 5M hydrochloric acid and stirred for 1 h. Water was added and the product recovered in ethyl acetate. Evaporation gave a gum which was purified by flash chromatography on a 20 × 3 cm column eluted with the following percentages of ethyl acetate in light petroleum containing 0.5% acetic acid: 50% (400 ml), 60% (200 ml), and 70% (200 ml). Fractions (32 × 25 ml) were collected. Fractions 18–29 (706 mg) contained the required 17-carboxygibberellin A_4 (**7**) as a 2:1 mixture of *E*:*Z* isomers (NMR and GLC on 2% SE33 at 240 °C as MeTMSi derivative; λ_{max}(EtOH) 222 nm (ε = 10 090) (Found: M^+, 376.152. $C_{20}H_{24}O_7$ requires M^+, 376.152); ^{1}H NMR (C_5D_5N) *E* isomer: δ 1.66 (3 H, s, 18-CH_3), 3.01 (1 H, dd, *J* 17, 2 Hz, 15β-H), 3.23 (1 H, d, *J* 11 Hz, 6-H), 3.51 (1 H, d, *J* 17, 15α-H), 3.94 (1 H, d, *J* 11 Hz, 5-H), 4.15 (1 H, br s, 3-H), and 6.21 (1 H, br s, 17-H). *Z* isomer: (C_5D_5N) δ 1.66 (3 H, s, 18-H_3), 3.21 (1 H, d, *J* 11 Hz, 6-H), 3.90 (1 H, d, *J* 11 Hz, 5-H), 3.90 (1 H, m, 13-H), 4.15 (1 H, br s, H-3) and 6.05 (1 H, br s, 17-H); *m*/*z* (%) 376 (M^+, 7%), 358 (100), 340 (4), 330 (15), 314 (40) and 268 (60). The dimethyl ester (**9**) was prepared with diazomethane (Found: M^+, 404.186. $C_{22}H_{28}O_7$ requires M^+, 404.184); ^{1}H NMR ($CDCl_3$) δ 1.16 (3 H, s, 18-H_3), 2.74 (1 H, d, *J* 11 Hz, 6-H), 2.92 (0.67 H, d, *J* 17 Hz, 15α-H, *E* isomer), 3.20 (0.33 H, d, *J* 11 Hz, 5-H, *Z* isomer), 3.23 (0.67 H, d, *J* 11 Hz, 5-H, *E* isomer), 3.69 and 3.72 (6 H, 2s, 2 × CO_2Me), 3.85 (1 H, br, s, 3-H), 5.73 (0.33 H, br s, 17-H, *Z* isomer) and 5.87 (0.67 H, br s, 17-H, *E* isomer); *m*/*z* (%) 404 (M^+, 7%), 372 (100), and 282 (45).

Formation of the Mixed Anhydride (**8**) *and Coupling to Glycine.*—17-Carboxygibberellin A_4 (**7**) (47 mg) in dry dioxane (2 ml) was treated with tributylamine (30 μl) and isobutyl chloroformate (16 μl). After 0.5 h at room temperature, TLC analysis indicated that the starting material had been converted to the less polar mixed anhydride. The reaction solution was added to a solution of glycine (90 mg) in dioxane (3 ml) and pH 8.1 sodium borate buffer (5 ml). After being stirred overnight the reaction solution was acidified to pH 3 with dilute hydrochloric acid and extracted with ethyl acetate and then with butanol. The combined organic extracts were then treated with ethereal diazomethane. After evaporation under reduced pressure, the resulting gum was fractionated by flash chromatography on a 20 × 1 cm column eluted with ethyl acetate–light petroleum (1:1, 30 ml) followed by ethyl acetate–light petroleum (3:2, 50 ml) and ethyl acetate (100 ml); 13 × 14 ml fractions were collected. Fractions 2–4 contained 17-carboxygibberellin A_4 dimethyl ester (**9**) (21 mg), identified by TLC comparison with that prepared above. Fractions 8–12 contained the 17-amide dimethyl ester (**10**) (10 mg) (Found: M^+, 461.204. $C_{24}H_{31}NO_8$ requires: M^+, 461.205); ^{1}H NMR ($CDCl_3$) δ 1.15 (3 H, s, 18-H_3), 2.73 (1 H, d, 6-H), 3.04 (0.67 H, d, *J* 17 Hz, 15α-H, *E* isomer), 3.20 (0.33 H, d, *J* 11 Hz, 5-H, *Z* isomer), 3.22 (0.67 H, d, *J* 11 Hz, 5-H, *E* isomer), 3.72 and 3.78 (6 H, s, 2 × CO_2Me), 3.83 (1 H, br s, H-3), 4.07 (2 H, d, *J* 5 Hz, –NHCH_2CO–), 5.68 (0.33 H, br s, 17-H, *Z* isomer), 5.84 (0.67 H, br s, 17-H, *E* isomer), and 5.92 (1 H, t, *J* 5 Hz, –NH–); *m*/*z* (%) 461 (M^+, 15%), 446 (2), 443 (1), 430 (9), 429 (9), 402 (3), 372 (100), and 142 (55).

Cleavage of the Amide with Osmium Tetraoxide/Sodium Periodate.—The above amide (**10**) (5 mg) in THF (1 ml) and

water (1 ml) was treated with osmium tetraoxide (1 crystal) and sodium periodate (10 mg) at room temperature overnight. The reaction was further diluted with water and the product recovered in ethyl acetate. Analysis by TLC showed a single spot which co-eluted with authentic GA_4 norketone methyl ester (**12**).

Preparation of [1,2-$^3H_2$]-17-*Carboxy* GA_4 (**7**).—(a) [1,2-$^3H_2$]-GA_4 norketone (**3**). To [1,2-$^3H_2$]-gibberellin A_4 (**1**) (450 mg, 2.42 × 10^6 dpm) in THF (40 ml) and water (40 ml) at 0 °C was added osmium tetraoxide (1 crystal) and sodium metaperiodate (600 mg). After being stirred overnight at room temperature the reaction mixture was concentrated under reduced pressure, further diluted with water, and extracted with ethyl acetate. The gum recovered from the ethyl acetate was purified by flash chromatography on a 20 × 2.5 cm column eluted with the following proportions of ethyl acetate in light petroleum containing 0.5% acetic acid: 50% EtOAc (100 ml), 60% (100 ml), 70% (100 ml), and 100% (100 ml), and 20 × 20 ml fractions were collected. The [1,2-$^3H_2$]-norketone (**3**) (434 mg, 1.80 × 10^6 dpm 0.64 μCi $mmol^{-1}$) was recovered from fractions 4–14 and identified by comparison with unlabelled material.

(b) [1,2-$^3H_2$]-17-carboxy GA_4 (**7**). The [1,2-$^3H_2$]-norketone above (434 mg) in pyridine (1.5 ml) with trimethylchlorosilane (1.5 ml) and hexamethyldisilazane (1.5 ml) was kept for 1 h at room temperature. The reaction mixture was then evaporated and filtered in dichloromethane as described above. The resulting [1,2-$^3H_2$]-GA_4 norketone $TMSi_2$ (**5**) in dry THF (10 ml) was added to a solution of $TMSi^-CHCO_2$-$2Li^+$ (**16**) prepared from methyl-lithium (10.4 ml; 1.5M solution), di-isopropylamine (2.19 ml) in THF (40 ml) at 0 °C and a solution of trimethylsilylethanoic acid (1.03 g) in THF (5 ml) as above. The reaction mixture was allowed to warm to room temperature over 4 h. Acetone (5 ml) was then added, followed by 5M HCl to pH 2. This mixture was kept at 4 °C overnight and was then diluted with water and extracted with ethyl acetate. Analytical TLC indicated the presence of two products. These were separated by flash chromatography on a 20 × 2.5 cm column eluted with the following proportions of ethyl acetate in light petroleum containing 0.5% acetic acid: 50% (100 ml), 60% (100 ml), 70% (100 ml), 80% (100 ml), 90% and 100% (100 ml); 28 × 21 ml fractions were collected.

Fractions 13–16 contained the required [1,2-$^3H_2$]-17-carboxy GA_4 (**7**) (120 mg, 3.74 × 10^5 dpm 0.52 μCi $mmol^{-1}$) identified by TLC and ^1H NMR comparison with that previously prepared. The *E*:*Z* ratio was 1:4 by NMR spectroscopy with λ_{max} 226 (ε = 10 090). Fractions 18–28 (216 mg) contained the *endo*-isomer (**13**) which was re-chromatographed in the same way to give pure (**13**) (127 mg); (M^+ − 18), 358.139 [$C_{20}H_{22}O_6$ (M − H_2O) requires 358.142] δ_H (HDA) 1.12 (3 H, s, 18-H_3), 2.50 (1 H, d, *J* 10 Hz, 6-H), 2.82 (1 H, dd, *J* 8, 5 Hz, 13-H), 3.18 (1 H, d, *J* 10 Hz, 5-H), 3.73 (1 H, br, s, 3-H), and 5.89 (1 H, s, 15-H); *m*/*z* (%) 376 (M^+, absent) 358 (13), 326 (4), 314 (5), 310 (10), 270 (8), and 268 (10). Dimethyl ester (**14**), prepared with diazomethane, (Found: M^+, 404.184. $C_{22}H_{28}O_7$ requires 404.184), $\delta_H(CDCl_3)$ δ 1.16 (3 H, s, 18-H_3), 1.96 (1 H, d, *J* 11 Hz, 17-H), 2.25 (1 H, d, *J* 11 Hz, 17-H), 2.59 (1 H, d, *J* 10 Hz, 6-H), 2.83 (1 H, dd, *J* 7, 5 Hz, 13-H), 3.16 (1 H, d, *J* 10 Hz, 5-H), 3.65 (3 H, s, OMe), 3.73 (3 H, s, OMe), 3.85 (1 H, br, s, 3-H), and 5.68 (1 H, s, 15-H); *m*/*z* (%) 404 (M^+, 24%), 372 (100), 344 (13), 342 (18), and 282 (40).

Coupling of 17-*Carboxy* GA_4 (**7**) *to Bovine Serum Albumin* (*BSA*).—[1,2-$^3H_2$]-17-Carboxy GA_4 (70 mg) was diluted with unlabelled material (36 mg) to give 2 083 dpm mg^{-1} (2.2 × 10^5 dpm total). This material (106 mg) in dioxane (5 ml) was treated with tributylamine (67 μl) and isobutyl chloroformate (37 μl). After 0.5 h this solution was added to BSA (110 mg) in borate buffer, pH 8.1 (15 ml) and dioxane (5 ml) at 4 °C. After being stirred for 12 h at 4 °C the solution contained precipitated protein, which failed to redissolve on the addition of a further 20 ml of borate buffer. The suspension was then dialysed against water–dioxane (9:1) and then against water. The product, which was still a suspension, was lyophilised and then resuspended in water (25 ml). The precipitate was collected by centrifugation at 18,000 rpm. The pellet was lyophilised to give 148 mg of GA_4-17-carboxy-BSA conjugate (94 dpm mg^{-1}, 14 047 dpm total) with a coupling ratio of GA:BSA of 8.6 (0.12 μmole GA_1 mg conjugate).

Acknowledgements

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