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GIBBERELLIN A_{43} AND OTHER TERPENES IN ENDOSPERM OF *ECHINOCYSTIS MACROCARPA*

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Key Word Index—*Echinocystis macrocarpa*; Cucurbitaceae; nucellus-endosperm; GC–MS; gibberellin A_{43}; two isomers of gibberellin A_{13}; *ent*-kaurene; *ent*-6α, 7α,16β,17-tetrahydroxykauranoic acid; *ent*-6α,7α,17-trihydroxy-16αH-kauranoic acid.

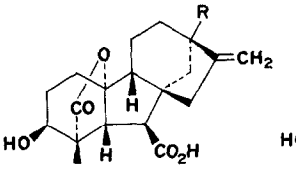
Abstract—By GC–MS the following acidic constituents of the endosperm of *Echinocystis macrocarpa* were identified: abscisic acid and its *trans,trans*-isomer, 4′-dihydrophaseic acid, GA_4, GA_7, iso-GA_7, GA_{24}, GA_{25}, two isomers of GA_{13}, GA_{43}, *ent*-6α,7α,17-trihydroxy-16αH-kauran-19-oic acid and *ent*-6α,7α,16β,17-tetrahydroxykauran-19-oic acid. The structures of the last three new natural products were confirmed by partial synthesis. *ent*-Kaurene was detected in the neutral fraction.

INTRODUCTION

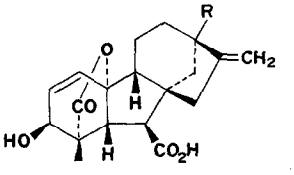
The presence of gibberellin-like (GA-like) compounds in the seed of *Echinocystis macrocarpa* Greene was demonstrated by West and Phinney [2]. and by Corcoran and Phinney [2]. The latter investigators found maximal biological activity in the liquid nucellus-endosperm of seed in which the cotyledons had developed to 50% of their final length. The identification of the GA-like compounds in the endosperm was first investigated by West and Reilly [3] who resolved an acidic extract into GA_3- and GA_7-like components by PC. Later Elson *et al.* [4] examined a similar extract from more mature seed by TLC, spectrofluorimetry, and bio-assay. They tentatively identified GA_1 (**1**), GA_3 (**3**), *iso*-GA_3 (**5**), GA_4 (**2**), GA_7 (**4**), and *iso*-GA_7 (**6**) plus an unidentified substance *f* with properties similar to GA_7. In view of the successful use of cell-free enzyme preparations from the endosperm of *E. macrocarpa* in the study of the early stages in GA-biosynthesis [5], a re-examination of the endogenous GAs in this endosperm was undertaken using integrated gas chromatography-mass spectrometry-computer data processing (GC–MS–C).

RESULTS AND DISCUSSION

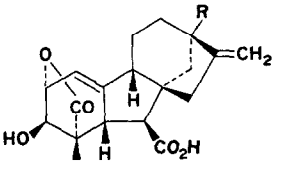
Four separate samples of liquid endosperm, supplied by Professor C. A. West and his associates, were examined with essentially the same results. The samples were prepared from seed collected in 1968, 1970, and 1971 in Santa Monica, California, at the stage of maturity routinely used [5] for the preparation of cell-free enzyme systems. The



(**1**) R = OH
(**2**) R = H



(**3**) R = OH
(**4**) R = H



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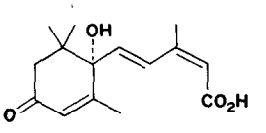

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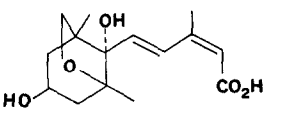
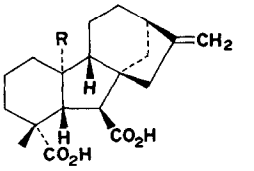


(**8**)



(**9**) R = CHO
(**10**) R = CO_2H

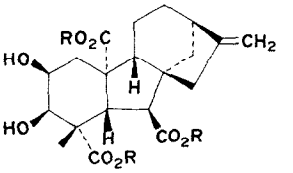
INTRODUCTION

EtOAc soluble extract from the endosperm was separated into acidic and neutral fractions which were bio-assayed using the mutant d-5 of *Zea mays* [6] (Table 1) before being sent to us for GC–MS–C. No detectable biological activity remained in the aqueous residue after extraction of the liquid endosperm by ethyl acetate and essentially all the biological activity was present in the acid fraction. Although the bio-assay data are of limited value because of the variable results obtained at different dilutions of a particular sample, the levels of GA-like compounds were very low compared to the maximal levels (*ca* 500 μg GA_3-equivalents per ml) obtained by Corcoran and Phinney [2].

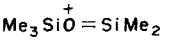
The acidic fraction was examined by GC–MS–C as the Me and MeTMSi derivatives without further purification and the following known compounds were identified by comparison with reference MS: [7, 8] abscisic acid (**7**) and its *trans,trans*-isomer, 4′-dihydrophaseic acid (**8**), GA_4 (**2**), GA_7 (**4**), *iso*-GA_7 (**6**), GA_{24} (**9**), and GA_{25} (**10**). In addition to these known compounds the following 5 compounds were detected: a dihydroxy-GA_{25}, 2 monohydroxy-derivatives of GA_{25}, and a trihydroxy- and a tetrahydroxy-derivative of *ent*-kauran-19-oic acid.

The dihydroxy GA_{25} was assigned the structure (**11**) from the MS of the MeTMSi derivative which showed M^+ 580 with fragment ions at *m/e* 147 (**13**) and 217 (**14**) indicative [7, 9] of a 2,3-dihydroxy-GA. The structure (**11**) for this new GA, allocated [10] the number A_{43}, was confirmed by partial synthesis of the tri-Me ester (**12**) from GA_{13} (**15**). Hydroxylation of the 2,3-dehydro-norketone (**17**), derived [11] from GA_{13} tri-Me ester (**16**), gave the 2,3-diol (**18**) which was protected as the *bis*-TMSi ether (**19**), then reacted with methylenetriphenylphosphorane. Mild hydrolysis of the Wittig product removed the TMSi-protecting groups and yielded GA_{43} tri-Me ester (**12**). While this work was in progress, GA_{43} (**11**) was identified as a major radioactive product from GA_{12}-aldehyde [^{14}C] in a cell-free system from the endosperm of *Cucurbita maxima*, and further investigation showed that GA_{43} also occurs endogenously in *C. maxima* endosperm [12].

The monohydroxy derivatives of GA_{25} were characterized by the MS of their Me and MeTMSi derivatives. The spectra of the triMe esters of both compounds showed the expected stepwise loss of



(**11**) R = H
(**12**) R = Me



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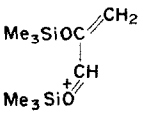
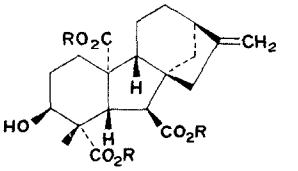
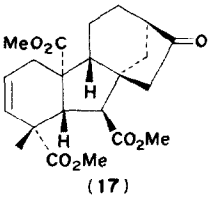


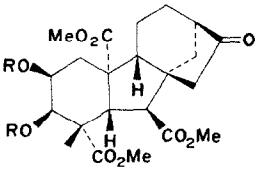
(**14**)



(**15**) R = H
(**16**) R = Me



(**17**)



(**18**) R = H
(**19**) R = $SiMe_3$

32 and 59/60 amu from the three ester functions and weak M^+-18 ions. The spectra of both derivatives of each compound contained intense highest hydrocarbon peaks [13] at *m/e* 223 with corresponding peaks at 59 and 60 amu higher. The MS of the MeTMSi derivatives do not show significant fragment ions at *m/e* 129 (typical [7, 8] of 1- and 3-hydroxy GAs), at *m/e* 207/8 (characteristic [7] of 13-hydroxy GAs), or at *m/e* 156 (typical [8] of 15-hydroxy GAs). Neither do the MS of the MeTMSi derivatives contain strong M^+-90 ions shown [8] by 11β- or 12α-hydroxy GAs. With the available reference spectra, it has not been possible to deduce structures for these two monohydroxy derivatives of GA_{25} and the partial synthesis of possible structures is in hand.

In contrast to the results of Elson *et al.* [4] GA_1 and GA_3 were not detected. The concs of the GAs in the acidic extract were determined by GLC and found to be in the range 0·5–2·0 μg per ml endosperm. Although the biological activities of GA_{43} and the 2 monohydroxy-derivatives of GA_{25} have not been determined, they would be expected to be low in the maize d-5 bioassay since GA_{13} [14] and GA_{17} [15] have low activities and the presence of a 2β-hydroxy group, as in GA_{43}, usually leads to

Table 1. Bio-assay Data (μg GA_3 Equivalents per ml)* for *E. macrocarpa* endosperm

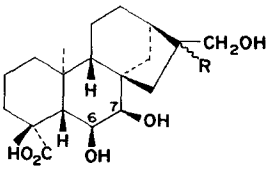
Fraction	Preparation 6–68	4–70	4–71
Neutral	1†	11†	0·07†
Acid	24‡	23†	6·9‡
Endosperm	4·3	54†	

* *Zea mays* mutant d-5 [6]. † Mean of 2 serial dilutions. ‡ Mean of 3 serial dilutions.

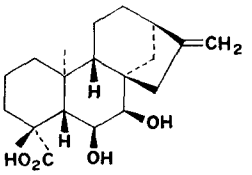
very low activity. Thus the concs of the detected GAs do not account for all the biological activity shown (Table 1) by the acidic fraction.

Structure (**20**) for the tetrahydroxy-derivative of *ent*-kauran-19-oic acid was determined as follows. The MS of the MeTMSi derivative was similar to that [16] of *ent*-6α,7α-dihydroxykaur-16-en-19-oic acid (**24**) showing a weak M^+ and intense ions at M^+-15, M^+-90, *m/e* 269(**25**), 209(**26**) and 147(**13**). In addition ions at *m/e* 103(**27**) and M^+-103 indicated a CH_2OTMSi group attached to a tertiary centre. These MS features suggested a 6,7,16,17-tetrahydroxy-derivative of *ent*-kauranoic acid. Direct comparison with *ent*-6α,7α,16α,17-tetrahydroxykauranoic acid (**21**), isolated by Murofushi *et al.* [17] from *Calonyction aculeatum*, showed that the MS of the MeTMSi derivatives were very similar but that their GC-retention times were different. However, hydroxylation of *ent*-6α,7α-dihydroxykaurenoic acid (**24**) with osmium tetroxide gave a product whose MeTMSi derivative was identical by GC-MS and GLC to that of the tetrahydroxy-compound from *Echinocystis* endosperm. The occurrence of the same compound (**20**) in extracts of immature seed of *Phaseolus coccineus* has also been demonstrated recently by Gaskin and MacMillan [19].

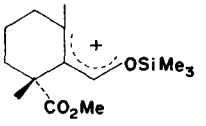
Structure (**22**) for the trihydroxy-derivative of *ent*-kauranoic acid was deduced from the MS of the MeTMSi derivative which showed the fragmentation ions at *m/e* 269(**25**), 209(**26**) and 147(**13**) characteristic of *ent*-6α,7α-dihydroxy- and *ent*-6α,7α,16β,17-tetrahydroxykauranoic acids. In contrast to the *ent*-6α,7α-dihydroxy-derivatives (**20**) and (**21**) the trihydroxy-derivative (**22**) showed weak M^+-103 and *m/e* 103(**27**) ions indicating that position-16 was not fully substituted. Hydroboronation of *ent*-6α,7α-dihydroxykaurenoic acid gave a minor (5%) and a major (80%) product which, from steric considerations, could be



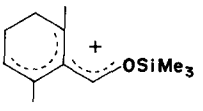
(**20**) R = 16α—OH
(**21**) R = 16β—OH
(**22**) R = βH
(**23**) R = αH



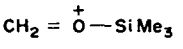
(**24**)



(**25**)



(**26**)



(**27**)

assigned structures (**22**) and (**23**). The MeTMSi derivatives of each product had MS indistinguishable from the trihydroxy-acid from *E. macrocarpa*. However, only the MeTMSi of the minor product had the same GLC retention time, confirming structure (**22**) for the natural compound.

The neutral chloroform extract from the *Echinocystis* endosperm was shown by GC-MS-C to contain *ent*-kaur-16-ene and a series of fatty acids.

EXPERIMENTAL

M.p.s. were determined on a Kofler hot-stage apparatus and are corrected. Layers (0·4 mm) of Kieselgel HF were used for PLC. NMR (τ, *J* in Hz) were obtained at 100 MHz for $CDCl_3$ solns with TMS as internal standard. Analytical GLC: glass columns 182·5 × 2 mm packed with 2% SE-33 on Gas Chrom Q, isothermal at 220°, N_2-flow rate 20 ml/min.

GC–MS–C. A double beam mass spectrometer, coupled to a GLC through a silicone-membrane separator, was used. The GLC-conditions were: 2% SE-33 on Gas Chrom Q packed in glass columns (182·5 cm × 2 mm) with He flow-rate of 25 ml/min and temp programmed from 180–260° at 3° per min. MS conditions were: 24 eV, source 210°, separator 190°, scan speed 6·5 sec per decade at 4 KV and 1000 resolution, scanned either manually or cyclically. Data were processed on line by a *Linc* 8 computer.

Extraction. Pooled liquid endosperm from separate collections was adjusted to pH 2·5 with 6 M HCl and extracted with EtOAc (× 3, equal vol). The combined EtOAc extracts were then extracted with 5% $NaHCO_3$ (× 3, 1/6 vol). Recovery of the EtOAc gave the neutral fraction. The $NaHCO_3$ extract was acidified to pH 2·5 with 6 M HCl and extracted with EtOAc (× 3, equal vol). Recovery of the EtOAc extract provided the acid fraction. One-tenth of each fraction was used for bio-assay.

Partial synthesis of gibberellin A_{43} methyl ester (**12**). (a) *The diol* (**18**). The norketone (**17**) (100 mg) of 2,3-dehydro-gibberellin A_{25} trimethyl ester, prepared from gibberellin A_{13} (**15**) [11] was treated for 4 days at room temp. with OsO_4 (100 mg) in C_5H_5N (0·3 ml) and $CHCl_3$ (0·3 ml). The reaction mixture was then stirred for 15 min with $Na_2S_2O_5$ (200 mg) in C_5H_5N–H_2O [18]. Recovery in EtOAc gave an oil (116 mg), purified by PLC on Si gel with EtOAc–petrol (4:1). Elution of the band at R_f 0·2–0·3 gave the *diol* (**18**) crystallising from Me_2CO–petrol as a flocculent microcrystalline powder (93 mg) m.p. 148–150° (Found: C, 60·3; H, 6·9%; M^+ 438·189. $C_{22}H_{30}O_9$ requires C, 60·2; H, 7·0% M 438·189); IR ν_{max} (nujol) 3540, 3460, 1745 and 1725 cm^{-1}; PMR τ 8·73 (*s*, 18-H_3), 7·34(*d* J 12 Hz, 5-H), 6·38, 6·34, 6·28 (each *s*, 3 × $-CO_2Me$), 6·16(*d* J 4Hz, 3-H), 6·05 (*d*, J 12 Hz, 6-H), 5·5(*dt*, J 4 and 12 Hz, 2-H); MS *m/e* 438 (M^+, 35%), 420(5), 406(100), 388(13), 374(19), 370(13), 361(56), 356(15), 346(21), 328(24), 318(16), 301(19), 241(20), 117(13), and 91(15).

(b) *Diol di-TMSether* (**19**). The diol (**18**)(80 mg) in C_5H_5N (1 ml) was stirred for 3 hr with Me_3SiCl (0·3 ml) and $(Me_3Si)_2NH$ (0·3 ml). The oil obtained by evaporation under vacuum was filtered in EtOAc through a short Si gel column. Recovery from the filtrate gave the gummy di-TMSi ether (**19**) (93 mg) which was used directly in (c) without further purification; MS *m/e* 582 (M^+, 12%), 567(6), 507(8), 492(3), 433(100), 372(7), 351(42), 217(52), 204(23), 188(51), 173(50), 147(23), 75(9), and 73(46).

(c) *Gibberellin A_{43} trimethyl ester* (**12**). Butyl lithium was added dropwise, and with stirring, to Me triphenylphosphonium iodide (370 mg) in THF (10 ml) until the yellow colouration persisted and the suspension had almost completely dissolved. The diol di-TMSi (**19**) (92 mg) in THF (2 ml) was added and the mixture was stirred at room temp. for 4 hr, then refluxed for 5 hr. After removal of the THF by distillation under vacuum, H_2O was added and the pH was adjusted to 2·5 with 2 M HCl. Recovery of the product in EtOAc gave a gum (244 mg) which was stirred for 2 hr with gentle warming with HOAc–H_2O (5:1). The recovered gum was purified by PLC on Si gel with EtOAc–petrol (3:1). Elution of the band at R_f 0·35–0·45 gave gibberellin A_{43} trimethyl ester (**12**) (46·5 mg) as a gum, homogeneous by TLC in two solvent systems and by GLC as the Me ester and Me ester di-TMSi ether, each on 2 columns. (Found: C, 62·9; H, 7·7%, M^+ 436·206. $C_{23}H_{32}O_8$ requires C, 63·3; H, 7·4% M 436·210); IR ν_{max} ($CHCl_3$) 3540br, 3400br, 1720 and 1655 cm^{-1}; PMR τ 8·72(*s*, 18-H_3), 7·36(*d*, J 12 Hz, 5-H), 6·40, 6·34, 6·28(each *s*, 3 × $-CO_2Me$), 6·16(*d*, J 4 Hz, 3-H), 6·14(*d*, J 12 Hz, 6-H), 5·60(*dt*, J 4 and 11 Hz, 2-H), 5·19 and 5·10(*m*, 17-H_2); MS *m/e* 436 (M^+, 13%), 418(2), 404(56), 386(3), 376(4), 372(4), 358(21), 344(100), 326(20), 316(39), 312(10), 298(28), 284(28), 267(10), 257(10), 239(28), 129(13), and 91(22). The Me ester di-TMSi ether had *m/e* 580 (M^+, 14%), 565(6), 548(2), 520(2), 505(9), 490(10), 431(100), 371(15), 349(20), 217(35), 204(15), 188(26), 173(25), 147(18), 75(16), and 73(48). The acetonide, prepared from gibberellin A_{43} trimethyl ester, Me_2CO, and a trace of *p*-$MeC_6H_4SO_3H$ at room temp, was obtained as a gum *m/e* 476 (M^+, 0·5), 461(44), 444(2), 418(25), 386(44), 358(39), 326(100), 299(51), 281(19), 267(16), 239(41), 228(13), 167(22), and 43(24).

MS data for the two monohydroxy-derivatives of GA_{25}. (a) *x-Hydroxy GA_{25}.* Me ester: *m/e* 420 (M^+, 45), 402(2), 388(66), 360(100), 356(52), 342(18), 329(93), 328(41), 326(30), 301(61), 300(52), 283(64), 282(27), 281(30), 241(36), 239(41), 223(52), 222(16), 221(23), 164(95), 155(34), 143(36), 131(34), 129(32), 121(34), 119(43), 105(45), 93(43), 91(54), and 81(39); MeTMSi 492 (M^+, 18), 477(2), 460(13), 432(22), 342(10), 311(13), 296(10), 283(34), 282(16), 281(9), 223(21), 222(9), 221(9), 164(22), 75(57), and 73(100). (b) *y-Hydroxy GA_{25}.* Me ester *m/e* 420 (M^+, 1), 418 (0·5), 388(31), 360(2), 366(10), 342(11), 328(100), 300(43), 283(39), 282(51), 241(22), and 223(46); MeTMSi: *m/e* 492 (M^+, 3), 477(1), 460(14), 400(18), 342(24), 311(13), 283(36), 282(36), 223(35), 173(42), 171(78), 164(15), 75(43), and 73(100).

ent-6α,7α,16β,17-*Tetrahydroxykauran*-19-*oic acid* (**20**) was characterised by the following GC-MS data for the MeTMSi derivative: *m/e* 670 (M^+, 0·3), 655(8), 580(1), 567(34), 565(3), 477(100), 417(5), 387(10), 361(1), 309(5), 269(32), 209(13), 191(13), 147(24), 129(15), 117(15), 103(15), 75(33), and 73(92). GLC R_t (17·95 min) and MS identical to the TMSi ether of the product from OsO_4 treatment of methyl *ent*-6α,7α-dihydroxykaurenoic acid (**24**). The MeTMSi of *ent*-6α,7α,16α,17-tetrahydroxykauranoic acid (21), provided by Professor N. Takahashi, had *m/e* 655(3), 567(32), 565(1), 477(100), 417(4), 387(9), 361(1), 309(4), 269(5), 209(4), 191(7), 147(14), 129(4), 117(7), 103(8), 75(11), and 73(44); it had GLC R_t of 16·8 min.

ent-6α,7α,17-*Trihydroxykauran*-19-*oic acid* (**22**) was characterised by the following GC–MS data for the MeTMSi derivative: *m/e* 582 (M^+, 0·6), 567(54), 523(3), 492(9), 479(2), 477(4), 361(18), 269(100), 209(24), 147(23), 103(14), 75(26), and 73(81). GLC R_t (19·45 min) and MS identical to the MeTMSi of the minor product from hydroboronation of *ent*-6α,7α-dihydroxykaurenoic acid.

Hydroxylation of ent-6α,7α-*dihydroxykaurenoic acid.* (a) *OsO_4.* The acid (*ca* 50 μg) in THF-H_2O (200 μl, 1:1) was treated with OsO_4 (*ca* 100 μg) for 2½ days at 20°. After addition of 5% aq. $NaHSO_3$ (1 ml), the mixture was extracted with EtOAc (3 × 1 ml). The recovered product was 90% pure by GLC and had the same R_t (17·65 min) as the MeTMSi of the tetrahydroxy-acid from *E. macrocarpa*. (b) *Hydroboronation.* The acid (*ca* 50 μg) in dry THF (50 μl) was treated with 0·5 M BH_3.THF in THF (50 μl) for 30 min at 20°. H_2O_2 (6% v/v) in 5% aq KOH (1 ml) was added, and, after 30 min, the soln was acidified to pH 2·5 and extracted with EtOAc (3 × 1 ml). Recovered material was shown by GLC to consist of a minor product (**22**, 5%) and a major product (**23**, 80%). The MeTMSi of the minor product had the same R_t (19·45 min) and MS as the MeTMSi of the trihydroxy-acid from *E. macrocarpa*. The MeTMSi of the major product (**23**) had a R_t of 22 min, and the same MS as the MeTMSi of the minor product.

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