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ENT-3β-HYDROXYKAUR-16-ENE AND *ENT*-17-HYDROXYKAUR-15-ENE IN PACLOBUTRAZOL-TREATED WHEAT SEEDLINGS

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Key Word Index—*Triticum aestivum*; *Hordeum vulgare*; Gramineae; wheat; barley; *ent*-kaurenols; paclobutrazol.

Abstract—Large quantities of *ent*-kaur-16-ene accumulated in the scutella of wheat and barley seedlings treated with the *ent*-kaurene oxidase inhibitor, (2*S*,3*S*)-paclobutrazol. In addition, *ent*-3β-hydroxykaur-16-ene and *ent*-17-hydroxykaur-15-ene were identified by full-scan GC mass spectrometric comparison with authentic samples in embryos of paclobutrazol-treated wheat. In four-day-old paclobutrazol-treated wheat, *ent*-3β-hydroxykaur-16-ene occurred in similar amounts to *ent*-kaur-16-ene in embryonic axes but was about 10-fold less than the quantity of *ent*-kaur-16-ene in scutella. By comparison, *ent*-17-hydroxykaur-15-ene was present at very low levels and only in scutella of treated wheat seedlings. Only trace amounts of *ent*-3β-hydroxykaur-16-ene were detected in scutella of paclobutrazol-treated barley.

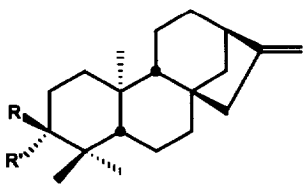
INTRODUCTION

The sequential hydroxylation of *ent*-kaur-16-ene (**1**) via *ent*-kaur-16-en-19-ol (**4**) and *ent*-kaur-16-en-19-al (**5**) to *ent*-kaur-16-en-19-oic acid (**6**), catalysed by *ent*-kaurene oxidase, is an early step in the biosynthetic pathway to the gibberellins (GAs) [1, 2]. The activity of *ent*-kaurene oxidase from cell-free preparations of *Cucurbita maxima* endosperm is inhibited by triazoles, such as paclobutrazol [3–5]. These triazoles are potent growth retardants, decreasing shoot height and GA content of cereal seedlings [6–8] and causing large amounts of **1** to accumulate in treated seedlings [9, 10]. Measurement of **1** by isotope-dilution GC mass spectrometry in inhibitor-treated barley seedlings provided a novel method for determining the spatial and temporal aspects of *de novo* GA biosynthesis in plants [9]. We have adopted this method to examine GA production in germinating wheat grains and have discovered that, in addition to **1**, significant amounts of two *ent*-kaurenols accumulated in certain tissues of inhibitor-treated seedlings. One of the compounds, *ent*-3β-hydroxykaur-16-ene (**2**), was shown to be identical to a reference compound [11] that had been synthesized from *ent*-kaur-16-en-3β, 19-diol (**7**). The other compound was identified as *ent*-17-hydroxykaur-15-ene (**8**), a known metabolite of **1** in *Zea mays* [12].

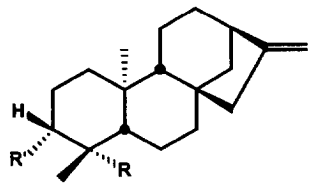
RESULTS AND DISCUSSION

Preliminary examination of derivatized fractions from wheat embryos by GC-selected ion monitoring (SIM) revealed the presence of two compounds that co-chromatographed with *ent*-kaur-16-en-19-ol (**4**) on silica gel columns and reverse-phase HPLC and increased in abundance, in a concentration-dependent manner, in paclobutrazol-treated seedlings. In a larger-scale work-up, the minor component was shown to have the same Kovat's Retention Index (KRI) and mass spectral fragmentation pattern as authentic *ent*-17-hydroxykaur-15-ene (**8**) (Table 1). The major component was suspected to be a 3-hydroxykaur-16-ene, due to the presence of a prominent $[M - 129]^+$ ion, at *m/z* 231, in the mass spectrum of the TMSi derivative. Oxidation of authentic *ent*-3β-hydroxykaur-16-ene (**2**) and Meerwein–Ponndorf–Verley reduction of the resulting 3-ketone (**9**) yielded a 1:1 mixture of 3α- and 3β-alcohols that were resolved by TLC and the structures assigned by ^{1}H NMR. As predicted, the resonance of the proton at C-3 (δ3.20) in the *ent*-3β alcohol (**2**) showed axial–axial coupling (J = 11 Hz) to the proton at C-2, whereas, in the 3-epimer (**3**), this signal was a narrow triplet (J = 2 Hz) at δ3.41. The metabolite present in paclobutrazol-treated wheat embryos had the same KRI and mass spectrum as authentic **2** (Table 1).

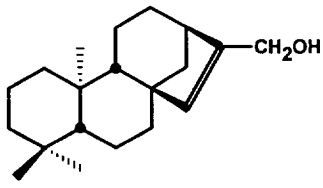
ent-Kaur-16-ene (**1**) was quantified in plant extracts by isotope-dilution GC-MS [9], whereas **2** and **8** were determined by GC-SIM, using calibration curves based on authentic compounds and *ent*-[^{14}C]kaur-16-en-19-ol (**4**). Compounds **2**, **8** and **4** cochromatographed on silica gel columns and reverse-phase HPLC. This method was validated for **2** by preparing [$^2H_1$]-labelled **2**, by borodeuteride reduction of the 3-ketone (**9**) (see Experimental) and constructing another calibration curve. The



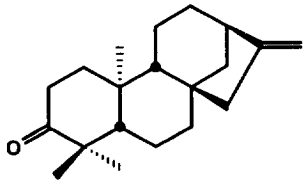
(1) R = R' = H
(2) R = H, R' = OH
(3) R = OH, R' = H



(4) R = CH_2OH, R' = H
(5) R = CHO, R' = H
(6) R = CO_2H, R' = H
(7) R = CH_2OH, R' = OH



(8)



(9)

Table 1. Comparison of mass spectra of the TMSi derivatives of authentic *ent*-kaurenols with those isolated from scutella of wheat seedlings (cv. Maris Huntsman) treated with 3 μM paclobutrazol for four days

Compound	KRI	Characteristic ions *m/z** (% base peak)
ent-3α-Hydroxykaur-16-ene (**3**) (standard)	2279	360[M]$^+$ (3), 345(1), 317(1), 270(68), 255(83), 231(84), 230(48), 227(49), 187(100), 129(46)
ent-3β-Hydroxykaur-16-ene (**2**) (standard)	2316	360[M]$^+$ (6), 345(4), 317(2), 270(39), 255(31), 231(100), 230(52), 227(23), 187(80), 129(38)
ent-3β-Hydroxykaur-16-ene (wheat)	2316	360[M]$^+$ (5), 345(3), 317(2), 270(35), 255(36), 231(100), 230(49), 227(35), 187(91), 129(61)
ent-17-Hydroxykaur-15-ene (**8**) (standard)	2330	360[M]$^+$ (24), 345(12), 270(38), 255(100), 222(7), 213(42), 201(18), 182(87), 156(71), 103(97)
ent-17-Hydroxykaur-15-ene (wheat)	2332	360[M]$^+$ (22), 345(14), 270(20), 255(78), 222(12), 213(25), 201(14), 182(71), 156(56), 103(100)

*Based on ions above *m/z* 100.

amounts of endogenous **2** in extracts of paclobutrazol-treated seedlings were similar using both calibration curves.

Initial comparison of wheat and barley showed that **1** accumulated mainly in the scutellum of paclobutrazol-treated seedlings (Table 2), suggesting that this tissue is the main site of *de novo* GA biosynthesis in untreated seedlings [9, 10]. However, such a conclusion is dependent on the hydrocarbon **1** being non-mobile and assumes that paclobutrazol penetrates all tissues uniformly and is not preferentially metabolized. It also depends on accumulated **1** not being further metabolized. Whilst this is nearly so in barley, with only small amounts of **2** accumulating, it is certainly not the case in wheat (Table 2). Similar amounts of **1** and **2** accumulated in axes and both **2** and **8** were present in scutella of wheat seedlings treated with paclobutrazol. Since **2** and **8** were not detected in untreated seedlings, we assume that they arise as metabolites induced by the large amounts of **1** that accumulate in paclobutrazol-treated seedlings. In this respect, it appears that barley can tolerate higher concentrations of **1** than wheat. Similar results were obtained when wheat and barley were germinated at 25° in the presence of paclobutrazol (data not shown).

In a further experiment with wheat, **2** was again shown to be a significant proportion of **1** in treated axes (Table 3), suggesting that quantification of **1** alone would have underestimated the GA-biosynthetic capacity of this tissue, although transport of **2** from scutella cannot be ruled out. Similarly, measurement of **2** and **8**, together with **1**, is required to obtain a more complete picture of potential GA production in wheat scutella.

EXPERIMENTAL

Authentic *ent*-3β-hydroxykaur-16-ene (**2**) [11] and *ent*-17-hydroxykaur-15-ene (**8**) [12] were generous gifts from Professor J. MacMillan, University of Bristol.

Table 2. Quantification of *ent*-kaurene and *ent*-kaurenols in wheat (cv. Maris Huntsman) and barley (cv. Himalaya) seedlings treated with 3 μM (2*S*,3*S*)-paclobutrazol for four days. The amounts of *ent*-kaurene present in untreated (control) seedlings are shown in parentheses

	Content (pmol per part)		
	Tissue		
Species/compound	Axis	Scutellum	Endosperm
Wheat			
ent-Kaurene (**1**)	0.56 (0.03)	5.52 (0.10)	1.07 (0.31)
ent-3β-OH Kaurene (**2**)	0.38	0.74	nd
ent-17-OH Kaurene (**8**)	nd	0.09	nd
Barley			
ent-Kaurene (**1**)	1.93 (0.09)	14.60 (0.78)	2.59 (2.16)
ent-3β-OH Kaurene (**2**)	nd	0.22	nd
ent-17-OH Kaurene (**8**)	nd	nd	nd

nd: Not detected.

Table 3. Accumulation of *ent*-kaurene and *ent*-kaurenols in the axis (shoot plus root) and scutellum of wheat (cv. Maris Huntsman) seedlings treated with 3 μM (2*S*,3*S*)-paclobutrazol

	Content (pmol per part)		
	Time (days)		
Tissue/compound	2	3	4
Scutellum			
ent-Kaurene* (**1**)	1.73	5.70	9.13
ent-3β-OH Kaurene (**2**)	0.30	0.51	0.86
ent-17-OH Kaurene (**8**)	nd	0.04	0.14
Axis			
ent-Kaurene† (**1**)	0.27	0.45	0.49
ent-3β-OH Kaurene (**2**)	0.14	0.25	0.44
ent-17-OH Kaurene (**8**)	nd	nd	nd

*Untreated controls < 0.11 pmol per part.
†Untreated controls < 0.12 pmol per part.
nd: Not detected.

Preparation of ent-*kaurene-16-en-3-one* (**9**). Compound **2** (60 mg) in Me_2CO (20 ml) was treated with Jones reagent until the soln remained yellow. After stirring for 20 min at room temp., MeOH (0.5 ml) and H_2O (3 ml) were added, the Me_2CO evapd *in vacuo* and H_2O added to a total vol. of 20 ml. Following partitioning with EtOAc (3 × 20 ml), the combined EtOAc phases were evapd to dryness. The product (60 mg) was pure by TLC [R_f 0.5 on silica gel developed in EtOAc–hexane (4:1)] and was identified as **9** by GC-MS. MS *m/z* (rel. int.): 286 ([M]$^+$, 70), 271 (21), 253 (4), 243 (100), 227 (22), 215 (7), 201 (50), 185 (16), 159 (12), 91 (27).

Meerwein–Ponndorf–Verley reduction of **9**. Al foil (200 mg) and $HgCl_2$ (3 mg) were refluxed in propan-2-ol (3 ml). CCl_4 (20 μl) was added and the mixt. refluxed for a further 4 hr, after which time the Al foil had dissolved. Compound **9** (35 mg) in propan-2-ol (200 μl) was added and refluxing continued for a further 2 hr. The reaction mixt. was poured into H_2O (20 ml), adjusted to pH 3 (2 M HCl) and partitioned with EtOAc (3 × 20 ml). The combined EtOAc extracts were evapd to dryness and the products purified by TLC on silica gel developed in EtOAc–hexane (7:3). The band at R_f 0.4 (8.3 mg) was identical to authentic **2**. ^{1}H NMR ($CDCl_3$): δ4.79 and 4.73 (each *br s*, H_2-17), 3.20 (*dd*, J = 11, 5 Hz, H-3), 1.02, 0.98, 0.78 (3 × *s*, H_3-18, H_3-19, H_3-20); MS *m/z* (rel. int.): 288 [M]$^+$ (86), 273 (31), 270 (22), 255 (100), 245 (44), 241 (4), 227 (72), 201 (21), 135 (23), 91 (47) (TMSi derivative, see Table 1). The band at R_f 0.56 gave *ent*-3α-hydroxykaur-16-ene (**3**) (8.3 mg). ^{1}H NMR ($CDCl_3$): δ4.79 and 4.73 (each *br s*, H_2-17), 3.41 (*t*, J = 2 Hz, H-3), 1.03, 0.95, 0.84 (3 × *s*, H_3-18, H_3-19, H_3-20); MS *m/z* (rel. int.): 288 [M]$^+$ (34), 273 (10), 270 (29), 255 (100), 245 (4), 241 (4), 227 (54), 201 (9), 135 (15), 91 (20) (TMSi derivative, see Table 1).

Sodium borodeuteride reduction of **9**. A soln of **9** (20 mg) and $NaBD_4$ (20 mg) in EtOH (2 ml) was stirred at room temp. for 1 hr and worked-up, as described above. The band at R_f 0.4 gave *ent*-[$^2H_1$]3β-hydroxykaur-16-ene (7.9 mg). The ^{1}H NMR was identical to **2** but with the absence of the signal at δ3.2 (H-3). Similarly, the MS was identical to **2** but increased by 1 mu, except for *m/z* 91. The band at R_f 0.56 gave *ent*-[$^2H_1$]3α-hydroxykaur-16-ene (1.5 mg). The ^{1}H NMR was identical to **3** but without the signal at δ3.4. The MS was identical to **3** but increased by 1 mu, except for *m/z* 91.

Identification of ent-3β-*hydroxykaur-16-ene* (**2**) *and* ent-17-*hydroxykaur-15-ene* (**8**) *in paclobutrazol-treated wheat seedlings*. Wheat (*Triticum aestivum*, cv. Maris Huntsman) grains were surface-sterilized in NaOCl soln (1% available Cl_2) for 10 min, rinsed × 6 in sterile distilled H_2O, dried and plated out (15 × 15 pattern) into sterile

Pyrex dishes containing two layers of 20 × 20 cm Whatman 3 MM filter paper and 70 ml filter-sterilized (0.2 μm) 3 μM (2*S*,3*S*)-paclobutrazol soln. The dishes were covered with a double layer of Al foil and incubated at 15° in darkness. After 4 days, the embryonic axis (shoot plus roots), scutellum and endosperm were dissected, plunged into liquid N_2, weighed and transferred to cold MeOH and stored at −25°.

After homogenization, *ent*-[^{14}C]kaur-16-ene (*ca* 800 Bq; 7.52 TBq mol^{-1}) and *ent*-[^{14}C]kaur-16-en-19-ol (*ca* 800 Bq; 8.70 TBq mol^{-1}), both prepd from (*R*)-[2-^{14}C] mevalonic acid using a cell-free system from *Cucurbita maxima* endosperm [9], were added and the tissue was extracted with MeOH. After filtration and re-extraction, the combined MeOH extracts were evapd under a stream of N_2. The sample was redissolved in hexane (5 ml) and applied to a 2 g silica gel column (40 μm, Analytichem Bondesil) that had been equilibrated previously with hexane. The sample was washed successively with hexane (2 × 5 ml), 2.5% EtOAc in hexane containing 1% HOAc (3 × 5 ml), 5% EtOAc in hexane containing 1% HOAc (3 × 5 ml) and 15% EtOAc in hexane containing 1% HOAc. Each 5 ml washing was applied individually to the column and was collected. Aliquots were removed for liquid scintillation counting to locate *ent*-kaur-16-ene (**1**) (hexane fr.) and *ent*-kaur-16-ene-19-ol (**4**) (5% EtOAc fr.). The 5% EtOAc fr. was evapd to dryness under a stream of N_2, redissolved in 100 μl EtOAc and purified by reverse-phase HPLC using a 5 μm ODS Hypersil column (250 mm × 4.5 mm id) eluted with a linear gradient of 80–100% MeOH in aq. HOAc (50 μl l^{-1}) over 20 min at a flow rate of 1 ml min^{-1}. The frs corresponding to **4** (20–22) were evapd to dryness, methylated (CH_2N_2) and repurified by HPLC, as described above, but using a linear gradient of 50–100% MeOH. Again, the required frs (27–29) were evapd to dryness and trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 90° for 30 min and the derivatized sample was analysed by full-scan GC-MS. Samples (1 μl in MSTFA-CH_2Cl_2 1:4) were coinjected with Parafilm in hexane (to determine KRI values) into an OV-1 fused-silica WCOT capillary column (25 m × 0.22 mm id, 0.25 μm film thickness) at an oven temp. of 50°. After 2 min, the splitter (50:1) was opened and the temp. increased at 10° min^{-1} to 150° then at 3° min^{-1} to 300°. The He inlet pressure was 0.08 MPa and the inj., interface and MS source temps were 280, 280 and 210°, respectively. The source was operated at 24 eV and the spectrometer at a resolving power of 1000. Positive EI-MS were acquired, scanning from 750 to 50 mu at 1 Hz. The MS data of derivatized samples from wheat scutella are given in Table 1.

Quantification of ent-*kaur*-16-*ene* (**1**), ent-3β-*hydroxykaur*-16-*ene* (**2**) *and* ent-17-*hydroxykaur*-15-*ene* (**8**) *in wheat and barley seedlings.* Wheat and barley (*Hordeum vulgare*, cv. Himalaya) were grown under sterile conditions, as described above. In addition to the paclobutrazol treatment, another batch of grain was germinated in sterile distilled H_2O (controls). In a further expt with wheat, samples were harvested on days 2–4 from both control and paclobutrazol-treated seedlings. In addition to the [^{14}C]-labelled int. standards **1** and **4**, *ent*-3β-hydroxy[$^2H_1$]kaur-16-ene was also added to the initial homogenate of one batch of paclobutrazol-treated wheat and was used as a second int. standard for quantification of **2**. Samples were worked-up, as described above, and were analysed by GC-SIM, as described previously [9, 10]. The concn of endogenous (**1**) was determined by isotope dilution analysis by monitoring ions at *m/z* 272, 274, 276, 278 and 280 [9]. The concns of endogenous **2** and **8** were determined from calibration curves of the peak area ratios of the authentic compounds (*m/z* 270) and *ent*-[^{14}C]kaur-16-en-19-ol (*m/z* 278), plotted against molar ratios of the compounds. A second calibration curve was also constructed for **2**, using *ent*-3β-hydroxy-[$^2H_1$]kaur-16-ene (*m/z* 271). Peak purity was checked by monitoring other relevant ions.

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