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Isolation of Gibberellin Precursors from Heavily Pigmented Tissues[1]

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ABSTRACT

The kauranoid precursors of gibberellins are difficult to isolate from heavily pigmented plant tissues. In this paper, we describe relatively simple and efficient procedures for the purification of these compounds from tissues containing chlorophyll and other high molecular weight pigments. Extracts of shoots from *Thlaspi arvense* L. were subjected first to size exclusion chromatography using ethyl acetate as the eluting solvent. This procedure resulted in the separation of kauranoids as a class of compounds from chlorophyll. Typically, a 90% reduction in mass of the kauranoid enriched-fraction was observed. This fraction was subjected to reverse phase high performance liquid chromatography and individual fractions analyzed by combined gas chromatography-mass spectrometry. Five kauranoids were identified in shoot extracts of *T. arvense*: *ent*-kaur-16-ene, *ent*-kaur-16-en-19-ol, *ent*-kaur-16-en-19-oic acid, trachylobanoic acid, and 7β,13-dihydroxykaurenolide. The metabolic relationships of these compounds to the gibberellins previously identified in this species (JD Metzger, MC Mardaus [1986] Plant Physiol 80: 396–402) are discussed. In addition, the utility of size exclusion chromatography in preparative situations is demonstrated by the purification of *ent*-kaurenoic acid in milligram quantities from the florets of *Helianthus annuus* L.

Studies on the regulation of plant hormone metabolism often involve two complementary lines of experimentation. First, endogenous levels of the hormone, its precursors, and metabolites are determined. Second, a plant's or a tissue's capability for hormone biosynthesis and metabolism is assessed through studies utilizing isotopically labeled compounds. For the results from both types of studies to be meaningful, determining the chemical identity of the compounds of interest is necessary. Most often this involves some physical-chemical technique such as GC-MS. These techniques are difficult to apply to hormone studies because hormones and their precursors usually comprise only a small proportion of the total mass contained in extracts of plant tissues. This necessitates subjecting an extract to several purification procedures.

In our studies on the thermoinductive regulation of GA metabolism in field pennycress, we have found it necessary to develop procedures to quantitate the endogenous levels of the kauranoid precursors to GAs as well as identify metabolites of labeled kauranoids following their application to field pennycress plants. Because vegetative tissues often contain large amounts of pigments, routine analytical scale chromatographic procedures such as reverse phase HPLC cannot be employed until significant reductions in the mass of the extract have been achieved. In green tissues this usually means separation of Chl and carotenoids from the compounds of interest. For GAs, both solvent partitioning with nonpolar solvents such as hexane and charcoal adsorption chromatography (9, 10) have proved satisfactory. However, both techniques are unsuitable for the kauranoid precursors of GAs because their nonpolar character bestows them with solubility properties similar to Chl. Thus, to circumvent these problems, a separation technique must be used that is based on chemical or physical properties other than relative polarities of compounds. One such chromatographic procedure is SEC[3] which exploits mol wt differences to effect separation. This technique was shown to be useful in the purification of various plant hormones from crude plant extracts (13). In this paper, we demonstrate the utility of SEC in the purification of kauranoid compounds from highly pigmented tissues and report the identification of several kauranoids in extracts of field pennycress shoots.

MATERIALS AND METHODS

Plant Material

Seeds of an inbred line (CR_1) of field pennycress (*Thlaspi arvense* L.) were germinated in Petri dishes lined with blotter papers saturated with deionized H_2O. Seedlings were transferred to 54 × 27 × 6 cm plastic flats divided into 48 equal compartments (Compaks, T. O. Plastics, Inc., Minneapolis, MN)[4] and grown in a greenhouse with a nominal temperature of 21°C. After 6 weeks, the plants were thermoinduced by subjecting them to a 4 week cold treatment at 6°C in a growth chamber. Following thermoinduction, the plants were re-

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[3] Abbreviations: SEC, size exclusion chromatography; EtOAc, ethyl acetate; K, *ent*-kaur-16-ene; Kol, *ent*-kaur-16-en-19-ol; Kal, *ent*-kaur-16-en-19-al; KA, *ent*-kaur-16-en-19-oic acid; 7β-OH KA, *ent*-7α-hydroxykaur-16-en-19-oic acid; MeOH, methanol; steviol, *ent*-13-hydroxykaur-16-en-19-oic acid; THF, tetrahydrofuran.

[4] Mention of trademark or proprietary product does not constitute a guarantee or warranty of that product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

turned to the greenhouse. After 10 d, the shoots were excised, frozen in liquid N_2, and lyophilized. The dried shoot material was stored in sealed containers at −15°C until used.

Sunflowers (*Helianthus annuus* L.) were also grown in a greenhouse. Flower heads with mature ray flowers were excised and the bracts removed. The remaining tissue (flowers plus small amounts of the capitulum) were frozen in liquid N_2 and lyophilized. The dried material was then ground to a fine powder with a Wiley mill and stored in sealed containers at −15°C until used.

Identification of Endogenous Kauranoids in Field Pennycress Shoots

Freeze-dried field pennycress shoots were extracted in 50 g lots with 1 L of ice-cold ethyl acetate in a large Waring Blendor. The extract was filtered, and the residue was extracted once more with another liter of ethyl acetate. Following filtration, the filtrates were combined and 50,000 dpm of ^{14}C-K (specific activity = 97 μCi μmol^{-1}) and 250,000 dpm of ^{3}H-KA (specific activity = 53 μCi μmol^{-1}) were added. The radiopurity of both compounds exceeded 99% as judged by HPLC. The volume of the extract was reduced to about 10 mL under reduced pressure at 30°C.

The concentrated extract was applied to a 50 × 6 cm column of Biobeads SX-8 (Bio-Rad) which has an exclusion limit of 1000. The column was eluted with ethyl acetate at 2 mL min^{-1}. Both the extraction procedures and SEC were performed under dim light. Fractions were collected every 5 min and the amount of ^{14}C and ^{3}H in each fraction determined. A 10% aliquot from each fraction was removed and applied to a Packard Combustocone filled with a compressed tissue. The solvent was allowed to evaporate and the residues combusted in a Packard model 306 sample oxidizer and the amount of trapped radioactivity determined by liquid scintillation spectrometry.

Fractions containing radioactivity were combined and subjected to reverse phase HPLC. The residue from the combined fractions was redissolved in about 150 μL of THF, centrifuged to remove any particulate material, and injected onto a 9 × 250 mm Magnum (Whatman) C_{18} reverse phase column. The HPLC system was comprised of two Waters model 6000A pumps controlled by a Waters Automatic Gradient Controller. The column was eluted with a water-methanol gradient; both solvents contained 1% (v/v) acetic acid. Following injection, the solvent composition was maintained at 30% methanol for 1 min. The percentage of methanol was then increased to 60% in 14 min using a convex gradient (gradient profile No. 5 on the Automatic Gradient Controller), whereupon the proportion of methanol was increased linearly in 10 min to 100% methanol. The flow rate was 5 mL min^{-1}. Fractions were collected every min and dried by blowing air from a fan over the collection vials. The gradient profile plus the elution volumes of reference kauranoids and several GAs are shown in Figure 1.

The reverse phase HPLC fraction containing ^{14}C-K was subjected to an additional chromatographic step because of the presence of small amounts of Chl. This fraction was dissolved in 50 μL of THF and injected onto a size exclusion column for HPLC packed with Ultrastyragel (Water Associ-

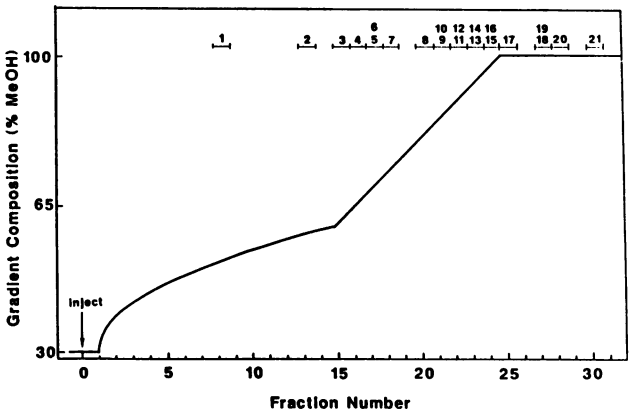

Figure 1. Profile of the water-methanol gradient used to separate kauranoids by reverse phase HPLC. Numbers indicate fractions containing authentic compounds. Fractions collected every min: 1, GA_1; 2, GA_{20}; 3, GA_{19}; 4, 6β, 7β, 13-trihydroxykaurenoic acid; 5, 7β,13-dihydroxykaurenolide; 6, 7β-13-dihydroxykaurenoic acid; 7, 13-hydroxylated seco-B ring di-acid; 8, GA_{53}; 9, GAg; 10, seco-B ring tri-acid; 11, 7β-hydroxykaurenolide; 12, seco-B ring diacid; 13, 6β, 7β-dihydroxykaurenoic acid; 14, 7β-hydroxykaurenoic acid; 15, GA_{12}; 16, steviol (13-hydroxykaurenoic acid); 17, GA_{12}-aldehyde; 18, kaurenoic acid; 19, trachylobanoic acid; 20, kaurenol; 21, kaurene.

ates) with a 100 Å pore size. The column was eluted with THF at 1 mL min^{-1}. The fraction containing K was collected by monitoring the elution of ^{14}C-kaurene with a Berthold model LB 503 radioactivity monitor. The solvent was reduced to incipient dryness at room temperature with a stream of N_2 immediately after the fraction was collected.

The entire extraction and fractionation process described above was repeated five times for a total of 300 g of shoot tissue that were extracted. Corresponding HPLC fractions from each of the six extracts were combined, methylated with ethereal diazomethane, and dissolved in 100 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. Four hundred μL of dry acetonitrile were added to the derivatized fraction containing K.

Purification of KA from Sunflower Florets

Dried and powdered sunflower florets (100 g) were extracted by stirring overnight at 4°C in 500 mL of acetone. The extract was filtered and the residue extracted twice more in an identical manner. The three filtrates were combined and the acetone removed *in vacuo* at 35°C. Fatty acids were removed by saponification followed by clatharization with urea (12) as follows: The remaining residue after evaporation of the acetone was dissolved in 30 mL of MeOH and 5 ml each of toluene and H_2O. KOH (5.5 g) was added, and the mixture was refluxed for 4 h. After cooling, 100 mL of H_2O and about 2 mL of acetic acid were added and the mixture partitioned three times against equal volumes of ether. The combined ether fractions were dried and dissolved in a mixture of boiling MeOH (60 mL) and urea (20 g). The mixture was allowed to cool and the clathrated fatty acids removed by filtration. The solvent was evaporated from the filtrate, and the residue was dissolved in 10 mL of 90% (v/v) aqueous MeOH. Other nonpolar compounds were removed by passing

Table I. *Elution Profile of Mass and Radioactivity following Application of an Ethyl Acetate Extract from 50 g of Field Pennycress Shoots to a 50 × 6-cm Column of Biobeads S×8*

Fraction[a]	Total Extract Mass	Recovered ^{3}H-Kaurenoic Acid[b]	Recovered ^{14}C-Kaurene[c]
		%	
1–4	0	0	0
5	14	0	0
6	28	0	0
7	21	0	0
8	16	1	0
9	9	3	0
10–12	8	96	100

[a] Each fragment represents a 5 min collection time. [b] 250,000 dpm added to extract. [c] 50,000 dpm added to extract.

this solution through a 14.5 × 3.0 cm column of Preparative C_{18} (Waters Associates) and eluting with 425 mL of 90% (v/v) aqueous MeOH.

After the solvent was removed, the residue was dissolved in a few mL of EtOAc and applied to a 48 × 3 cm column of Biobeads SX-8 size exclusion gel. The column was eluted with EtOAc at a flow rate of 2 mL min^{-1}. Fractions were collected every min. A small aliquot from each fraction was taken and subjected to TLC on silica gel G using toluene:ethanol:ammonia (65:35:1, v/v) as the developing solvent system. Fractions containing KA were identified by comparing the chromatographic behavior with that of the authentic substance. Compounds were visualized by exposing developed plates to I_2 vapors for 5 to 10 min.

Those fractions with KA were combined and subjected to reverse phase HPLC using the same column and HPLC system as described earlier. Samples were injected onto the column in about 150 μL THF. The column was eluted with 90% (v/v) MeOH in H_2O (no acetic acid added) at 5 mL min^{-1}. The column effluent was split in two streams with 10% going to a UV monitor (Kratos Spectroflow 757) set at 208 nm and 2.0 absorbance units full scale. Both streams were recombined and collected. The retention time of KA (7.7 min) was determined with injection of an authentic sample. Trachylobanoic acid, a C/D ring isomer that was present in levels roughly half that of KA (1, 12) had a retention time of 8.3 min. Since there were significant amounts of KA in the peak of trachylobanoic acid, this fraction was collected and subjected to HPLC once more. Purity was assessed with TLC as described earlier and by GC-MS.

GC-MS

Derivatized samples were subjected to capillary GC using a Hewlett-Packard 5890 instrument containing a 30-m × 0.32-mm i.d. fused silica column with a methyl silicone stationary phase (Hewlett-Packard). Samples (1 μL) were introduced onto the column via a cool-on-column injector. The column temperature was 150°C at injection, and after a 1-min isothermal hold, the temperature was increased to 300°C at 7°C min^{-1}. The column head pressure of the carrier gas (He) was maintained at 35 kPa. The column was coupled directly to the ion source of a Finnegan Ion Trap mass spectrometer. Positive ions were generated with a stream of electrons at 80 μA. Full scan mass spectra (100–600 amu) were recorded at a rate of 2 scans s^{-1}.

Reference Compounds

K, KA, and ^{3}H-KA were gifts from Abbot Laboratories, Dr. Ghisalberti—University of Western Australia, and Dr. T. Gianfagna—Rutgers University, respectively. Large scale incubations of 2-^{14}C-mevalonic acid with pumpkin endosperm preparations (4, 8) were used to generate ^{14}C-K, ^{14}C-GA_{12}, ^{14}C-GA_{12}-aldehyde, and ^{14}C-7β-OH-KA. Kol was synthesized from methyl kaurenoate by refluxing the latter with lithium aluminum hydride in tetrahydrofuran for 2 h (12). Culture filtrates of the fungus *Gibberella fujikuroi* strain LM 45-399 were used as a source for numerous diterpenes metabolically related to GAs (3). This strain was a gift from Dr. J. A. D. Zeevaart, Michigan State University, and was originally obtained from Dr. B. O. Phinney, UCLA. Analogs of these compounds containing a C-13 hydroxyl group were obtained by feeding steviol to cultures of *G. fujikuroi* cultured in a medium with 2-chlorocholine chloride to inhibit endogenous diterpene synthesis (2, 7). Steviol was obtained by treating aqueous extracts of dried leaves of *Stevia rebaudiana* (Sigma) with a pectinase (Dr. T. Gianfagna, personal communication).

RESULTS

Identification of Endogenous Kauranoids in Field Pennycress Shoots

Subjecting an EtOAc extract of field pennycress shoots (50 g) to SEC resulted in the effective separation of the bulk of the mass, including the Chl, from the fractions containing ^{14}C-K and ^{3}H-KA (Table I). Chl was first detected in the column effluent at the end of fraction 4. The green color of the effluent was visually most intense in fraction 6 which also contained the greatest mass. The intensity of the green color declined in fraction 7 and was replaced by a dark brown color in fraction 8. However, when SEC fractions containing almost all of the radioactive K and KA (fractions 10–12) were subjected to reverse phase HPLC, small amounts of Chl were observed in the HPLC fraction containing ^{14}C-K. Therefore, this fraction was purified further with size exclusion HPLC. This resulted in the total separation of the two compounds.

The overall recoveries for the two radioactive compounds following all chromatographic procedures were good: 75 to 85% for ^{3}H-KA and 45 to 55% for ^{14}C-K. Moreover, most of the observed losses occurred during the manipulations that followed SEC; the recoveries of radioactivity immediately following SEC exceeded 90% for both compounds.

Individual reverse phase HPLC fractions were derivatized and analyzed by GC-MS. Each fraction was examined by GC-MS for specific kauranoids or GAs based on the chromatographic behavior of reference compounds (Fig. 1). Five kauranoid compounds were identified in extracts of field pennycress shoots based on comparisons of HPLC and GC retention times, and mass spectra between the endogenous substances and authentic compounds: K, Kol, KA and its C/D ring isomer, trachylobanoic acid, and 7β,13-dihydroxykaurenolide

(Table II). In addition, many of the GAs previously identified in field pennycress shoots (9) were also detected in the extracts initially fractionated by SEC (data not shown). However, several kauranoids that might also be expected to be present because of metabolic considerations (3, 6) were absent. These include Kal, 7β-OH KA, kauradienoic acid, 7β-hydroxykaurenolide, and iso-K. Moreover, GA_{12}-aldehyde, the first compound in the GA biosynthetic pathway with the *ent*-gibberellane skeleton (3, 6) was also not detected. No polyhydroxylated kaurenoic acid derivatives were detected either, including C-13 hydroxylated compounds such as steviol (13-hydroxykaurenoic acid), nor was there any evidence for the presence of seco B ring compounds in which ring B of the *ent*-kaurane skeleton is opened via oxidative cleavage.

Because many of these compounds have significantly higher mol wt than either K or KA, it is possible that they eluted earlier on SEC than the two reference kauranoids. Therefore, SEC fractions 8 and 9, which separated the bulk of the Chl from most of the radioactivity, (Table I) were combined, subjected to reverse phase HPLC, and the resulting HPLC fractions analyzed by GC-MS. No kauranoids or GAs were detected in these fractions.

Purification of Kaurenoic Acid from Sunflower Florets

In our work on thermoinductive regulation of GA metabolism in field pennycress, we found it necessary to have access to relatively large amounts of KA to assess its biological activity, prepare labeled forms for metabolism studies and internal standards, and for the synthesis of other GA precursors such as Kol and Kal. Sunflower florets are reportedly a rich source of KA (1, 12). This proved to be the case in our initial attempts to purify KA from this source. However, despite following the procedures described in the literature (1, 12), we were unable to separate KA from an unidentified yellow oil. Additional purification procedures such as reverse phase HPLC, SiO_2 partition chromatography, and recrystallization did little to increase its purity. Therefore, a chromatographic technique was needed that utilized some different chemical or physical property to effect separation. Although the identity of the contaminating substance(s) was not known, it apparently had a rather high boiling point (as indicated by its behavior on GC), suggesting a higher mol wt than KA. This mol wt difference was exploited by applying SEC to a partially purified sunflower extract.

Figure 2 shows that when SEC was omitted from the purification process, the KA-containing fraction resulting from reverse phase HPLC contained several contaminating substances as judged by TLC. These compounds were totally eliminated when SEC was employed. Moreover, the yellow color was also absent. Analysis of SEC-purified sunflower extract by GC-MS revealed that it contained only one major component that had a mass spectrum identical to authentic KA (Fig. 3). Only a trace of trachylobanoic acid was detected in the purified KA preparation; this compound was recovered in a later eluting isocratic reverse phase HPLC fraction.

Typically, around 400 mg of highly purified KA were obtained from 100 g of dried sunflower florets (Table III). This table also shows the reduction in the mass of the extract following each step in the purification procedure. Although mass reduction resulting from SEC was relatively small when compared to the other purification steps, the contaminating substances removed by SEC could not be separated from KA by the other procedures. Trachylobanoic acid comprised a large portion of the mass removed by isocratic reverse phase HPLC, the final step in the purification process.

DISCUSSION

Using SEC and reverse phase HPLC, five kauranoids were identified in extracts of field pennycress shoots: K, Kol, KA, trachylobanoic acid, and 7β,-13-dihydroxykaurenolide (Table II). To date, there are few reports in the literature dealing with the qualitative and quantitative analysis of the kauranoid precursors of GAs in vegetative (chlorophyllous) tissues. This is most certainly due to the lipophillic nature of both Chl and the kauranoids, precluding easy separation. In addition to good resolution, the high concentrations of Chl in vegetative tissues demand that the separation technique must also have a great sample loading capacity. To isolate and quantitate K in vegetative rice shoots, Moore *et al.* (11) used a series of partition steps followed by adsorption chromatography on silica and reverse phase HPLC. In this case, Chl was separated from K during the silica chromatography step. However, this procedure is not suitable in situations when other kauranoids such as KA are also of interest. In contrast, our results demonstrate that SEC is capable of separating a significant number of kauranoids as a class of compounds from Chl. The SEC technique also satisfies the requirements for resolution

Table II. *Mass Spectral Data for Authentic Kauranoids and Endogenous Compounds Isolated from Field Pennycress Shoots*

Sample	HPLC Fraction	Principal Ions and Relative Abundance
		% base peak
Authentic kaurene	30	272 (M^+, 54), 257 (100), 229 (48), 213 (79), 105 (91)
Field pennycress compound	30	272 (M^+, 52), 257 (88), 229 (40), 213 (72), 105 (100)
Authentic TMS-kaurenol	28	360 (M^+, 9), 270 (76), 257 (100), 255 (80), 227 (71)
Field pennycress compound	28	360 (M^+, 10), 270 (72), 257 (100), 255 (82), 227 (69)
Authentic Mekaurenoic acid	27	316 (M^+, 57), 301 (29), 273 (41), 257 (100), 241 (52)
Field pennycress compound	27	316 (M^+, 64), 301 (23), 273 (42), 257 (100), 241 (31)
Authentic Metrachylobanoic acid	27	316 (M^+, 79), 301 (28), 260 (100), 257 (58), 241 (21)
Field pennycress compound	27	316 (M^+, 81), 301 (14), 260 (100), 257 (64), 241 (47)
Authentic TMS-7β,13-dihydroxykauenolide	17	476 (M^+, 100), 267 (17), 208 (12), 193 (12), 109 (27)
Field pennycress compound	17	476 (M^+, 100), 267 (15), 208 (18), 193 (12), 109 (22)

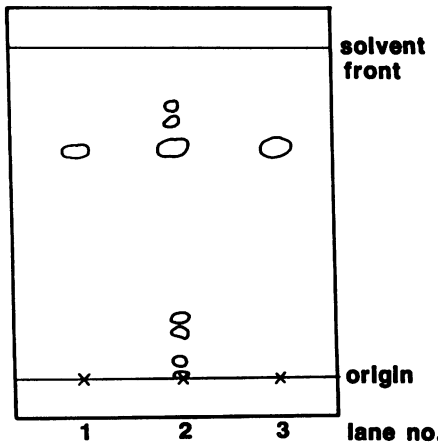

Figure 2. Thin layer chromatogram of sunflower extracts purified with or without size exclusion chromatography. Thin layer plates of silica gel G (0.25 mm thickness) were spotted with solutions of authentic kaurenoic acid (lane 1), sunflower extract purified without SEC (lane 2), and sunflower extract purified with SEC (lane 3). Chromatograms were run to 12 cm in toluene:ethanol:ammonia (65:35:1, v/v). The plates were allowed to dry, and compounds detected by exposing the plate to I_2 vapor for 10 min.

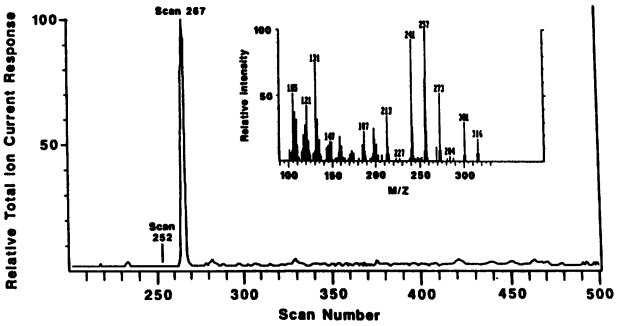

Figure 3. Total ion current trace following injection of 160 ng of a highly purified methylated extract from sunflower florets. GC and MS conditions are described in "Materials and Methods." Inset, Mass spectrum at scan 267. Arrow indicates the retention time (scan 252) of methyl trachylobanoic acid.

Table III. *Reduction in Mass Resulting from Each Procedure Used in the Purification of KA from 100 g of Dried Sunflower Florets*

Procedure	Mass of Dried Fraction	Mass Reduction from Previous Step
	g	%
Start	100.00	
Acetone extract	11.31	88.7
Saponification and clatharization	2.48	78.1
Preparative reverse phase chromatography	1.10	55.6
SEC	0.96	12.7
Isocratic reverse phase HPLC	0.44	54.3

and large sample loading capacity. In addition, the technique is relatively fast and losses are small.

SEC is also useful in preparative scale separations. The purification of about 400 mg of KA from 100 g of sunflower florets (Table III) clearly shows the utility and versatility of the technique to solve a variety of chromatographic problems. Thus, use of SEC may be of considerable value in the large scale isolation of other small mol wt natural products from plant sources.

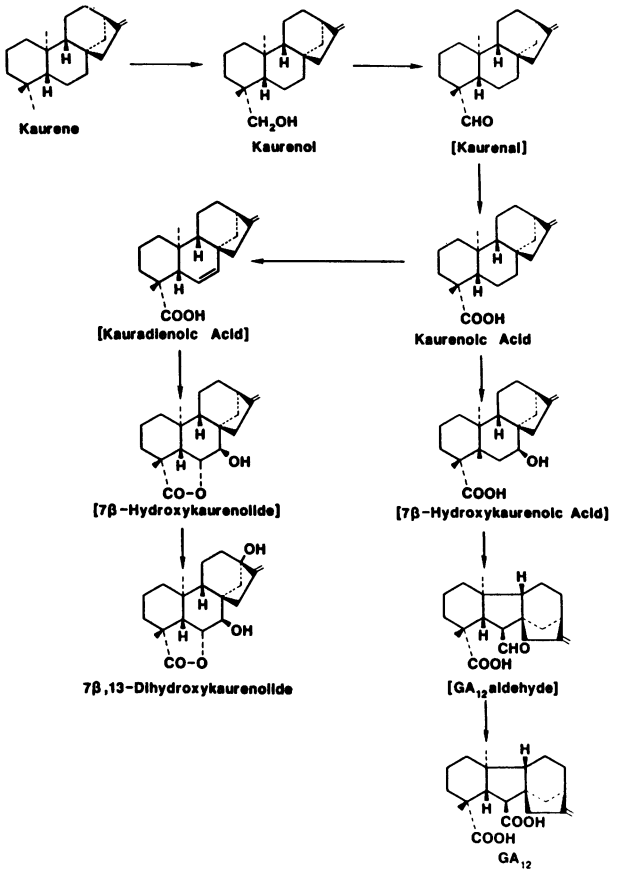

Figure 4. Possible metabolic relationships between the kauranoids identified in field pennycress shoots. Compounds enclosed in brackets were not detected but probably intermediate (3, 6).

The probable metabolic relationships between the five kauranoids identified in extracts of field pennycress shoots and the GAs previously identified in this species (9) are shown in Figure 4. It is interesting that three of the intermediates between K and GA_{12} were not detected in extracts from 300 g of shoot material: Kal, 7β-hydroxykaurenoic acid, and GA_{12}-aldehyde. The reasons for this are not entirely clear, but several possibilities exist. It may be that considerable separation of kauranoids occurred during SEC because of mol wt differences so that compounds with higher mol wt eluted in fractions earlier than those containing the radioactive standards. However, this does not appear very likely for the column used in the present work. First, when the two fractions collected just prior to those containing K and KA were analyzed by GC-MS, no kauranoids were detected. Second, 7β,13 dihydroxykaurenolide (mol wt 332) eluted in the same fractions as K (mol wt 272) and KA (mol wt 302) (Table I). Compounds with intermediate mol wt and similar three-dimensional configurations such as 7β-OH KA would therefore not be expected to separate from these three compounds.

Another possibility is that the extraction and purification procedures cause chemical changes resulting in losses of the compounds to levels below detectable limits. This would appear more likely for compounds with reactive functional groups such as aldehydes, *viz*, Kal and GA_{12}-aldehyde. However, inclusion of butylated hydroxytoluene as an antioxidant to the extraction solvent did not result in detection of these two compounds (data not shown). It is also possible that turnover of these compounds is so great that they do not accumulate to significant levels.

The presence of side branches from the main GA biosynthetic pathway that lead to the kaurenolides and polyhydroxylated KAs (Fig. 4) are also of interest since the points of divergence may represent regulatory steps in GA biosynthesis. The presence of a competing, alternate route for the metabolism of a precursor could serve as a mechanism for regulating the synthesis of GAs. In field pennycress, at least two metabolic pathways diverge from KA: one to 7β-13-dihydroxykaurenolide and the other to GAs. Thus, the conversion of kaurenoic acid to 7β-OH KA may be a controlling reaction in GA biosynthesis. Consistent with this is our recent observation that the conversion of kaurenoic acid to GAs is under thermoinductive control in field pennycress shoot tips (5). Future work will be directed toward examining in more detail this possible aspect of regulation in GA biosynthesis.

LITERATURE CITED

1. **Beale MH, Bearder JR, MacMillan J, Matsuo A, Phinney BO** (1983) Diterpene acids from *Helianthus* species and their microbiological conversion by *Gibberella fujikuroi*, mutant B1-41a. Phytochemistry **22:** 875–881
2. **Bearder JR, MacMillan J, Wels CM, Phinney BO** (1975) The metabolism of steviol to 13-hydroxylated *ent*-gibberellanes and *ent*-kauranes. Phytochemistry **14:** 1741–1748
3. **Bearder JR** (1983) *In vivo* diterpene biosynthesis in *Gibberella fujikuroi*: The pathway after *ent*-kaurene. *In* A Crozier, ed, The Biochemistry and Physiology of Gibberellins, Vol 1. Praeger Publishers, New York, pp 251–388
4. **Birnberg PR, Maki SL, Brenner ML, Davis GC, Carnes MG** (1986) An improved enzymatic synthesis of labeled gibberellin A_{12}-aldehyde and gibberellin A_{12}. Anal Biochem **153:** 1–8
5. **Hazebroek JP, Metzger JD** (1988) Comparison of metabolism of exogenous ^{2}H-kaurenoic acid in induced and noninduced *Thlaspi arvense* L. (abstract No. 221) The 13th International Conference on Plant Growth Substances, Calgary, Canada
6. **Hedden P** (1983) *In vitro* metabolism of gibberellins. *In* A Crozier, ed, The Biochemistry and Physiology of Gibberellins, Vol 1. Praeger Publishers, New York, pp 99–150
7. **Gianfagna T, Zeevaart JAD, Lusk WJ** (1983) Synthesis of [^{2}H] gibberellins from steviol using the fungus *Gibberella fujikuroi*. Phytochemistry **22:** 427–430
8. **Graebe JE, Bowen H, MacMillan J** (1972) Biosynthesis of gibberellins A_{12}, A_{15}, A_{24}, A_{36}, and A_{37} by a cell-free system from *Cucurbita maxima*. Phytochemistry **13:** 1433–1440
9. **Metzger JD, Mardaus MC** (1986) Identification of endogenous gibberellins in the winter annual weed *Thlaspi arvense* L. Plant Physiol **80:** 396–402
10. **Metzger JD, Zeevaart JAD** (1980) Identification of six endogenous gibberellins in spinach shoots. Plant Physiol **65:** 623–626
11. **Moore TC, Yamane H, Murofushi N, Takahashi N** (1988) Concentrations of *ent*-kaurene and squalene in vegetative rice shoots. J Plant Growth Regul **7:** 145–151
12. **Pyrek JST** (1970) New pentacyclic diterpene acid, trachyloban-19-oic acid from sunflower. Tetrahedron **26:** 5029–5032
13. **Reeve DR, Crozier A** (1976) Purification of plant hormone extracts by gel permeation chromatography. Phytochemistry **15:** 791–793