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Differential accumulation of isoprenoids in glanded and glandless *Artemisia annua* L

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Abstract

The relative abundance of steam-distillable isoprenoids and other volatile compounds in leaves of glanded and glandless *Artemisia annua* L. was investigated. Steam distillation of leaves bearing glandular trichomes yielded 0.24% essential oil on a fresh weight basis. Monoterpenes were predominant in this essential oil, of which α-pinene (26.7%), pinocarvone (15.8%), and artemisia ketone (11.0%) were the major constituents. The essential oil distilled from glandless leaves amounted to 0.06% of fresh weight, and consisted mostly of sesquiterpenes, of which germacrene-D (49.8%) and β-caryophyllene (25.1%) were the major components. Only one monoterpene, β-ocimene, present in trace amounts, was detected in the oil distilled from the glandless tissue. The sesquiterpene artemisinin, found only in the glanded biotype, is heat-labile and cannot be extracted by steam distillation, during which it decomposes into non-distillable products. Published by Elsevier Science Ltd.

Keywords: Artemisia annua; Asteraceae; Annual wormwood; Artemisinin; Glandular trichome; Isoprenoids

1. Introduction

Artemisia annua produces a group of medicinally useful sesquiterpene lactones. Foremost is artemisinin, which has anti-microbial activity against chloroquine-resistant strains of Plasmodium falciparum, the malaria parasite (Loevinsohn, 1994; Klayman, 1985). Interest in the production of artemisinin and related compounds for use as an alternative treatment of malaria continues to increase, as the resistance of the parasite to traditionally used drugs becomes more widespread. Production of artemisinin rarely exceeds 0.8% of dry weight (Delabays, Jenelten, Paris, Pivot & Galland, 1994). In addition to artemisinin and related sesquiterpenes, *A. annua* yields an aromatic essential oil that is rich in monoterpenes (Ahmad & Misra, 1994).

Specialized plastids present in the apical and subapical cells of capitate glandular trichomes of *A. annua* have been proposed as the site of artemisinin synthesis (Duke & Paul, 1993). Artemisinin accumulates in the subcuticular space of capitate glandular trichomes that are abundantly present on the surfaces of leaves and flower organs (Duke, Paul, Elsohly, Sturtz & Duke, 1994). This compound, as well as the characteristic aroma of glanded plants, is absent in the foliar tissues of plants of a biotype of *A. annua* lacking glandular trichomes. The glandless biotype of *A. annua* arose spontaneously among field- cultivated plants, and is being used as a research tool to study the biosynthesis of artemisinin and other isoprenoids. The anatomy of the glandless biotype is virtually indistinguishable from its glanded counterpart except for the absence of peltate, secretory glands. No ultrastructural differences were found in the mesophyll cells of the two biotypes (Duke & Paul, 1993). As part of our efforts to characterize the glandless biotype, we have analyzed the composition of its essential oil. In this report, we present evidence that the monoterpenes of *A. annua* accumulate almost exclusively in the glandular trichomes.

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Table 1
Constituents of the essential oils of a biotype of *Artemisia annua* with glandular trichomes and of a biotype without glandular trichomes

RI[a]	RT[b]	Compound		Glanded[c]	Glandless[d]
				R.A.[e]	R.A.
800	2.88	4-methyl-3-penten-2-one		2.7	0.5
833	3.33	furfural		t[f]	–[g]
840	3.43	4-hydroxy-4-methyl-2-pentanone		2.2	t
854	3.63	*E*-2-hexenal		0.2	0.4
856	3.67	*Z*-3-hexenol		0.9	t
867	3.87	*n*-hexanol		t	t
927	5.09	tricyclene	MH[h]	t	–
931	5.17	α-thujene	MH	t	–
939	5.36	α-pinene	MH	26.7	–
954	5.73	camphene	MH	0.6	–
959	5.86	thuja-2,4(10)-diene	MH	t	–
962	5.93	benzaldehyde		t	t
977	6.37	sabinene	MH	t	–
981	6.48	β-pinene	MH	1.6	–
992	6.84	dehydro-1,8-cineole	MO	0.2	–
999	7.05	yomogi alcohol	MO[i]	0.2	–
1005	7.27	*E*-3-hexenol acetate		1.4	0.34
1019	7.65	α-terpinene	MH	0.1	–
1027	7.89	*p*-cymene	MH	0.3	–
1034	8.03	benzyl alcohol		–	0.1
1034	8.12	1,8-cineole	MO	8.4	–
1038	8.25	santolina alcohol	MO	t	–
1044	8.44	benzene acetaldehyde		t	0.2
1051	8.68	$E-\beta$-ocimene	MH	t	t
1061	9.04	γ-terpinene	MH	0.1	–
1063	9.12	artemisia ketone	MO	11.0	–
1066	9.23	acetophenone		t	t
1069	9.34	*cis*-sabinene hydrate	MO	0.2	–
1072	9.43	*n*-octanol		–	t
1084	9.93	artemisia alcohol	MO	0.4	–
1088	10.07	*o*-guaiacol		t	–
1090	10.14	terpinolene	MH	t	–
1097	10.47	*trans*-sabinene hydrate	MO	0.2	–
1103	10.69	*n*-nonanal		t	0.3
1119	11.24	isophorone		0.7	t
1127	11.53	α-campholenal	MO	0.5	–
1137	11.92	nopinone		t	–
1141	12.06	*trans*-pinocarveol	MO	9.0	–
1161	12.88	*trans*-pinocamphone	MO	t	–
1163	12.98	pinocarvone	MO	15.8	–
1166	13.13	borneol	MO	0.5	–
1177	13.60	terpin-4-ol	MO	0.6	–
1181	13.79	*p*-methylacetophenone		t	–
1183	13.88	*p*-cymen-8-ol	MO	t	–
1189	14.13	α-terpineol	MO	0.1	–
1191	14.24	methyl salicylate		t	–
1193	14.33	myrtenal	MO	0.7	–
1194	14.38	myrtenol	MO	0.4	–
1205	14.84	verbenone	MO	t	–
1217	15.30	*trans*-carveol	MO	0.1	–
1230	15.79	*cis*-carveol	MO	t	–
1243	16.30	carvone	MO	t	–
1259	16.98	*trans*-myrtanol	MO	t	–
1288	18.26	indole		t	0.1
1292	18.45	tridecene		0.2	–
1297	18.72	*trans*-pinocarvyl acetate		t	–
1299	18.78	carvacrol	MO	t	–
1351	20.84	α-cubebene	SH[j]	t	0.1

Table 1 (*continued*)

RI[a]	RT[b]	Compound		Glanded[c]	Glandless[d]
				R.A.[e]	R.A.
1356	21.06	eugenol		t	–
1376	21.92	α-copaene	SH	0.4	1.9
1389	22.50	β-cubebene	SH	t	t
1391	22.60	β-elemene	SH	t	t
1394	22.74	*Z*-jasmone		t	–
1399	22.96	*n*-tetradecane		t	t
1410	23.35	α-cedrene	SH	t	t
1418	23.67	β-caryophyllene	SH	2.6	25.1
1428	24.04	coumarin		0.3	t
1453	25.04	α-humulene	SH	0.1	1.3
1458	25.24	*E* − β-farnesene	SH	0.7	5.4
1473	25.87	β-cadinene	SH	–	0.3
1476	25.99	γ-muurolene	SH	t	t
1480	26.15	germacrene-D	SH	6.1	49.8
1484	26.32	*E* − β-ionone		t	–
1485	26.34	β-selinene	SH	t	–
1494	26.76	bicyclogermacrene	SH	0.3	3.5
1498	26.92	α-muurolene	SH	t	0.4
1502	27.09	germacrene-A	SH	–	0.1
1508	27.27	*E*, *E* − α-farnesene	SH	0.1	0.1
1512	27.43	γ-cadinene	SH	t	0.3
1523	27.82	δ-cadinene	SH	t	1.2
1531	28.13	cadina-1,4-diene	SH	–	0.2
1531	28.15	*trans*-calamene	SH	t	–
1537	28.34	α-cadinene	SH	–	t
1563	29.37	*E*-nerolidol	SO[k]	t	t
1575	29.82	spathulenol	SO	–	t
1580	30.03	caryophyllene oxide	SO	–	0.3
1605	31.01	β-oplopenone	SO	–	t
1638	32.18	epi-α-cadinol	SO	–	t
1640	32.25	epi-α-muurolol	SO	–	0.3
1641	32.30	cubenol	SO	–	t
1644	32.39	α-muurolol	SO	–	t
1652	32.69	α-cadinol	SO	–	0.3

[a] RI = retention index as determined on DB-5 using the homologous series of *n*-hydrocarbons.
[b] RT = retention time in minutes.
[c] glanded refers to a biotype of *A. annua* possesing glandular trichomes.
[d] glandless refers to a biotype of *A. annua* with no glandular trichomes.
[e] R.A. = relative area (peak area relative to total peak area).
[f] t = trace (< 0.01%).
[g] – = not found.
[h] MH = monoterpene hydrocarbon.
[i] MO = mono-oxygenated monoterpene.
[j] SH = sesquiterpene hydrocarbon.
[k] SO = mono-oxygenated sesquiterpene.

2. Results and discussion

Steam distillation of 12.7 and 20.0 g of leaves from 3 glanded and 3 glandless plants, respectively, produced clear, colorless essential oils. The glandless leaves yielded 10.7 mg (0.06% of fresh weight), while the glanded material yielded 28.1 mg (0.24% of fresh weight). Gas-chromatographic analysis of the composition of these essential oils revealed very contrasting profiles of isoprenoid content (Table 1). Seventy-eight compounds were identified in the oil of the glanded biotype and 46 in the oil of the glandless biotype, accounting for over 96% and 92% of the composition of each oil, respectively. Forty-seven compounds identified in the oil of the glanded biotype oil were not found in that of glandless biotype. Noteworthy among these compounds are a group of monoterpenes that make up over 75% of the glanded biotype oil, and were absent, with the exception of trace amounts of β-ocimeme, in the glandless biotype oil. Conversely, 15 compounds were identified only in the oil distilled from the glandless leaves, including trace amounts of

benzyl alcohol and *n*-octanol, as well as 12 sesquiterpenes present in less than 0.4% yield each (for a total of less than 3.5%).

The essential oil of the biotype of *A. annua* with glandular trichomes was characterized by the presence of large amounts of α-pinene (26.7%), pinocarvone (15.8%), artemisia ketone (11.1%), and 1,8-cineole (8.4%). The oil components included 11 monoterpene hydrocarbons (29.4%), 24 mono-oxygenated monoterpenes (48.2%), 17 sesquiterpene hydrocarbons (10.2%), and trace amounts of a mono-oxygenated sesquiterpene.

The essential oil of the biotype of *A. annua* without glandular trichomes was characterized by the presence of large amounts of germacrene-D (49.8%) and *β*-caryophyllene (25.1%). The oil components included 19 sesquiterpene hydrocarbons (89.7%), 10 mono-oxygenated sesquiterpenes (1.0%), and traces of a monoterpene hydrocarbon.

Direct correlation between equivalent peaks between the two biotypes cannot be made because percentages given are in terms of peak area relative to total area. Quantitation using a calibration curve obtained with the pure compound as a standard showed that glandless and glanded leaves contain 59 and 25 μg of *β*-caryophyllene per g of fresh weight, respectively. We roughly estimated total sesquiterpene content by correlating peak area with essential oil content in fresh plant material. In this way, glandless leaves were estimated to contain more than twice as much total sesquiterpenes per unit of fresh weight as the glanded leaves. Using this method, *β*-caryophyllene was estimated to be 2.9-fold more abundant in the glandless leaves, which is fairly close to the 2.4-fold determined by the use of the calibration curve. The relative abundance of the major sesquiterpene components is similar in the two types of leaves. Germacrene-D and *β*-caryophyllene account for approximately half and a quarter of the total sesquiterpene content, respectively in both oils; *E*-β-farnesene amounts to 5–6%.

The virtual absence of monoterpenes in the steam distilled oil of the glandless biotype is a good indication that the monoterpenes accumulate exclusively in the glandular trichomes of *A. annua*, as occurs with artemisinin (Duke & Paul, 1993; Duke et al., 1994). To corroborate this finding, we proceeded to perform chloroform extractions of leaves bearing glandular trichomes, prior to steam distillation. Duke et al. (1994) established that immersion in chloroform causes the collapse of the subcuticular space of the glands without damaging the rest of the foliar tissues. Therefore, we expected that a short exposure to chloroform would preferentially extract the monoterpenes from *A. annua*'s leaves. First, we extracted 20 g of whole leaves by swirling in 400 ml of chloroform for 5 s. The chloroform-extracted leaf material was then immediately steam distilled; a fraction was collected after 2 h, which contained residual chloroform, followed, after another 5 h, by collection of the pentane fraction. Most of the monoterpenes present in the tissue were extracted by the 5-s chloroform wash. Quantitation using a calibration curve obtained with pure 1,8-cineole as a standard showed that 96.5% of this compound was extracted by chloroform.

The observation that a small portion of its monoterpene content remained associated with the leaf material after the initial chloroform extraction prompted us to try a more exhaustive protocol. We hypothesized that these monoterpenes were carried over to the steam-distillation step by the residual chloroform and/or were trapped within folds of the collapsed cuticle. We carried out a second experiment consisting of four consecutive 5-s extractions of 10 g of leaves with 200 ml of chloroform, followed by steam distillation. We observed the presence of decreasing quantities of monoterpenes in the first three chloroform extracts, which contained 89.1, 9.1, and 1.4% of the total amount of 1,8-cineole, respectively. We could not detect the presence of monoterpenes in the fourth extract, while no appreciable amounts ($<0.2\%$) of the steam-distillable sesquiterpenes could be found in any of the extracts. Steam distillation of the leaf material subjected to multiple chloroform extractions yielded a 2 h distillate with a high ratio of sesquiterpenes to monoterpenes, and a 7 h distillate consisting almost exclusively of sesquiterpenes. The detection of small amounts of monoterpenes, including 0.4% of the total content of 1,8-cineole, in the leaf material after 4 rounds of chloroform extraction suggests that these compounds may be present within the secretory cells of the glandular trichomes.

Finally, we tested the hypothesis that the steam distillable volatile sesquiterpenes were unavailable for chloroform extraction because they were contained within structures that physically protected them from the solvent. For this purpose, we crushed the gland-bearing leaves under liquid nitrogen, prior to extraction with chloroform. Both the steam distillable mono- and sesquiterpenes were found in the chloroform extract of the crushed leaves. This observation, along with the detection of large amounts of steam distillable sesquiterpenes in the glandless leaves, indicates that the foliar tissues of *A. annua* accumulate steam distillable volatile sesquiterpenes in structures other than the glandular trichomes. No efforts were made to identify or quantify non-steam-distillable sesquiterpenes, although their presence was noted in this last extract, and as trace components in each of the 5-s extracts. Coumarin was also fairly ubiquitous among the extracts and the distillations.

Artemisinin was not found among the chromatographic profiles of the essential oils of either biotype.

This is not surprising since it has been reported that artemisinin undergoes decomposition under prolonged heat (Lin, Klayman & Hoch, 1985) and that it can be quantified by gas chromatography by its decomposition products (Sipahimalani, Fulzele & Heble, 1991). In order to establish whether any of the components in our essential oil profiles were derived from artemisinin, we investigated its fate under our steam distillation conditions. HPLC analysis established the absence of artemisinin in the distilled oil and in $CHCl_3$ extracts of the salted out (NaCl) aqueous residue or of the plant residue. GC–MS analysis of pure artemisinin using our conditions revealed three decomposition products at 47.18, 50.17, and 51.01 min (RI 2089, 2191, and 2221). These products, however, were absent from our profiles of the essential oils of *A. annua*. A sample of artemisinin (2.8 mg) was, therefore, steam distilled under the same conditions as the plant material with the exception that the distilling flask had a 250 ml capacity and was loaded with 100 ml of H_2O. After the distillation, the aqueous residue, a $CHCl_3$ extract of the aqueous residue, and the distillate were examined. Neither artemisinin nor by-products were observed by GC–MS analysis in the distillate or in the $CHCl_3$ extracted aqueous residue. The $CHCl_3$ extract of the aqueous residue, however, yielded 2.6 mg of the same mixture (by GC–MS) of decomposition products as that of pure artemisinin (subjected to GC–MS) except that the ratios of products were different, containing a much higher proportion of the earliest eluting compound. HPLC analysis of the $CHCl_3$ extract showed the presence of three main components and only trace amounts of artemisinin. These results indicate that artemisinin decomposes under the steam distillation conditions rather than upon analysis and that these decomposition products are not steam distilled.

Our findings constitute evidence that the subcuticular space of the glandular trichomes is the site of monoterpene storage in the foliar tissues of *A. annua*, and that steam distillable sesquiterpenes are not present in this subcuticular space, but elsewhere in the leaves. We have not directly addressed the question of where these groups of isoprenoids are synthesized, but it is well established that glandular trichomes do possess the biosynthetic machinery to produce secondary metabolites, including isoprenoids (Clark, Hamilton, Chapman, Rhodes & Hallahan, 1997; Guo, Severson & Wagner, 1994), and flavonoids (Schopker, Kneisel, Beehues, Robenek & Wiermann, 1995). Furthermore, glandular trichomes appear to be the only sites of synthesis of monoterpenes and secondary diterpenes in spearmint and tobacco, respectively (Gershenzon, Maffei & Croteau, 1989; Keene & Wagner, 1985; Gershenzon, McCaskill, Rajaonarivony, Mihaliak, Karp & Croteau, 1992).

The absence of trichomes and certain terpenoids could be due to different genetic factors. Indeed, genetic variations have been reported for plants that retain glands, but lack certain terpenes (Nielsen, Akers, Jarlfors, Wagner & Berger, 1991). However, in the only other case of which we are aware, in which, glanded and glandless variants of a species are compared, five genetically similar pairs of glanded and glandless cotton cultivars give similar results (Elzen, Williams, Bell, Stipanovic & Vinson, 1985) to what we have reported with *A. annua*. They found glandless cultivars to be devoid of many monoterpenes and some sesquiterpenes and triterpenes. Differential micropipette extraction confirmed that many of the compounds absent in the glandless varieties are found only in the glands of the glanded varieties. These findings, along with the results presented here and in our previous paper (Duke et al., 1994) suggest that the synthesis and storage of many of these compounds is often trichome localized, although further study is required to establish that the glands are indeed the site of biosynthesis of these compounds in *A. annua*. The implications of trichome localized accumulation of certain terpenes from a chemical ecology standpoint have been reviewed previously (Duke, 1994).

3. Experimental

Seeds of *Artemisia annua* were obtained from Aromagen (Albany, Oregon) and germinated on moist Miracle–Gro professional potting soil in a CU-32LX growth chamber (Percival Scientific, Boone, Iowa) under a 14 h light regime and constant temperature of 24°. Four-week-old seedlings were individually transplanted to 1.75 l pots and allowed to develop under the conditions described above. Plants were watered twice a week; fertilizer was applied once a week. Leaves were collected from four-month-old plants by cutting them off at the proximal end of their pedicels; extreme care was taken to avoid touching the gland-bearing surfaces to keep the glands intact. When necessary, crushing of *A. annua* leaves was performed in liquid nitrogen using mortar and pestle; the frozen ground material was transferred to a 150 ml beaker and allowed to warm to room temperature before addition of chloroform. Whenever leaves were harvested from more than one plant of a given biotype, a single composite sample was prepared.

Steam distillation was conducted in a Nickerson–Likens type apparatus (Nickerson & Likens, 1966; Maarse & Kepner, 1970). Freshly cut leaves were placed in a 2 l round bottom flask along with 1 l of H_2O. The distillate was continuously extracted during a 7 h distillation with 10 ml of pentane (b.p. 35–36°) into a 10 ml pear shaped flask heated with a water bath maintained at ca 65°. The two pentane fractions

along with two pentane rinses of the apparatus, were combined and dried over anhydrous $MgSO_4$ overnight and filtered. Analyses were performed directly on these two solutions (before addition of standards) to determine if naturally occurring plant components coeluted with the octane and eicosane standards. Octane and eicosane standards were used only for determination of retention times and not for quantitation purposes: to a 180 μl aliquot each, was added 10 μl of octane in pentane (1 mg/ml) and 10 μl of eicosane in pentane (1 mg/ml) at a final concentration of 50 μg/ml of each alkane before injection. The solvent was removed from each of the remaining filtrates under reduced pressure at 1° using a rotary evaporator.

Extraction with $CHCl_3$ was always carried out using a ratio of 100 ml of $CHCl_3$ per 5 g of leaf material. Concomitant extraction of glandular and non-glandular parts of the leaves of glanded *A. annua* was performed by crushing 5.0 g of leaf material under liquid N_2 with a mortar and pestle, warming to room temperature and extracting with $CHCl_3$. Exclusive extraction of the components of the glandular trichomes from whole leaves of glanded *A. annua* was performed by swirling the leaf material in $CHCl_3$ for 5 s either once or four times, decanting off the $CHCl_3$ into round bottom flasks using a funnel fitted with a cotton plug and removing the solvent under reduced pressure. The remaining plant material was then immediately steam distilled as above, except that the continuous extraction zone was drained after 2 h to remove the distilled $CHCl_3$ phase and additional pentane added to make up the necessary organic solvent phase.

Terpene analyses were performed by GC–MSD (EI, 70 eV) with a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness 0.25 μm) using He as carrier gas (1 ml/min), 1 μl injection size and a programmed (injector temp.: 220°, transfer line temp.: 240°, initial column temp.: 60°, final column temp.: 240°, 3°/min) temp. run (Adams, 1995). Identifications of oil components were performed by a comparison of mass spectra with literature data (Adams, 1995; NIST/EPA/NIH, 1990) and by a comparison of their relative retention times with those of authentic compounds, or by comparison of their retention indices with those in the literature (Adams, 1995). The relative amounts (RA) of individual components of the oil are expressed as percent peak area relative to total peak area.

Quantitation of artemisinin followed the procedure of Zhao and Zeng (1985) with minor modifications. Dried extracts were dissolved in MeOH to a concentration of 10 mg/ml. A 50 μl aliquot was taken and 420 μl of 0.2% NaOH was added. The mixture was placed in a heating block at 50° for 30 min, then 30 μl of 20% methanolic HOAc was added. Analyses were performed by HPLC with the following conditions: column was a C-18 Waters μBondapak™ 3.9 × 300 mm; mobile phase was 0.01 M NaH_2PO_4 buffer (pH 7.00): MeOH eluted in gradient from 59:41 to 55:45 over a period of 25 min; flow rate was 1 ml/min; injection volume was 100 μl. The retention time of artemisinin was 9.7 min. Detection was at λ260 nm.

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