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Enantioselective monoterpene alcohol acetylation in *Origanum*, *Mentha* and *Salvia* species

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ABSTRACT

Selected plants within the *Origanum*, *Mentha* and *Salvia* genera, that contain significant amounts of chiral volatile alcohols and their related acetates, exhibit remarkable enantioselectivity of alcohol acetyl transferase (AAT) activity and particularly can discriminate between linalool enantiomers. *Origanum dayi* AAT produced almost enantiomerically pure (*R*)-linalyl acetate by enzymatic acetylation of racemic linalool, whereas the closely related *O. majorana* AAT produced a mixture of (*R*)- and (*S*)-linalyl acetate with a ratio of 6:4. V_{max} of *O. dayi* acetylation activity was 30-fold higher for (*R*)-linalool, whereas in *O. majorana* no such differences were found.

1. Introduction

Esters are important constituents of herbs, flowers and fruits imparting unique aromas and constituting key compounds of essential oils. The enzymatic formation of volatile esters is well documented in the literature. These reactions are catalyzed by alcohol acetyl transferases (AAT), which are able to transfer an acyl moiety from a CoA thioester to an alcohol acceptor. Many genes encoding AATs have been isolated from fruits such as apple (Souleyre et al., 2005), melon (Yahyaoui et al., 2002), strawberry (Aharoni et al., 2000) and flowers such as rose (Shalit et al., 2003) and *Clarkia breweri* (Dudareva et al., 1998). AAT enzymatic activity has been studied in cell-free extracts or after expression of AAT in yeast or bacteria. Generally AATs can use a broad spectrum of alcohols as substrates including straight and branched chain aliphatic and aromatic alcohols. These enzyme activities cannot efficiently accept linalool or other tertiary monoterpene alcohols as substrates. Recently, an enzyme activity able to esterify linalool has been evaluated in *Mentha citrata*, a plant that produces copious amounts of linalyl acetate (Zaks, personal communication). However, the stereoselectivity of this enzyme has not been evaluated.

In the present study we report on the enzymatic and stereoselective formation of linalyl acetate in eight selected species of the *Lamiaceae* family, including six *Origanum* species, *M. citrata* and *Salvia dominica*. We have also evaluated the formation of additional tertiary acetates such as *cis*- and *trans*-sabinene hydrate acetates, α-terpinyl acetate and terpinen-4-yl acetate that are known constituents of these species. Since the substrate alcohols have asymmetric carbon atoms, the possibility of stereoselective acetate formation was investigated.

2. Results and discussion

2.1. Enantiomeric distribution of chiral monoterpene alcohols and acetates

The enantiomeric distribution of the chiral monoterpene alcohols (Fig. 1) and their corresponding acetates in *Origanum*, *Salvia* and *Mentha* species are listed in Table 1. The results indicate different patterns of stereoselective acetate accumulation according to the species analyzed. The first pattern of acetate accumulation followed an enantioselective pattern, in which both alcohol enantiomers were present, but only one acetate enantiomer was accumulated. This type of enantioselective pattern was observed for linalyl acetate in *O. dayi* and in *O. syriacum*; for *trans*-sabinene hydrate acetate in *O. dayi*, *O. vulgare* and *O. onites* and for *cis*-sabinene hydrate acetate in *O. dayi* (Table 1). In the second pattern of enantiomeric distribution both enantiomers of the alcohols and both of their corresponding acetates were found. This pattern was observed for linalyl acetate in *O. vulgare* and in *O. majorana*, and for terpinen-4-yl acetate in *O. dayi* and in *O. vulgare*. A third

Abbreviations: AAT, alcohol acetyl transferase; ee, enantiomeric excess; HS-SPME, head space-solid phase micro extraction.

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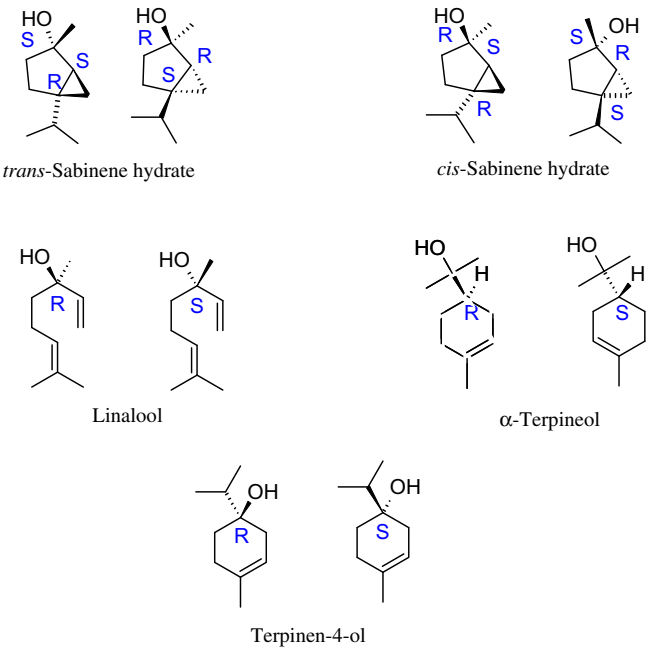

Fig. 1. Tertiary monoterpene alcohol structures.

pattern of enantiomeric distribution was found in the majority of herbs in which only one alcohol enantiomer was present and only one corresponding acetate (if any) was accumulated (Table 1).

Indications of possible enantioselective acetate formation in *O. dayi* were obtained from analyses of the enantiomeric composition of 58 accessions of ten populations originating in the Judean Desert. The chiral GC–MS analyses of such accessions, which grew under natural conditions with different soil characteristics, allowed us to conduct correlation analyses of enantiomeric excesses of alcohols vs. enantiomeric excesses of their corresponding acetates. The same pattern 1 of enantioselective acetate formation observed before (Table 1) was observed for linalyl acetate and *trans*-sabinene hydrate acetate in the wild 58 accessions (Figs. 2A and B). *cis*-Sabinene hydrate acetylation was stereoselective in 21 plant accessions that were characterized by considerably higher total amounts of formed acetate compared with the other 37 plant accessions which contained negligible amounts of this acetate and did not show the same enantioselectivity (Fig. 2C). Substrate-correlated acetate formation (Pattern 2) was observed for terpinen-4-yl acetate (Fig. 2D).

2.2. Enzymatic acetylation activity of soluble cell-free extracts

2.2.1. Radioactive AcCoA as substrate

In order to clarify whether enzymatic acetylation activity is responsible of acetate accumulation, we conducted enzymatic acetylation reactions with [^{14}C]AcCoA as an acetyl donor utilizing desalted soluble cell-free extracts from the species of interest with several alcohol substrates. The aliphatic alcohol hexanol was acetylated to hexyl acetate by all of the soluble cell-free extracts (Fig. 3). Linalyl acetate was formed only by soluble cell-free extracts from *O. dayi*, *O. syriacum*, *O. majorana*, *O. vulgare*, *S. dominica* and *M. citrata*, which accumulated significant amounts of linalyl acetate in their essential oils. *O. ramonense*, which did not accumulate linalyl acetate and *O. dayi* (clone No.2), which contained only trace amounts of linalyl acetate (data not shown), showed negligible acetylation activity when (*RS*)-linalool was offered as a substrate. Interestingly, only *S. dominica*, a plant that accumulates significant amounts of α-terpinyl acetate in its essential oil, showed α-terpineol acetylation activity. The plants that lack α-terpinyl acetate also failed to acetylate α-terpineol in vitro (Fig. 3).

2.2.2. Chiral analyses of acetate formation

To further investigate whether the enzymatic acetylation observed is specific towards a particular linalool enantiomer, we conducted experiments with non-radioactive AcCoA and determined the enantiomeric composition of the produced linalyl acetate by chiral analyses. The results showed that when racemic linalool was given as a substrate, soluble cell-free extracts from different plants utilized the two linalool enantiomers at different rates (Fig. 4). (*R*)-linalool was preferably converted to (*R*)-linalyl acetate with enantiomeric excesses ranging from 87% (*O. dayi*) to 15% (*O. onites*). Soluble cell-free extracts from *O. dayi* preferentially acetylated (*R*)-linalool with high stereoselectivity when linalool was offered at various (*R*)/(*S*) ratios (Fig. 5A). In contrast, it seems that both enantiomers are acetylated at similar rates by extracts of *O. majorana* (Fig. 5B).

In order to study the differences in the rates of the acetylation activity of *O. dayi* and *O. majorana* enzymatic preparations, we determined the kinetic parameters for both (*R*)- and (*S*)-linalool with saturated AcCoA and for AcCoA with saturated (*R*)- and (*S*)-linalool. The K_m values for (*R*)- and (*S*)-linalools were similar both in *O. dayi* and *O. majorana* (0.02 and 0.03 mM for (*R*)- and (*S*)-linal-

Table 1
Enantiomeric composition of monoterpene alcohols and their corresponding acetates in six *Origanum* species, *M. citrata* and *S. dominica*

Monoterpene		Species							
		O. dayi	*O. syriacum*	*O. majorana*	*O. vulgare*	*O. ramonense*	*O. onites*	*S. dominica*	*M. citrata*
Linalool	Alcohol	(*R*) 21 (t)[a]	(*R*) 45	(*S*) 10	(*S*) 45	n.d.[b]	(*R*) 99 (t)	(*R*) 99 (t)	(*R*) 99
	Acetate	(*R*) 99	(*R*) 99	(*R*) 48	(*S*) 22	n.d.	(*R*) 99	(*R*) 99	(*R*) 99
(*trans*)-Sabinene hydrate	Alcohol	(*S*) 88	(*R*) 54	(*R*) 53	(*R*) 42	(*R*) 92	(*R*) 37	n.d.	n.d.
	Acetate	(*S*) 99	n.d.	n.d.	(*R*) 99	n.d.	(*R*) 99	n.d.	n.d.
(*cis*)-Sabinene hydrate	Alcohol	(*R*) 10	(*R*) 99	(*R*) 99	(*R*) 96	(*R*) 96	(*R*) 96	n.d.	n.d.
	Acetate	(*R*) 96	n.d.	(*R*) 99	(*R*) 97	(*R*) 99	(*R*) 98	n.d.	n.d.
Terpinen-4-ol	Alcohol	(*S*) 22	(*S*) 69	(*S*) 88	(*S*) 74	(*S*) 57	(*S*) 46 (t)	n.d	n.d.
	Acetate	(*S*) 29 (t)	n.d.	n.d.	(*S*) 22 (t)	n.d.	n.d.	n.d	n.d.
α-Terpineol	Alcohol	(*S*) 10	(*R*) 20	(*R*) 26	(*R*) 20	(*S*) 91	(*R*) 26 (t)	(*S*) 71(t)	n.d.
	Acetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.s. [c]	n.d.
Borneol	Alcohol	(*S*) 99	(*S*) 99	(*S*) 99	(*S*) 99	(*S*) 99	(*S*) 99 (t)	n.d.	n.d.
	Acetate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.d.

Single plants were examined at two months intervals for at least 1 year. The enantiomeric composition remained constant through the year in the case of enantiomerically pure compounds. In other cases the variability was ± 4% of ee.
Enantiomeric composition expressed in enantiomeric excess (ee, %).
[a] (t), Low relative abundance (<1%).
[b] n.d., Not detected (<0.05%).
[c] n.s., Enantiomers were not separated.

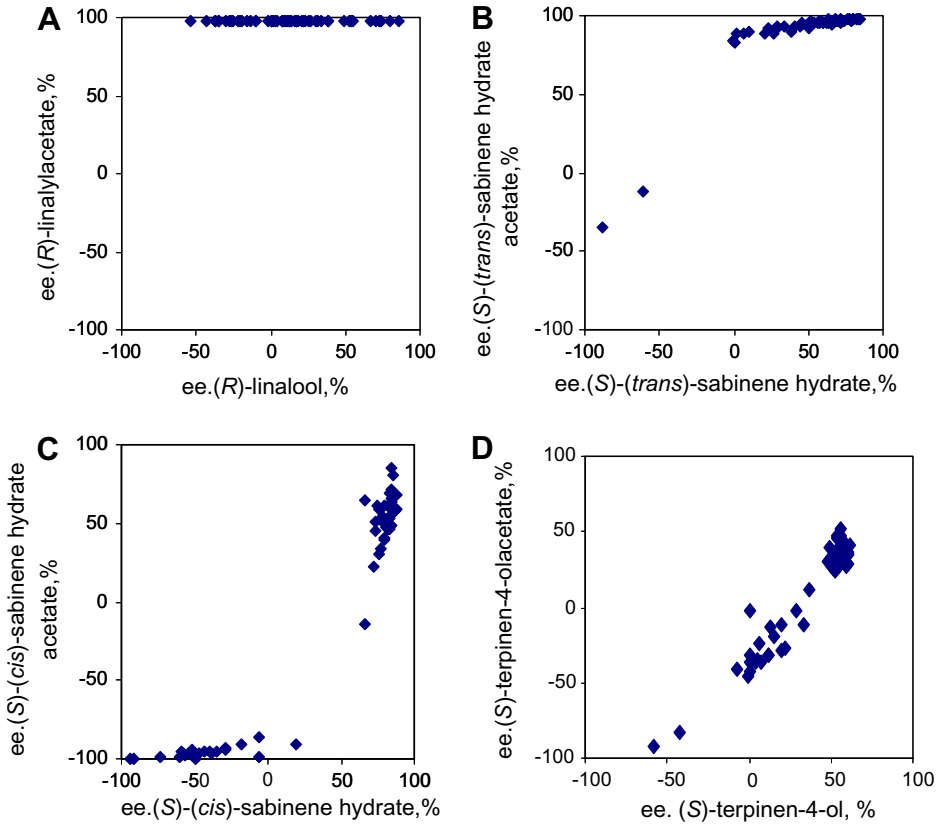

Fig. 2. Correlation between enantiomeric composition (expressed as enantiomeric excesses [ee], %) of alcohols and their corresponding acetates in 58 accessions of *O. dayi*. (A) (*R*)-linalool/acetate; (B) (1*S*)-(*trans*)-sabinene hydrate/acetate; (C) (1*S*)-(*cis*)-sabinene hydrate/acetate; and (D) (*S*)-terpinen-4-ol/acetate. Negative numbers are shown when the opposite enantiomer is predominant.

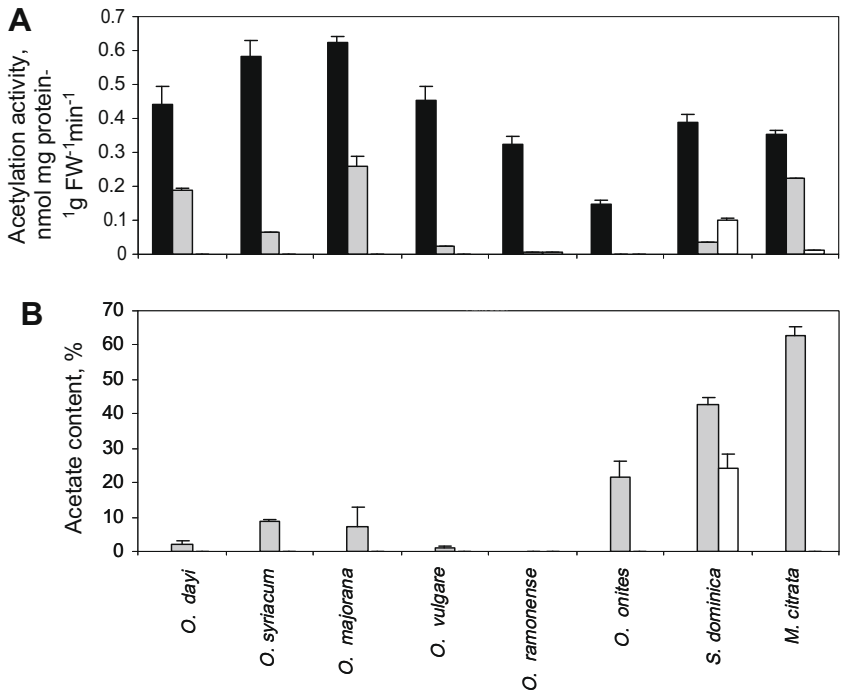

Fig. 3. (A) AAT activity of cell-free protein extracts from *Oregano*, *Salvia* and *Mentha* spp. with [14C]acetyl-CoA as acyl donor. Substrates used: hexanol (black bars), (*RS*)-linalool (grey bars) and (*RS*)-α-terpineol (white bars). Averages of two replicates + STDEV are shown. The experiment was replicated two times with similar results. The control using AcCoA alone has been included in the calculations. (B) Ester content (%) in SPME extracts from plants used for protein extraction. Acetates shown: linalyl acetate (grey bars) and α-terpinyl acetate (white bars). (Hexyl acetate was not found).

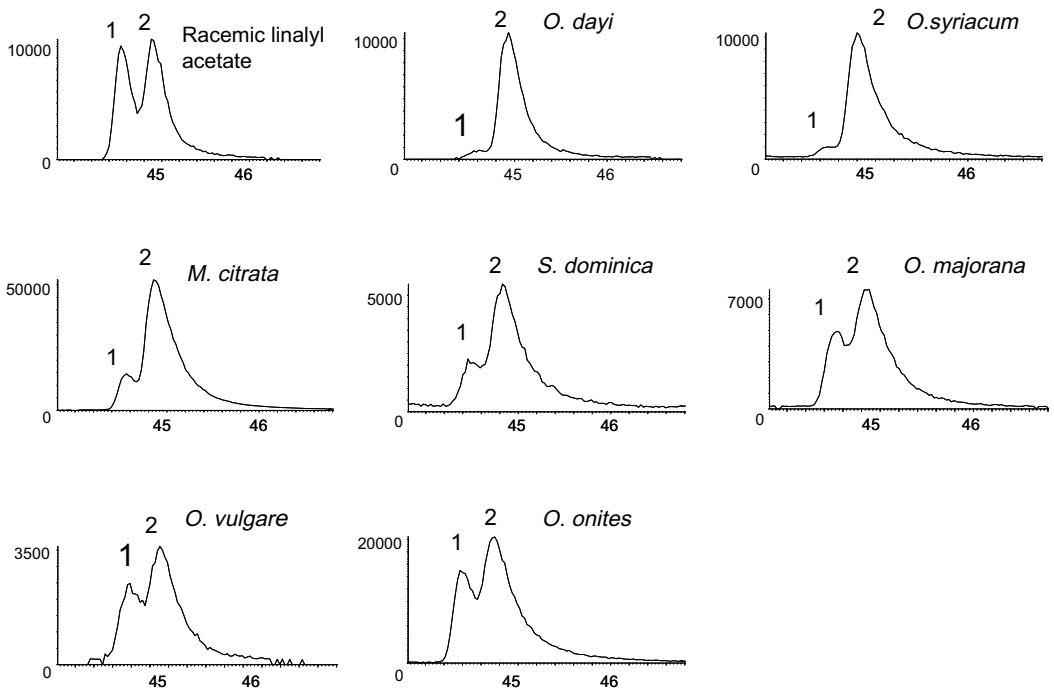

Fig. 4. GC–MS analyses of linalyl acetate enantiomers generated in vitro by AAT activity of desalted protein extracts with racemic linalool as a substrate (1.6 μM) and non-radioactive AcCoA as an acyl donor (0.1 mM). 1-(*S*)-linalyl acetate; 2-(*R*)-linalyl acetate. The enantiomeric excess (%) of (*R*)-linalyl acetate was 87 (*O. dayi*); 82 (*O. syriacum*); 56 (*M. citrata*); 41 (*S. dominica*); 22 (*O. majorana*); 18 (*O. vulgare*); 15 (*O. onites*). *x*-axis – retention time (min), *y*-axis – abundance of *m/z* 80 ion.

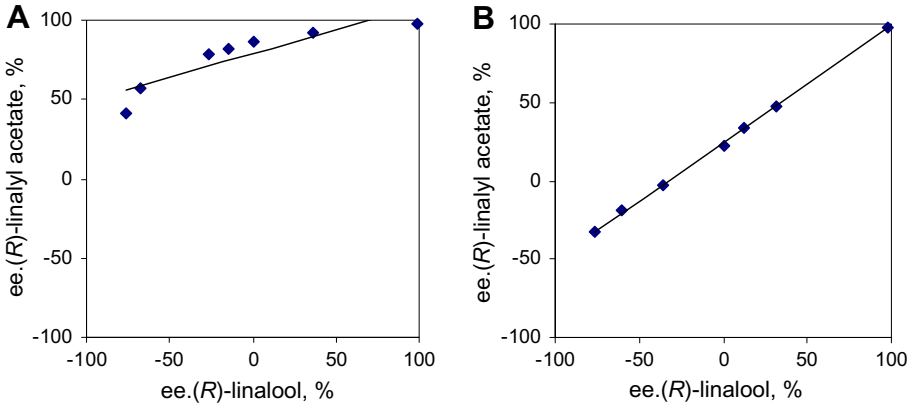

Fig. 5. Correlation between enantiomeric excesses of the substrate (*R*)-linalool and in vitro produced (*R*)-linalyl acetate. (A) *O. dayi* cell-free protein extract; (B) *O. majorana* cell-free protein extract.

ool in *O. dayi* and 0.18 and 0.05 mM in *O. majorana* for (*R*)- and (*S*)-linalool, respectively) at a fixed AcCoA concentration of 0.7 mM (Table 2). The V_{max} value for (*R*)- linalool in *O. dayi* cell-free extracts was much higher compared to (*S*)-linalool (3.96 and 0.14 nmol min^{-1} mg $protein^{-1}$, respectively). In contrast, no differences in V_{max} of (*R*)- and (*S*)-linalool were observed in *O. majorana* (0.66 and 0.51 nmol min^{-1} mg $protein^{-1}$, respectively). Values of the K_m's for both linalool enantiomers at acetyl CoA saturation are lower than the K_m for acetyl CoA when alcohol substrates are saturated (0.02 and 0.03 mM for (*R*)- and (*S*)-linalool, respectively, comparing to 0.8 and 0.6 mM). In spite of the very similar K_m values observed for both linalool enantiomers, large differences were seen between (*R*)- and (*S*)-linalool enantiomers in terms of V_{max} values. When (*R*)-linalool was the alcohol substrate, whether at saturation concentrations or not, the V_{max} observed was approximately 30-fold higher compared to that observed when (*S*)-linalool was used as a substrate. The lack of differences in K_m and in V_{max} values in *O. majorana* may confirm the non-selective model of linalyl acetate formation observed in enzymatic reactions. However, we cannot exclude the possibility that *O. majorana* possesses more than one activity, each specific for a certain enantiomer and the values we measure reflect an average of the kinetic parameters of each activity. Indeed since we did not use a purified enzyme preparation it is likely that enzyme extract contained a number of AAT activities. The differences in V_{max} when linalool and AcCoA were used either at saturating or non-saturating concentrations could be due to various reasons including inhibition by high substrate concentration. Such differences have been reported for example by Souleyre et al. (2005).

To further evaluate the substrate specificity of the enzymes studied we tested additional chiral alcohol substrates. The primary aliphatic alcohol 2-methyl butanol was readily acetylated in this reaction by both *O. dayi* and *O. majorana* enzymatic preparations. No preference towards a specific enantiomer of 2-methyl butanol

Table 2
Kinetic parameters of AAT activities in cell-free extracts of *O. dayi* and *O. majorana*

Protein source	Co-substrate *S*1 (variable concentration)	Co-substrate *S*2 (saturating concentration)	K_m (mM)	V_{max} (nmol min^{-1} mg $protein^{-1}$)	V_{max}/K_m (10^{-6} L min^{-1} mg $protein^{-1}$)
O. dayi	(*R*)-linalool	AcCoA	0.02	3.96	198.0
	(*S*)-linalool	AcCoA	0.03	0.14	4.7
	AcCoA	(*R*)-linalool	0.80	0.90	1.1
	AcCoA	(*S*)-linalool	0.60	0.03	0.1
O. majorana	(*R*)-linalool	AcCoA	0.18	0.66	3.7
	(*S*)-linalool	AcCoA	0.05	0.51	10.2
	AcCoA	(*R*)-linalool	0.06	0.12	2.0
	AcCoA	(*S*)-linalool	0.16	0.18	1.1

AAT activity was measured using non-radioactive assay and various concentrations of alcohol substrates (0.1 μM to 0.3 mM) at saturated concentration of AcCoA (0.6 mM) or various concentrations of AcCoA (0.02–1 mM) at saturated concentration of alcohols (0.3 mM).

Table 3
Enantiomeric composition of acetates produced by cell-free extracts with different racemic alcohol substrates

Enantiomeric ratio of produced acetate (%)	Racemic alcohol substrate									
	2-Methyl butanol		Lavandullol		Neomenthol		Isopulegol		Borneol	
	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)
O. dayi	50	50	50	50	71	29	99	1	50	50
O. majorana	50	50	n.d.	n.d.	23	77	n.d.	n.d.	50	50

was observed (Table 3). The enantiomers of the primary monoterpene alcohol lavandullol were also acetylated by *O. dayi* soluble preparations at similar ratios. Therefore, *O. dayi* AAT could not differentiate between primary alcohol enantiomers with a hydroxyl group in α-position to the chiral center. Similarly, other highly enantioselective enzymes, such as lipases, do not necessarily show any enantioselectivity toward primary alcohols. Few cases of primary alcohol enanioselective acetylation have been described (Oda et al., 1999).

O. dayi and *O. majorana* enzymatic preparations acetylated the secondary alcohol neomenthol (Table 3). Neomenthyl acetate with an enantiomeric ratio of 77 (*S*):23 (*R*) was produced from racemic neomenthol by an *O. dayi* enzymatic preparation, whereas an *O. majorana* enzymatic preparation acetylated racemic neomenthol to a mixture of neomenthyl acetate with the almost reverse enantiomeric ratio of 27 (*S*): 73 (*R*). Racemic isopulegol was acetylated to an enantiomerically pure (*R*)-isopulegyl acetate by *O. dayi* AAT, but we were unable to obtain a product using *O. majorana* ATT. *O. dayi* and *O. majorana* enzymatic preparations acetylated the secondary alcohol borneol (Table 3). When two pure borneol enantiomers were used as substrates separately, similar amounts of bornyl acetate enantiomers were obtained. We therefore assume that AATs of *O. dayi* and *O. majorana* either have no preference for naturally occurring (*S*)-borneol or that two separate AAT's are present in this plant. These results are different from the results of the acetyl transferase activity of *M.x piperita* (Croteau and Hooper, 1978). They reported that both neomenthol, menthol and isomenthol enantiomers were equally acetylated by acetyl transferase preparations of *M.x piperita*.

Our results indicate that some plants within *Origanum*, *Mentha* and *Salvia* genera exhibit remarkable enantioselectivity of alcohol acetyl transferase activity and can particularly discriminate between linalool enantiomers. This enantioselectivity was observed by chiral analyses of the essential oil of these plants and demonstrated in enzymatic reactions (Table 1, Figs. 4 and 5). Few stereoselective acetyltransferases have been reported to date. Salutaridinol 7-*O*-acetyltransferase from *Papaver somniferum*, an enzyme involved in alkaloid biosynthesis, showed a high stereoselectivity toward salutaridionol and did not accept its diastereomer 7-epi-salutaridinol as a substrate (Lenz and Zenk, 1995). Alcohol acetyl transferase from *Pichia kluyveri* bacteria was capable of enantioselective conversion of racemic citronellol to (*S*)-citronellyl acetate with an *E*-value of more than 20 (Oda et al., 1999).

2.2.3. Enantiomeric specificity of AAT

Our findings are of interest because known alcohol acetyl transferases involved in volatile ester biosynthesis in flowers and fruits have been reported to exhibit a broad substrate specificity, capable of accepting a wide range of alcohol substrates (Souleyre et al., 2005; Shalit et al., 2001; Beekwilder et al., 2004; D'Auria, 2006; Guterman et al., 2006). The limited range of esters found in the plants was attributed to the lack of availability of the endogenous substrates. Our work also shows that simple aliphatic alcohols hexanol and 2-methyl butanol were acetylated with ease by soluble cell-free extracts. However, specificity was observed when more complex monoterpenols such as α-terpineol or linalool were used as a substrates. In this work we also show that the availability of AAT may limit the formation of monoterpene acetates in the *Lamiaceae*. Substrate availability may also have a crucial role in determining the composition of acetates in the essential oil. Many monoterpene synthases are reported as stereospecific enzymes providing enantiomerically pure substrates for subsequent acetylation and thus determining the stereochemistry of the acetylation (Köllner et al., 2004). Linalool is formed from the achiral precursor geranyl diphosphate by various linalool synthases (LS) that are highly stereospecific. (*R*)-linalool synthases were identified in *M. citrata* (Crowell et al., 2002), *Ocimum basilicum* (Iijima et al., 2004) and *Lavandula angustifilia* (Landmann et al., 2007). (*S*)-linalool synthases were identified in *C. breweri* (Pichersky et al., 1995), *Cereus peruvianus* (Sitrit et al., 2004) and *Arabidopsis thaliana* (Aharoni et al., 2003).

3. Concluding remarks

Enzymatic reactions utilizing chiral alcohol substrates showed remarkable stereoselectivity towards the (*R*)-linalool enantiomer in *O. dayi*. This enantioselective pattern was observed in *O. dayi* plant extracts when these were examined by chiral GC analyses. Both enantiomers of linalool and *cis*- and *trans*-sabinene hydrate were present but only one acetate enantiomer was accumulated. The AAT stereoselectivities in various plants of the *Lamiacea*e family were found to be different.

4. Experimental

4.1. Plant material

Leaves of *Origanum* spp., *M. citrata* and *S. dominica* (Table 4) were harvested from a collection of live material maintained at Newe Ya'ar Research Center in Israel. Large experimental plots were kept under field conditions and selected plants were transferred to the

Table 4
Origins of the investigated plants

Accession	Genus/section	Origin	Main monoterpene acetates detected
O. dayi Post	*Origanum/ Campanulaticalyx*	Four selected clones from a wild population in the Judean Desert , Israel 58 accessions collected in the wild	(*trans*)-Sabinene hydrate acetate (*cis*)-sabinene hydrate acetate linalyl acetate (Dudai et al., 2003 ;Amzallag et al., 2005)
O. syriacum L. ssp. syriacum	*Origanum/ Majorana*	Selected clone from Newe Ya'ar, Israel	(*cis*)-Sabinene hydrate acetate (Larkov et al., 2005) linalyl acetate
O. majorana L.	*Origanum/ Majorana*	A commercial variety provided by CN Seeds Company, UK	(*cis*)-Sabinene hydrate acetate (Larkov et al., 2005) linalyl acetate
O. vulgare L. ssp. vulgare	*Origanum/ Origanum*	Selected clone from a wild population near Kalamaki, Greece	linalyl acetate
O. ramonense Danin	*Origanum/ Campanulaticalyx*	Selected clone from a wild population in the Ramon Heights , Israel	(*cis*)-Sabinene hydrate acetate (Larkov et al., 2005)
O. onites L.	*Origanum/ Majorana*	Selected clone from a wild population near Kalamaki , Crete, Greece	Linalyl acetate
S. dominica L.	*Salvia*	Selected clone from seeds collected in a wild population on the Gilboa mountains.	Linalyl acetate, α-terpinyl acetate (Ravid and Putievsky, 1985)
M. citrata L.	*Mentha*	Plants grown at the Newe Ya'ar herbs collection	Linalyl acetate (Ravid et al., 1994)

greenhouse. *O. ramonense*, that cannot survive under open field conditions, and *O. majorana* were cultivated in a greenhouse.

Additionally 58 *O. dayi* plant accessions from 10 wild populations originated in the Judean desert in Israel were investigated. The plants were collected and extracted as described by Amzallag et al. (2005) and analyzed by chiral chromatography.

4.2. Chemicals

(*R*)-linalool ex. bois de rose (enantioneric purity 99%, R.C. Treatt & Co., England), (*S*)-linalool (enantioneric purity 88%, ex. coriander oil prepared by flash chromatography as described by Larkov et al., 2005) and (±)-linalool (Roth Chemical Co., Karlsruhe, Germany); (*1R,3R,4S*)-(−)-isopulegol (Aldrich) and (*1S,3S,4R*)-(+)-isopulegol (Aldrich); (*S*)-(−)-2-methyl-1-butanol (Aldrich) and (±)-2-methyl-1-butanol (Aldrich); (±)-lavandulol (Roth), (±)-neomenthol (Fluka), (*R*)-borneol (Fluka), (*S*)-borneol (Fluka), (−)-terpineol (Fluka), (+)-terpineol (Fluka), (+)/(−)-terpinen-4-ol (60/40) (Aldrich) were utilized as substrates.

(*R*)-(−)-linalyl acetate (enantioneric purity 99%, R.C.Treatt & Co., ex petitgrain) and (±)-linalyl acetate (Fluka); (*S*)-(−)-2-methyl-1-butyl acetate (Aldrich); (±)-lavandulyl acetate (Fluka), (1*S*)-(+)-neomenthyl acetate (Fluka), (*1R*)-(−)-neomenthyl acetate (Fluka) were used as standards.

α-Terpinyl acetate and terpinen-4-yl acetate were not commercially available. They were obtained by acetylation with Ac_2O/pyridine (Larkov et al., 2005). When only minute amounts were needed, a fast on-fiber acetylation procedure was followed. Alcohol (1 μl) was placed in a 2 ml autosampler vial. A SPME fiber was inserted into the headspace to adsorb alcohols for 10 min at ambient temperature. The fiber with the adsorbed alcohol was inserted into a second 2 ml vial containing Ac_2O (10 μl) and pyridine (10 μl) for 10 min. The fiber with the newly formed acetates and the residual alcohols was inserted into the GC injection port for analyses. The conversion rate was 5–20% and depended on the type of acetylated alcohol.

4.3. Extraction of volatiles

4.3.1. Solvent extraction

Solvent extraction was carried out as described by Larkov et al. (2005). Fresh young leaves were extracted for 2 h by gentle shaking with *tert*-butyl methyl ether (MTBE) (5 ml per gram of fresh weight) at room temperature. The extracts were purified by passing them through a Pasteur pipette containing anhydrous Na_2SO_4 and Silica gel 60 (230–400 mesh, Merck) to dry the sample and to remove polar substances of high molecular weight that might interfere with the GC–MS analyses.

4.3.2. Hydrodistillation

Samples of at least 250 g of fresh plant material were hydrodistilled for 1.5 h in a modified Clevenger apparatus. The essential oil was cooled and separated from the water.

4.3.3. Solid-phase microextraction (SPME)

Fresh plant material or enzyme preparations were placed in sealed vials. The volatiles were extracted from the headspace using a 65 μm PDMS/DVB fiber [polydimethylsiloxane/divinylbenzene], (Supelco, PA, USA)] for 30 min. Desorbtion time was 5 min. The injections were performed with an autosampler (a GC PAL or Combi PAL systems, CTC Analytics) or manually.

4.4. Chiral chromatography

Linalyl acetate enantiomers were separated on a FS-Lipodex E (Macherey-Nagel,Germany) cyclodextrin column (25 m × 0.25 mm × 0.25 μm) using an Agilent GC-MSD system (CA, USA). Injector and transfer line temperatures were 230 °C. The following temperature program was used: 50 °C for 20 min, 50–70 °C at 1 °C/min; 70–180 °C at 5 °C/min; carrier gas was He at constant pressure 8 psi, linear velocity 44 cm/s. Enantiomeric ratio or excess were calculated using peak height.

Other chiral separations were performed on an Rt-βDEXsm (Restek, PA, USA) cyclodextrin column (30 m × 0.25 mm × 0.25 μm) on a Hewlett-Packard GCD gas chromatograph apparatus. The injector and transfer line temperatures were 230 °C. Mass range *m/z* 41–350. Carrier gas was He at a constant flow of 0.8 ml/min, linear velocity 32 cm/s. The temperature program was as follows: 50 °C for 1 min, 50–200 °C at 1.5 °C/min. In some plant extracts (1*R*,4*R*,5*S*)-(*cis*)-sabinene hydrate interfered with (*S*)-linalool and we used either a temperature program of 50 °C for1 min, 50–200 °C at 20 °C/min or the chiral column Hydrodex-b-TBDAc (Macherey-Nagel) (25 m × 0.25 mm × 0.25 μm) with a program of 50 °C for 20 min, 50–70 °C at 1 °C/min; 70–180 °C at 5 °C/min; carrier gas was He at a constant pressure of 8 psi, linear velocity 44 cm/s. Splitless or split modes with split ratios 5–50 were used. For separation of lavandullol/lavandullyl acetate enantiomers we used a program of 50 °C for 20 min, 50–200 °C at 3 °C/min.

4.5. Enzyme extraction

Fresh plant samples (1 g of 2–3 young leaf pairs) were ground with a pestle in a chilled mortar in liquid nitrogen, 0.5 g sand and 0.1 g PVPP insoluble until a uniform powder was obtained.

Ice-cold extraction buffer (1:5 w/v), consisting of 50 mM bis-Tris-propane pH 6.9, 10% (v/v) glycerol, 10 mM dithiothreitol

(DTT), 5 mM $Na_2S_2O_5$ and 1% (w/v) PVP-40 was added (buffer A). The slurry was centrifuged at 14,000*g* for 15 min at 4 °C. The supernatant (2 ml) was next passed through a P-6 column (1 × 10 cm) (Bio-Rad, Bio-Gel desalting gel 90–180 μm, Bio-Rad, Germany) to remove interfering low-molecular-weight compounds. We used buffer B (buffer A without PVP-40, pH 7.5) for column equilibration and protein elution. Protein concentration in 1 ml fractions was detected by the Bradford protein assay (Bradford, 1976) utilizing bovine serum albumin as a standard. GC–MS analyses were performed to ensure that interfering essential oil compounds were removed. Fractions were kept at −20 °C for further analysis.

Mentha citrata enzyme preparations were obtained from young leaves peltate glandular trichomes and isolated as previously described (Alonso et al., 1992). Isolated glandular trichomes were ground with buffer A, and the trichome extract was desalted on P6 column as described above.

4.6. Enzyme assay

4.6.1. Radioactive assay

Small-scale assays were performed by mixing 20 μL of a desalted cell-free extract, 0.33 mM alcohol substrate, and 23 μm (7.8 μCi μmol-1 [14C]acetyl-CoA, Amersham) into a final volume of 100 μL of assay buffer B. The assays were incubated for 30 min at 30 °C. One milliliter of hexane was added to each tube, which was then vigorously vortexed and spun for 1 min at 5000*g* to separate phases. 0.8 ml of the upper hexane layer, containing the newly formed radiolabeled alcohol acetate esters, was placed in a scintillation vial containing 3 ml of Ultimagold non-aqueous scintillation fluid (Packard Bioscience, The Netherlands). Radioactivity was counted by Tri Carb 2800TR liquid scintillation counter (Perkin–Elmer, The Netherlands). Boiled enzyme extracts and reaction with no enzymes were used as controls. Enzyme activity was calculated based on the specific activity of the substrate and using appropriate correction factors for the counting efficiency of the scintillation machine (Shalit et al., 2001).

4.6.2. GC–MS assay

The assay was performed in 2 ml vials at 30 °C and containing 40 μl desalted supernatant (containing 20–60 μg protein), 40 μl non-radioactive acetyl CoA (0.1 mM) and 5 μl of the appropriate alcohol (1.6 μM) in sample buffer B (pH 7.5) in a total volume of 300 μl. We used alcohol solutions in *n*-hexane at 0.5 M and then diluted them with buffer B. After 30 min 100 μl satr. $CaCl_2$ was added to stop the reaction. Products were analyzed by GC–MS using automated or manual HS-SPME as described above. Appropriate controls included the omission of substrate, omission of enzyme extract and the use of heat-inactivated enzyme extracts.

Comparative alcohol substrate preference assays were performed as described above using non-radioactive acetyl CoA. Enantiomerically pure (*R*)-linalool and (*S*)-linalool (88% enantiomeric purity) were used. All reactions were done in duplicates. Linalyl acetate concentration was calculated by external standard calibration. A calibration curve for linalyl acetate was obtained within the range of 0.02–0.6 μM and was linear with $R = 0.993$. The V_{max} and K_m were calculated from non-linear regressions of the Michaelis–Menten plot using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA).

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