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Location and biosynthesis of monoterpenyl fatty acyl esters in rose petals

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Abstract

The upper epidermal layer of cells and the epicuticular wax surface of Lady Seton rose petals are sites of biosynthesis and accumulation, respectively, of a family of terpenyl fatty acyl esters. These esters are based mainly on the acyclic monoterpene alcohol geraniol coupled primarily to fatty acids of chain lengths 16–20 and in mass terms represent from 14% to 64% of the total monoterpenes present in the petals. The lipophilic nature of these non-volatile esters of the monoterpene alcohols contrasts with that of the lipophilic volatile parent alcohols themselves and with the hydrophilic, non-volatile, glucoside derivative of the other principal petal fragrant compounds, the phenylpropanoids, β-phenyl ethanol and benzyl alcohol. These latter compounds are also synthesised and are resident in the petal. Biosynthetic studies confirmed that the petal upper epidermal cell layer has the capacity to incorporate mevalonic acid into the monoterpene component of the fatty acyl ester. The biosynthesis of the monoterpene component of the fatty acyl ester occurs via the mevalonic acid pathway in Lady Seton as well as in the hybrid tea rose Fragrant Cloud. In the latter flower the biosynthesis of geraniol was biosynthetically *trans* as was the formation of nerol and citronellol. Both geraniol and nerol were shown to be precursors of citronellol via an NADPH dependent reductase reaction. Oleic acid is assimilated into the acyl moiety of the terpenyl ester in Lady Seton isolated petal discs. It is probable that the lipophilic non-volatile terpenyl fatty acyl esters represent a stable storage form of the corresponding alcohols from their residency within the epicuticular wax layer. These acyl esters may realise, on hydrolysis, additional aroma notes from the living flower and potentially commercially significant quantities of the fragrant terpenols during oil of rose essence production.

Keywords: Monoterpenyl fatty acyl esters; Biosynthesis of terpenols and esters; Epidermis; Rose petals

1. Introduction

The petals of the hybrid tea rose Lady Seton have been shown to contain the monoterpene alcohols geraniol, nerol and citronellol and the phenylpropanoids β-phenyl ethanol and benzyl alcohol (Francis and Allcock, 1969). These compounds occur both as the free alcohols and as the glycosides, the former being lipophilic in nature and the latter water-soluble and primarily conjugated to glucose. In addition to the above components rose petals also contain a complex series of neutral lipids, including sterols, sterol esters, alkanes, alkenes, ketones, γ-lactones and longer chain wax esters (Kovats, 1987; Babu et al., 2002).

Radio-labelling experiments demonstrated the incorporation of (2)-^{14}C mevalonic acid (MVA), in 10.8% yield, into the glycosides of the monoterpene alcohols geraniol, nerol and citronellol in petals of the hybrid tea rose Lady Seton. Labelling into the ''free'' monoterpene alcohols was of the order of 1% (Francis and O'Connell, 1969). There was a significant initial flux of carbon through the glycoside fraction peaking at 60 min then declining rapidly. The free monoterpene fraction on the other hand peaked at ca. 40 min and exhibited a much less rapid decline. These results demonstrated a rapid turnover of the monoterpene pool and were suggestive of a precursor product relationship between

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the alcohols and their glycosides and additional further transformation of the glycosides and/or their transport out of the petal. The latter seems unlikely however since there was no evidence for the presence of the free monoterpenes or their glycosides, at least in leaves. In the above studies incorporation of ^{14}C from MVA into sesquiterpenes (ca. 0.1%) and into undefined higher terpenes was observed but not persued.

This communication describes the isolation, identification and distribution of a further group of non-volatile derivatives of the terpene alcohols, namely their long chain fatty acyl esters as well as some aspects of their biosynthesis and tissue localisation.

2. Results and discussion

2.1. Structure and distribution of the terpenyl fatty acyl esters

Lady Seton petals (700 g f.w.) were extracted with chloroform/methanol and further fractionated as described in 3.3 and 3.4 to yield 53 mg of a colourless waxy solid.

The IR spectrum of this material exhibited typical ester absorption with bands at 1735 cm^{-1}(C=O stretching), and 1175 cm^{-1} (–C–O–C– stretching). Weak bands were also present at 1674 (unconjugated double bond) and 842 cm^{-1} (trisubstituted ethylene). The allylic nature of the ester was evident by a medium band at 990 cm^{-1} and the paraffinic fatty chain by absorption at 728 cm^{-1}.The infrared spectrum was indicative of a terpenyl long chain ester. The EI mass spectrum of a sample of the ester gave a peak at m/z at 420, this was the most abundant ion in the high mass region and corresponded to the molecular formula $C_{28}H_{52}O_2$ equivalent to the isomeric compounds geranyl or neryl stearate. Ions in the higher mass region at m/z 448 and 476 corresponded to the terpenyl arachidate (C_{20}) and behenate (C_{22}), respectively. Molecular ions corresponding to palmitate and linoleate esters were also observed but were more difficult to distinguish due to interference from fragment ions from higher mass compounds Alkaline hydrolysis of a sample of the ester gave an acid and alcohol fraction (Table 1).The alcohol fraction was a mixture of geraniol, nerol and citronellol (ratio 42:7:1). Gas chromatography (GC) of the methyl esters of the fatty acids shows 18:0 to be the major carbon chain length accounting for 49% of the total terpene bound fatty acid. Similar analysis of lipid extracts of petals of *Rosa demascena trigentipetala* and Fragrant Cloud yielded an ester fraction which again contained geraniol, nerol and citronellol with 16:0 being the major fatty acid esterified to the terpene alcohol in *Rosa damascena* whilst in Fragrant Cloud the predominant acid in the ester fraction was 18:0. In the petals of three roses examined from 62% to 85% of the fatty acids esterified to monoterpenes were saturated and these included a considerable proportion of longer chain acids (from C_{20} to C_{26}). Unsaturated fatty acids esterified to monoterpene alcohols varied from a ratio of 16:0 + 18:0:16:1 + 18:1 + 18:2 + 18:3 from 4.1:1 to 1.6:1 to 1.9:1 for Lady Seton, *Rosa damascena trigentipetala* to Fragrant Cloud, respectively. GC of the intact terpenyl fatty acyl esters resolved them according to the chain length of the fatty acid as expected and to a lesser extent to the structure of the terpenol component. Each ester mixture was resolved into a number of groups with the fatty acid carbon number dominating and with each group further subdivided according to the terpene alcohol composition, the retention times increasing in the order citronellol, nerol, geraniol. A plot of log retention time vs. carbon number of the fatty acids esterified was linear in agreement with a similar plot for the isolated fatty acids (methyl esters). The individual members of each ester group were identified by comparison with synthetic standards. The predominance of the 18:0 and 20:0 esters in Lady Seton and 16:0 and 18:0 in *R. damascena* was confirmed by reversed-phase TLC. For the same fatty acid carbon number it was not possible by this TLC method to resolve the geranyl and neryl esters though these were separated from the corresponding citronellyl derivatives. The presence of unsaturation in the fatty acid moieties complicated separation further since citronellyl esters were not resolved from the geranyl/neryl esters of the next highest fatty acid.

Sterol esters, which were present at a level of about 17% of the terpenyl ester fraction in the petals, were readily separated from the latter by adsorption and reversed-phase TLC as well as by GC.

Table 1
Terpenol and fatty acid composition of the monoterpene fatty acyl esters from rose petals

	% Composition		
	Lady Seton	*Rosa damascena* trigentipetala	Fragrant Cloud
Fatty acid			
12:0	1.4	nd	nd
14:0	2.2	5.2	1.3
16:0	6.6	34.4	21.6
16:1	1.4	9.2	3.0
18:0	48.9	14.9	33.3
18:1	2.9	9.4	10.3
18:2	7.7	6.4	3.4
18:3	1.6	6.1	11.9
20:0	21.3	3.5	15.2
20:1	nd	nd	nd
20:2	0.3	0.9	–
20:3	0.3	0.8	–
22:0	3.2	1.9	–
22:1	0.4	3.8	–
22:2	0.2	1.1	–
22:3	0.4	nd	–
24:0	1.0	2.4	–
26:0	0.2	nd	–
Terpenol			
Geraniol	84	57	29
Nerol	14	11	11
Citronellol	2	32	60

nd, not detected.

As noted above the fatty acids esterified to terpenols were characteristically low in unsaturated fatty acids with the major contribution from saturated longer chain acids of C_{18}, C_{20} and above especially in the case of Lady Seton. Fragrant Cloud and *Rosa damascene trigentipetala* differed from this position by containing relatively high levels of 16:0 and unsaturated C18 fatty acids. Sterol esters and triglyceride on the other hand showed a pattern of fatty acids similar to that of the total fatty acid pattern of the tissue where 18:2 was the major acid and 18:3 occurred in significant amounts (6% and 10% esterified to sterol and glycerol, respectively). Fatty acid esterified to sterol showed some similarities to the terpenyl ester fatty acids insofar as it contained a high proportion of 20:0, somewhat lower 18:2 and 18:3 than triglyceride or the total fatty acid picture. The high content of longer chain saturated acids in petal esters bears similarity to the general pattern of alkanes, esters, alcohols, fatty acids and aldehydes/ketones found in plant surface cuticular waxes (Somerville et al., 2000). These latter usually contain *n*-alkanoic acids and *n*-alkanols mostly with even numbers of carbon atoms usually in the range C_{12} to C_{32} with branched chains and unsaturation in this group being uncommon. Xanthophyll fatty acyl esters isolated from the petals of *Tagetes erecta* showed a fatty acid pattern similar to the terpenyl fatty acyl esters of Lady Seton in containing mostly saturated fatty acids (mainly 16:0) with only a trace of 18:1 esterified to the hydroxy carotenoid (Alam et al., 1968).

The distribution of the terpenes and phenylpropanoids between the unbound, fatty acyl ester and glycoside fractions in the petals was determined to clarify the quantitative importance of these terpenyl esters relative to the glycosides and free alcohols. The results of the analysis for flowers at the development stage, ranging from 7 to 9 days from splitting of the calyx, are shown in Table 2. Benzyl alcohol and β-phenyl ethanol did not occur to any significant extent esterified to long chain fatty acids; though these phenylpropanoids existed at a low levels in the acetate form. The majority of these benzenoids accumulated as the glycosides. The monoterpenes on the other hand appear to be distributed between all three fractions. There is clearly considerable variation in the percentage of the terpene fatty acyl ester in the petal. There was a trend for the larger, more mature flowers to contain a higher proportion of terpene (as ester) though a flower development vs. ester content exercise was not included in this study. When compared to the terpenoids, the phenylpropanoid components appeared to show no such variation in distribution. An exercise of this type merits further consideration in view of the potential significance of these findings to the commercially important rose varieties as sources of essential oil such as *R. damascena* var. *bulgaria*.

The waxy surfaces of rose petals as indicated above contains a high proportion of long chain odd carbon number mostly saturated hydrocarbons of mainly C_{21} to C_{35} (Marekov et al., 1968), and it was possible that the terpenyl fatty acyl esters might be associated with this layer in the rose petal. Pentane washings of petals of Lady Seton were examined for their content of terpene alcohols and fatty acyl esters and compared with the residual tissue lipids (Table 3). It is clear from the analysis that the majority of the terpene alcohols are readily extracted from petals by pentane washing. Similarly the terpenyl esters were present mostly in the pentane extract. β-Phenyl ethanol and its acetate were similarly disposed. There was no evidence for glycosidically bound terpene alcohols in the pentane extract. This finding supports the general validity of the pentane extraction procedure since terpenyl glucosides would not normally be extracted from the tissue using pentane as the solvent. GLC analysis showed that the pentane fraction contained only the C18 and C20 monoterpenyl fatty acyl esters while the subsequent chloroform/methanol extract contained in addition to the above the palmitate, myristate and laurate esters. The significance of this latter finding is not clear but it may reflect poorer solubility of the shorter chain esters in pentane rather than a different tissue distribution. Further work should be conducted to clarify this observation. Hydrocarbons were similarly distributed, (data not shown), occurring almost exclusively in the pentane extract whilst the more structural sterols were absent from the non-polar solvent washings and only extracted with chloroform/methanol. The specific distribution of the hydrocarbons, present in the pentane extract, and sterols, absent from the same, indicated that hydrocarbon solvent extraction procedure had removed predomi-

Table 2
Distribution of terpenols and aromatic compounds in Lady Seton petals

Flower	Components	% Composition			
		Alcohol	Glucoside	Terpenyl fatty acyl ester	Content[a]
1 (5.7 g)	Terpenols[b]	40	46	14	191
	Aromatics[c]	7	89	4	146
2 (8.8 g)	Terpenols	41	29	30	168
	Aromatics	15	79	6	137
3 (10.7 g)	Terpenols	19	41	40	165
	Aromatics	10	86	4	222
4 (13.1 g)	Terpenols	18	18	64	81
	Aromatics	12	85	3	95

[a] μg/g fresh weight.
[b] Average composition geraniol (83%), nerol (14%), citronellol (3%).
[c] Average composition β-phenyl ethanol (89%), benzyl alcohol (11%).

Table 3
Distribution of lipids in rose petal surface and epidermal layer

Lipid	% Distribution	
	Surface wax layer	Petal tissues
Citronellol	75	25
Nerol	78	22
Geraniol	78	22
β-Phenyl ethanol	65	35
β-Phenyl ethyl acetate	92	8
Terpenyl fatty acyl esters	67	33

nantly epidermal and not tissue lipids. Thus it appears that the free terpene alcohols and the terpenyl fatty acyl esters are localised mainly in the waxy cuticle of the petal. In this context therefore the site of biosynthesis of the terpene alcohols and their fatty acyl esters is of interest with the epidermal layer of cells as potential candidates (see later).

2.2. Biosynthesis of terpenyl fatty acyl esters

A single intact Lady Seton flower in an inverted position was incubated with 10 μC (2)-^{14}C-MVA dissolved in 100 μl water as described in Section 3.5. The substrate was taken up in 10 min, when further water was added via a silicone tube sleeved onto the cut stem to maintain the transpiration stream for a further 50 min. A the end of the incubation period the petals (3.4 g f.w.) were removed, immediately frozen in liquid nitrogen, ground to powder and finally steam distilled (see 3.3). The summed radioactivity in the four fractions from the incubation, namely SD_1, SD_2, L_1 and LH^+ on a yield basis was 47.2% based on 10 μC of ^{14}C-(±)-MVA substrate since only the (*R*)-enantiomer of MVA is utilised for monoterpene/polyterpene biosynthesis. The individual fractions were assayed for radioactivity; the majority resided in the non-volatile lipid fraction (L_1) this accounting for 43.5% of the recovered ^{14}C. The "free"and glycoside bound fractions (SD_1 and SD_2 respectively) retained 26.9% and 23.2% of the recovered activity, respectively. The acidified lipid fraction (LH^+) made up the balance of 6.4%. On a yield basis the fractions SD_1, SD_2, L_1 and LH^+ accounted for an uptake of label of 12.6%, 11.0%, 20.6% and 3.0%, respectively. All four fractions were analysed further to establish the composition of the major labelled components.

Adsorption TLC was employed to separate the non-volatile lipid fraction into its individual functional types (see Section 3.4). The distribution of ^{14}C (Table 4) confirmed that the main contributors to label uptake were the triterpene alcohol/sterol fraction (38% of the radioactivity), the sterol precursor squalene (27.2%) and the monoterpenyl fatty acyl esters (18.7%).The isolated monoterpenyl ester fraction was diluted with 10 mg neryl/geranyl palmitate, hydrolysed in a mixture of toluene/15% KOH in 85% ethanol (1:1) and extracted and steam distilled to isolate the volatile fraction as described in Section 3.4. The cooled reaction mixture was acidified to pH 2.0 and extracted with diethyl ether followed by solvent evaporation; 88.8% of the initial radioactivity of the sample was recovered in the distillate and with an overall recovery of label of 99%. The distillate was separated by silver ion TLC, Section 3.4, into its monoterpene components citronellol, nerol and geraniol; these compounds accounted for 99% of the label with the distribution 1.6%, 7.7% and 90.7%, respectively (68% of the activity was recovered from the TLC plate). It is clear from the above that the labelling in the terpenyl ester fraction is almost exclusively resident in the steam distillable components and that this is dominated by geraniol as the principal labelled compound with significantly less incorporation of carbon into nerol and the 2,3-dihydro derivative citronellol. The isolated hydrocarbon fraction was also chromatographed by silver ion TLC and resolved into alkanes, alkenes and the squalene sub-fractions. Of the total hydrocarbons ^{14}C resided only in squalene. This is anticipated in view of the known different biogenetic origins of the alkanes/alkenes and squalene (Kolattukudy, 1996; Lemieux, 1996; Chappell, 1995). Due to the low uptake of labelled carbon into the LH^+ fraction this material was not analysed further.

Table 4
Incorporation and distribution of ^{14}C from mevalonic acid into the non-volatile lipid components of Lady Seton rose petals

Compound	% of ^{14}C[a]
Squalene	27.2
Triterpene alcohol/sterol esters[b]	3.5
Monoterpene fatty acyl esters	18.7
Uncharacterised fraction	7.8
4,4- and 4α-methyl sterols	31.9
4-Desmethyl sterols	6.1
Polar fraction	4.8

[a] % of recovered of radioactivity.
[b] 4,4-Dimethyl, 4α-methyl and 4-desmethyl sterol esters.

Silver ion TLC of the SD_1 and SD_2 fraction followed by isolation of the individual monoterpene alcohols established the pattern of distribution of label between the three monoterpene alcohols and the aldehydes neral/geranial. This group of monoterpene derivatives accounted for 79% of the label in the "free" fraction (SD_1) and 94.7% in the SD_2 fraction. In both cases geraniol was the major labelled monoterpene alcohol accounting for 69.7 and 89.7% of the recovered radioactivity in the SD_1 and SD_2 fractions, respectively.

Recent evidence has emerged indicating that two pathways operate in higher plants for the biosynthesis of terpenoids. Plant isoprenoid synthesised in the cytosol and endoplasmic reticulum, e.g. phytosterols, are generally produced via MVA derived isopentenyl pyrophosphate (IPP) (McGarvey and Croteau, 1995) Plastid isoprenoids on the other hand such as the terpenoid moieties of phylloquinone, the tocopherols and tocotrienols are derived from IPP produced via the Rohmer pathway itself initiated by the condensation of pyruvate with D-glyceraldehyde-3-phosphate (Rohmer, 1999) The results obtained above with Lady Seton incubated with 2-^{14}C-MVA suggested that the prevalent pathway in rose flower and petal was the MVA rather than the Rohmer route. Support for this supposition was provided by double labelling experiments conducted on the rose Fragrant Cloud. This latter was chosen in view of the fact that monoterpenyl fatty acyl esters were also identified in this tissue and the fact that it contained significantly higher content of citronellol than Lady Seton petal tissue. Incubation experiments were carried out as described in Section 3.5 using MVA stereo-specifically labelled with (4*R*-4-^{3}H)- or (4*S*-^{3}H)-MVA mixed with 2-^{14}C-MVA. It has been shown that in the conversion of MVA to IPP there is specific loss of a proton originally

on C-4 of MVA, which becomes C-2 of IPP. Conversion of IPP to dimethylallyl pyrophosphate (DMAPP) and subsequent condensation of IPP with DMAPP does not change this proton further. The stereochemistry of this proton elimination is related to the geometry of the resulting double bond formed in IPP (Popjak and Cornforth, 1966) Thus H_s is eliminated if a *trans* double bond is formed as in the case of *all trans*-farnesol as does the converse apply for the *cis* double bond formation for poly-*cis* isomer found in rubber (Archer et al., 1966) The results of this experiment are shown in Table 5. The free monoterpene alcohols only were isolated in this case rather than the esterified or glucosylated C-10 fractions since the free alcohol fractions were responsible for ca. 54–58% of the ^{14}C incorporated into these three fractions. It is clear from the ratio of $^{3}H/^{14}C$ in the isolated monoterpene alcohols that the formation of geraniol, nerol and β-citronellol involves the elimination of the 4-S proton of MVA; the average ratio over the three alcohols compared to the MVA substrate ratio being 0.09 versus 1.85 for $^{3}H/^{14}C$, respectively. Conversely the 4-*R* proton is retained with average ratios of $^{3}H/^{14}C$ for the combined alcohols to MVA being 2.32 versus 2.32, respectively. This data confirms that the formation of geraniol involves proton elimination that results in the formation of a *trans* double bond. Of equal importance is the fact that both *cis*-nerol and the 2,3-dihydro compound β-citronellol are also produced by a *trans* mechanism presumably from geraniol or a derivative of the same. Potential candidates are geraniol, the glycoside or an esterified form such as the pyrophosphate or even the fatty acyl ester. In the case of nerol formation an isomerisation reaction may be invoked whilst the formation of citronellol is likely to proceed via a reduction probably utilising reduced nucleotide. Based on the above double labelling experiments β-citronellol may originate from either geraniol or nerol. Further experimentation with acetone powder extracts of Fragrant Cloud in the presence of NADPH or NADH confirmed that the former was the preferred reducing agent. Substrate specificity for the reduction reaction confirmed that both alcohols geraniol and nerol were equally effective substrates and that the product in each case was β-citronellol (Table 6). There was no evidence for the reduction of the double bond at the 6,7 position to form 6,7-dihydronerol or the corresponding 6,7-dihydrogeraniol. The enzyme preparation appeared to be specific for primary allylic terpene alcohols whilst tertiary allylic alcohols, acetate or pyrophosphate esters or cyclic terpenols were not reduced to any significant extent (see also Table 6). The enzymatic preparation exhibited optimum reductase activity at pH 8.0. The reductase reaction was completely inhibited by pre-incubation with 5 mM *p*-chloromercuriphenyl sulphonate which inhibition was totally reversed by pre-incubation with 5 mm reduced glutathione. Iodoacetic acid and *n*-ethylmaleimide were without effect on the reductase capability of the enzyme preparation. The above series of experiments verify the MVA rather than the Rohmer route to the monoterpene alcohols in whole rose flowers as well as petal disc and petal epidermal tissue for both Lady Seton and Fragrant Cloud varieties. It is equally clear that geraniol or a functional derivate thereof is the biogenetic precursor of nerol. Evidence from enzyme studies demonstrate that both geraniol and nerol are capable of conversion to β-citronellol by an enzyme preparation probably with a reactive site –SH group that catalyses the NADPH reduction of the 2,3-*trans*- or *cis*-double bonds. This data is consistent with comparable experiments with doubly labelled MVA incubated with whole flowers of Lady Seton where the biosynthesis of geranol and nerol and their corresponding glucosides were biogenetically formed by elimination of the 4-S proton of MVA, a *trans* process (Francis et al., 1970).

Table 6
Substrate specificity of the terpenoid reductase from petals of the hybrid tea rose Fragrant Cloud

Substrate	Product	Relative reductase activity
Nerol	Citronellol	100
Geraniol	Citronellol	100[a]
Geranial	Citronellol, geraniol[b]	90
Geranyl acetate	–[c]	0
Geranyl pyrophosphate	–	0
Geranic acid	–	0
Linalool	–	0
cis/*trans*-Farnesol	2,3-Dihydrofarnesol	34
Limonene	–	0
Carveol	Dihydrocarveol	12
(±)-Carvone	–	0
Pulegone	–	0
trans-2-Hexenol	*n*-Hexanol	8

[a] Activity corresponds to 57% conversion of geraniol to citronellol after 60 min at 30 °C using substrates and cofactors at levels given in Section 3.8.
[b] Both geraniol and citronellol produced by enzymatic reduction.
[c] 30% hydrolysis to geraniol occurred.

Table 5
$^{3}H/^{14}C$ ratios for monoterpene alcohols derived from incubation of (4*R*)- and (4*S*)-4-$^{3}H/2^{14}C$ mevalonate[a] with an intact flower of the hybrid tea rose Fragrant Cloud

Compound	(4*R*)-4-^{3}H- + 2-^{14}C MVA			(4*S*)-4-^{3}H- + 2-^{14}C MVA		
	dpm ^{14}C	dpm ^{3}H	$^{3}H/^{14}C$	dpm ^{14}C	dpm ^{3}H	$^{3}H/^{14}C$
Geraniol	67,370	159,600	2.37	36,400	2910	0.08
Nerol	10,370	23,020	2.22	6730	605	0.09
β-Citronellol	9500	22,620	2.38	7900	870	0.11

[a] Radioactive mevalonate was added as follows: (4*R*)-4-^{3}H-MVA (4.64 μC) + 2-^{14}C-MVA (2 μC), $^{3}H/^{14}C = 2.32$ or (4*S*)-4-^{3}H-MVA (3.70 μC) + 2-^{14}C-MVA (2 μC), $^{3}H/^{14}C = 1.85$.

Preliminary studies had demonstrated that isolated petal discs of rose petals were capable, of the biosynthesis of squalene, phytosterols, the terpene moiety of the monoterpene fatty acyl esters and the glycosides. This indicated the presence in the petal tissue of the enzyme systems for the conversion of MVA into monoterpene alcohols, the terpenoid component of the glycosides and the fatty acyl esters as well as the higher terpenes squalene and the phytosterols. Small strips of upper epidermal cell from Lady Seton petals, essentially free of underlying mesophyll cells, as observed by light microscopy, were collected and immediately placed in water (2 ml) containing 1 μC 2-^{14}C MVA, vacuum infiltrated as described in Section 3.5 and incubated at 22 °C for 3 h. At the end of the incubation period 2 g of untreated petals were added to the incubated epidermal cells and the material extracted to yield as previously an SD_1, SD_2, and an L_1 fraction. The yield of label from ^{14}C-MVA into these three fractions was 6.6%, approximately 14% of the uptake found for whole flowers. It should be borne in mind that in the case of whole flowers there was minimum tissue damage and probably more importantly that all of the radio-label was taken up by the whole flower during transpiration whilst in the case of the petal tissue bathed in solution only a fraction of the total radioactivity would be assimilated. It is clear however that petal epidermal tissue has the biosynthetic capacity to produce monoterpenes from MVA.The distribution of label between the fractions SD_1. SD_2 and L_1 was 28.4%, 26.9% and 44.7%, respectively. Further analysis of the lipid fraction (L_1) by TLC confirmed a similar pattern of distribution to that found in the whole flower experiment (see Table 4). These findings demonstrate that the biosynthesis of the whole family of the monoterpene alcohols and their functionalised derivatives (fatty acyl esters and glycosides) are synthesised in the specialised cells of the petal epidermis. In situ studies on the protein associated with the enzyme *S*-adenosyl-L-methionine:benzoic acid carboxymethyltransferase (BAMT) which catalyses the formation of methyl benzoate in snapdragon flowers has demonstrated its localisation primarily in the inner epidermal cells of the upper petals (Dudareva, 2001).

Despite the relatively low level of longer chain unsaturated fatty acids esterified to monoterpene alcohols in rose petals (oleic acid at 2.9% of the total esterified fatty acids in Lady Seton – see Table 1) incubation experiments were carried out with ^{14}C oleic acid (18:1). To 1 g petal discs of Lady Seton was added 10 μC of 1-^{14}C-18:1 as prepared in 3.5. After vacuum infiltration of the substrate into the discs the reaction was allowed to proceed for 6 h at 22 °C. After this time the discs were harvested, washed with water and lipids extracted with chloroform/methanol. The incorporation of label into the neutral lipid fraction (NL) was 1.5%. This fraction was separated into its principal lipid groups by TLC as described for the fractionation of the L_1 lipid extract above. The primary labelled contributors were triglyceride (78.3%) with 9.3% in the terpenyl fatty acyl ester fraction (Table 7). There was virtually no incorporation (0.2% of label) into the petal hydrocarbon fraction. The terpenyl fatty acyl ester fraction was isolated by preparative TLC and the purified ester was hydrolyses in ethanolic alkali followed by work-up to yield the alcohol and fatty acid soaps, the latter isolated as the free acids. The TLC of this hydrolysed fraction yielded 83% of the label in the free fatty acid with approx 16% co-chromatographing with geraniol. The latter may be due to contaminating non-terpenoids migrating, on TLC, with geraniol and/or to enzymatic oxidative degradation of the 18:1 substrate to acetate during the incubation phase which can then form MVA and thus the monoterpenes. Alkaline hydrolysis of the isolated triglyceride fraction yielded label only in the fatty acids with 18:1 accounting for 86% of the label with the remaining 14% associated with linoleic acid (18:2). In this latter case it is likely tha18:1 is converted to 18:2 by de-saturation (Somerville et al., 2000) though this would need to be confirmed by more detailed chemical analysis of the position of the radio-label in the diene fatty acid. The evidence from these labelling studies confirm that 18:1 is esterified to the terpene alcohol geraniol by petal tissues though the main focus for labelling from substrate 18:1 was into triglycerides.

Table 7
Distribution of label from ^{14}C 18:1 in the neutral lipids of Lady Seton petals

Compound group	% of ^{14}C label
Hydrocarbons	0.2
Sterol ester	0.7
Monoterpenyl fatty acyl esters	9.3
Triglyceride	78.3
Sterols	3.4
Polar lipids	8.1

The above finding of terpene alcohols esterified to long chain saturated fatty acids poses a number of interesting features from both their biogenetic origin, localisation of the enzymes within the petal epidermis, their functional role in the lifetime of the flower and finally the possible commercial value of terpenyl fatty acyl ester in rose essential oil production. The biosynthetic pathways to glycosides is well established, UDP glucose being the glucose donor, the reaction being catalysed by specific UDP glucose:glucosyl transferases. In tobacco leaves (*Nicotiana tabacum* L) UDP-glucose:salicylic acid 3-*O*-glucosyltransferase catalyses the conversion of salicylic acid to β-*O*-D-glucosyl salicylic acid in response to infection by tobacco mosaic virus (Enyedi and Raskin, 1993; Lee et al., 1995). Ester formation particularly volatile esters (e.g. acetates) impart distinct and characteristic floral scents in many plants. In this context low levels of volatile esters have been isolated from *R. damascena* Miller oil (Bayrak and Akgul, 1994). Geranyl acetate was the dominant ester in the oil, at 0.36 to 2.27%, with lower levels of citronellyl acetate and formate. These short chain esters were reported to contribute fresh top notes to the oil. The mechanism of formation of esters of this type is via alcohol acyl transferases (AAT).

The enzyme isolated from *Clarkia breweri* petals, acetyl-CoA:benzyl alcohol acetyl-transferase (BEAT) catalyses the formation of benzyl acetate from benzyl alcohol and acetyl-CoA (Dudareva et al., 1998). A similar mechanism exist for the biosynthesis of triglycerides in oil bodies where acyl-CoA:glycerol acyltransferases couple phosphorylated glycerol or its partially acylated derivative to the corresponding long chain esters (Budziszewski et al., 1996). In the case of the monoterpene alcohols in rose petals the reaction is likely to be between the monoterpene alcohol and the acyl-CoA, in the case of Lady Seton petals geraniol and stearoyl-CoA catalysed by the punative stearoyl-CoA:geraniol transferase. This reaction parallels that of BEAT in producing the scent compound benzyl acetate in petals of *C.breweri.* Although this enzyme has not been demonstrated, as yet, in rose petals the incorporation of radioactivity from oleic acid and MVA gives tacit support for this position.

Plants produce a more varied collection of terpenoids than animals or microorganisms a situation reflected in the complex organisation of plant terpenoid biosynthetic pathways at the cellular, sub-cellular and genetic levels. In plants biosynthesis, accumulation and secretion of terpenoids is usually associated with the presence of specialised anatomical structures. These include glandular trichomes, secretory cavities of leaves and the specialised epidermal cells of flower petals (Turner et al., 1999). This specialisation and complexity is further extended in the organisation of the formation pathway to isopentenyl pyrophosphate (IPP) the universal C_5 terpenoid precursor. In plants this compound is derived via two distinct and independent routes; the MVA or the more recently discovered deoxyxylulose-5-phosphate (DXP) or Rohmer pathway as described above. Rose petal epidermal cells clearly represent non-photosynthetic tissue that has retained the MVA pathway for the biosynthesis of monoterpene alcohols as well as the monoterpene components of the lipid-soluble long chain fatty acyl esters and the water-soluble glycosides. Squalene and phytosterols are formed in the flower petal via the same route.

The presence of a second derivative of the monoterpene alcohols namely the long chain fatty acyl esters provides the plant with a lipophilic, non-volatile, precursor form of this volatile C_{10} family. This derivative is present in the waxy surface layer of the petal epidermis and is probably capable of acting as a source of the terpene alcohols following lipase or esterase hydrolysis. The effect of this fraction on the potential for scent release merits further consideration. It is interesting to note in this context that feeding cut roses with β-phenylethyl-β-D-glucopyranoside, at 0.5% in solution, results in a 500 times elevation of the hydrolysis product β-phenyl ethanol in the florets (Ikemoto et al., 2004). It is quite possible therefore that the both glucoside and fatty acyl esters may provide facile availability of the terpene alcohols by enzymatic hydrolysis. The different solubilities of ester and glucoside and residence of the former in the petal surface extractable layer may add a site specific element to this process. The water-soluble glucoside may function as a transportable or storage form of the terpenoids and the benzenoids. As noted above β-phenyl ethanol and benzyl alcohol occurred only to any significant extent as the free alcohols and glycosides with only very low levels as the volatile acetate but with virtually none esterified to long chain fatty acids. Studies on aroma evolution during flower opening in *R. damascena* Mill provided some support for a differential functionalisation of the phenylpropanoids as represented by 2-phenyl ethanol and the monoterpenes typified by geraniol (Oka et al., 1999). It appears that the early formed β-D-glucopyranoside of 2-phenyl ethanol, which accumulates at least 12 h before flower opening may act as a reservoir of the benzenoid alcohol, the latter being liberated during the later opening bud stage and coincidental with a five fold increase in petal β-glucosidase activity. The glycosylated terpenols on the other hand accumulated maximally at the partly opened flower stage and were much less significant in quantitative terms than the free terpenols. If this situation is extrapolated to Lady Seton flowers it is interesting to speculate that the glycosides may act principally as reservoirs of the phenylpropanoids whilst the fatty acyl esters could act as a sink and source of the terpenols. This differentiation between phenylpropanoids and terpenols with respect to their possible precursor forms could be a consequence of either or both of their biogenetic origins and the sites of biosynthesis of the parent alcohols and their derivatisation to the appropriate glucosides or fatty acyl esters. The evidence in Table 2 supports the quantitative differences between the two families regarding the importance of the sugar or fatty acyl bound terpene alcohols. This situation provides complex options for the management and control of scent release by the plant from the flower by manipulation of the two distinct biosynthetic pathways of the volatiles as well as the liberation of the same compounds from the differently localised and solubilised bound scent precursors. The recent advent of real time aroma release measurement by proton transfer reaction mass spectrometry makes the monitoring of release of aroma compounds from intact flowers possible. Application of this technology to the release of aroma compounds from flowers in their natural state and under the influence of specific treatment is now possible (Dunphy, 2003).

From a commercial perspective the esterified form of the terpene alcohols is capable of providing an additional and significant contribution to the volatiles yield following suitable treatment. The terpenoid precursor fraction is not steam distillable and would require selective extraction with solvent from petal tissue followed by hydrolysis or recovery directly from the tissue by steam distillation preceded by chemical or enzymatic hydrolysis. Such a process could supplement the free fraction and the aroma compounds derived from the water transportable glycosidically bound components after glucoside hydrolysis thus increasing the economic and commercial yield of rose essential oils.

3. Experimental

3.1. Plant material

The hybrid tea rose Lady Seton was obtained from W. P. Vincent, Scarisbrick, Southport, UK, and was grown in a greenhouse at 22 °C with 16 h days provided by unscreened mercury vapour lamps at a distance of about 1metre. Flowers were selected for experiment at the "partly opened" stage, approximately 7 days after breaking of the calyx (Francis and Allcock, 1969).

3.2. Chemicals

Pure monoterpene and aromatic alcohols were obtained from Aldrich as were myristoyl, palmitoyl and stearoyl halides. Sodium dihydro-bis-(2 methoxyethoxy)-aluminate was purchased from Charles Druce, London. Acid washed alumina was purchased from M. Woelm, Eschwage, Germany. β-Glucosidase from almonds was obtained from Sigma, London. Radio-labelled substrates used for incubation experiments were (2)-^{14}C-(±)-mevalonic acid as the dibenzylethylene diamine salt, 5.3 mC/mM, (New England Nuclear, Boston, Mass., USA). (4*R*-4-^{3}H)- or (4*S*-^{3}H)-MVA as the lactones and 1-^{14}C oleic acid were purchased from the Radiochemical Centre, Amersham International, Bucks., UK.

3.3. Extraction of lipids

Free monoterpenes were isolated from rose petal tissue by steam distillation as described (Francis and Allcock, 1969). Petals were ground to a fine powder, after freezing in liquid nitrogen. Steam distillation followed by extraction of the aqueous distillate with diethyl ether gave free terpenols and benzenoid alcohols (SD_1 fraction). The residue, on cooling, was adjusted to pH 5.0 with sodium acetate buffer (0.1 M) and incubated with β-glucosidase at 40 °C for 3 h. Repetition of the distillation and solvent extraction yielded volatile organics originally bound as their glycosides (SD_2 fraction). Other non-volatile lipids were extracted from the residual tissue with 2:1 v/v chloroform/methanol (the L_1 fraction) (Folch and Lees, 1957). The residual aqueous/methanol fraction was adjusted to pH 2.0 with hydrochloric acid (0.2 N) then extracted with diethyl ether to yield an acidified lipid extract (LH^+ fraction). All four fractions were dried over anhydrous sodium sulphate to remove water then passed through a bed of silica gel H (4 × 1 cm) eluting with 25 ml diethyl in order to recover the medium polarity lipids whilst retaining polar lipids and any residual radioactive substrate on the silica bed. Epidermal lipids were isolated by gently stirring the fresh petals, 10 g, with 2 × 400 ml petroleum ether, b.p. 40–60 °C for 15 s at room temperature. The hydrocarbon solvent containing the epidermal lipids was filtered off and the solvent removed by evaporation. The residual tissue was extracted with 300 ml chloroform/methanol, 2:1, to isolate the remaining lipids.

3.4. Lipid fractionation

Terpene and benzenoid alcohols were separated by GC on 10% FFAP (on 100–200 mesh celite) with N_2 as carrier gas at a flow rate of 60 ml/min, isothermally at 145 °C using an FID detector. Injector and detectors were set at 200 °C. Identification and quantitation was by comparison with standards run under the same conditions. Terpenyl fatty acyl esters were isolated from lipid extracts by column chromatography on acid washed alumina (deactivated with 6% water). Petroleum ether (b.p. 40–60 °C) eluted hydrocarbons, 2% v/v diethyl ether in petroleum ether eluted fatty acyl esters while sterols were completely removed from the column with 20% diethyl ether in petroleum ether. For each g of lipid chromatographed 100 g of alumina was used and 500 ml of each eluting solvent was applied. Esters were further purified by preparative TLC on silica gel H (solvent system 5% diethyl ether in petroleum ether, v/v). Resolution of terpenyl fatty acyl esters into their individual components was achieved by GC on a 5 ft column of 3% silicone OV17 (on gas chrom Q, 60–80 mesh), carrier gas nitrogen, flow rate 60 ml/min isothermally at 230 °C or programmed from 200 to 260 °C at 2 °C/min. Adsorption TLC was used to separate the non-volatile lipids into their individual functional types. On silica gel H the L_1 fraction was fully developed with the solvent mixture petroleum ether (b.p. 40–60 °C)/toluene (1:1 v/v), to separate the hydrocarbon and ester groups then chromatographed twice more in succession to approximately 30% of the plate length with diethyl ether/petroleum ether (3:7 v/v) to develop the more polar sterol family. Compound identification at this stage was achieved by co-chromatography with authentic standards and by the characteristic colour developed on staining with the vanillin/sulphuric acid reagent (Krebs et al., 1969). Fractionation of esters was also possible by reversed-phase TLC on paraffin impregnated silica gel H using 5% aqueous acetone, saturated with liquid paraffin, as developing solvent. Esters were quantified on GC by comparison with known amounts of geranyl stearate or by reduction of the esters followed by quantification of the liberated terpene alcohols, by GC. Esterified fatty acids were analysed following alkaline hydrolysis, then acidification and conversion of the acids to the methyl esters using diazomethane or by transmethylation of the terpenyl fatty acyl esters with a mixture of benzene/methanol/sulphuric acid (10:20:1 v/v). GC on FFAP at 190 °C gave good resolution of the methyl esters of fatty acids ranging from C_2 to C_{26}. Samples of individual monoterpenyl fatty acyl esters were synthesised by reaction of the terpene alcohol with the appropriate acyl halide in the presence of a trace of pyridine at 20 °C for 16 h. Acyl halides not readily available, e.g. arachidyl chloride, were produced by reacting the fatty acid with excess oxalyl chloride reagent and removal of any unreacted reagent, under vacuum (Pinter et al., 1964). The free terpene alcohols citronellol, nerol and geraniol were separated from each other by TLC on $AgNO_3$-Silica gel H developing with ethyl acetate/toluene (30/70 v/v).

3.5. ^{14}Carbon incubation experiments

Incubation experiments were carried out on intact flowers of Lady Seton, petal discs (0.75 mm diameter cut with a cork borer) or an epidermal cell layer. The epidermal tissue was obtained from the adaxial petal surface by carefully tearing off pieces of the epidermal cell sheet. For whole flower experiments the flower stem was cut under water, to avoid air incursion into the transpiration stream, and narrow bore silicone tubing was fitted over the cut end of the stem. The tubing was then filled with water and the flower inverted. The flower stem was then used as the route for introduction of radioactive substrate via the transpiration stream. For MVA experiments both singly and double labelled (^{14}C or ^{14}C/^{3}H) radio-chemicals were applied. Following uptake of the label, ca. 15 min for 100 μl solution with the intact flowers, water was added to the silicone tube to maintain the fluid flow for the remainder of the experiment. For petal only experiments the discs were placed in the medium containing the labelled substrate and exposed to a slight vacuum, via a water pump for 2 s to displace air from the petal discs and to infiltrate the substrate. Epidermal cell layers were treated also in this way.

The radio-labelled oleic acid was prepared for incubation by adding it to 6 ml water containing 30 μl of 1% sodium carbonate and 70 μl of Tween 20. The aqueous mixture was sonicated for 15 s before adding the petal discs at room temperature.

3.6. ^{14}C counting

All samples were assayed in a Packard Tricarb Liquid Scintillation Counter. Radioactive materials were transferred directly to scintillation vials and any solvent removed in a stream of nitrogen at 20 °C. The scintillation mixture was 2,5-diphenyloxazol (5 g) as the primary and dimethyl-1,4-bis-[2(5-phenoxazol)-benzene] (0.3 g) as the secondary scintillant, these both dissolved in toluene (1 L). Samples from TLC were added directly to the above scintillant using the suspending agent Cab-O-Sil at the 4% level based on toluene. This procedure achieved high ^{14}C counting efficiency with no observed quenching up to 100 mg silica gel/15 ml scintillant (Packard Instrument Company Incorporated, Box 428, La Grange, IL, USA).

3.7. Preparation of enzymes

The petals from freshly picked Fragrant Cloud flowers were frozen in liquid nitrogen and ground to a fine powder which was washed four times with cold acetone. This dehydration step in addition to producing an acetone powder also removed terpenoids and other lipid compounds. Residual acetone was removed in vacuo and the powder stored at −8 °C under dry conditions until required. A soluble enzyme extract was prepared by stirring 1 g of acetone powder with 25 ml sodium phosphate buffer (0.1 M, pH 8.0) containing 25 mM 2-mercaptoethanol for 30 min at 2 °C. The suspension was filtered through two layers of cheesecloth and the residue discarded. To the clear supernatant was added ammonium sulphate to 70% saturation and the mixture stirred for 30 min then centrifuged at ca. 2000*g*. The precipitated protein was collected and re-suspended in sodium phosphate buffer (0.1 M, pH 8.0) and centrifuged at 1700*g* for 15 min to remove insoluble polysaccharides. The resulting clear colourless solution was used for incubation studies.

3.8. Enzyme incubations

These were carried out at 30 °C using 2 ml of the above enzyme preparation (approx. 2 mg protein/ml) with added nucleotide (1.2 μmol/ml) and terpene (124 μmol/ml) the latter added as a 5% solution in acetone. Reaction products were extracted with diethyl ether and analysed by GLC as described in Section 3.4. Reaction products were identified by comparison with known standards and by combined GC–mass spectrometry.

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