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GERANIOL β-D-GLUCOSIDE; OCCURRENCE AND SYNTHESIS IN ROSE FLOWERS

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Abstract—The β-D-glucosides of geraniol, nerol and citronellol have been isolated from the flowers of the Hybrid Tea rose "Lady Seton". The formation of both free monoterpenes and of the monoterpene β-D-glucosides was followed as a function of flower maturation. Little free or combined monoterpene is present until the flowers begin to open when accumulation of both free and bound monoterpene occurs. Both forms are present at maximum levels of 100–200 μg monoterpene per gramme wet weight petals although the maximum levels of monoterpene β-D-glucosides occur at an earlier stage of flower maturity than do those of the free monoterpenes.

INTRODUCTION

A NUMBER of plant species have been found to contain monoterpene β-D-glucosides.[1–4] The monoterpene moieties vary with the plant species but all the structures which have been determined include a cyclized carbon skeleton based on a fused cyclopentane-tetrahydropyran ring system.[1–4] The biosynthesis of one member of this group, plumierin, has been studied and the carbon skeleton shown to be derived from mevalonic acid.[5] In addition indirect evidence has suggested that monoterpene β-D-glucosides (bound monoterpenes) are present in *Pelargonium odoratissimum*[6] and in the flowers of *Rosa damascena* Mill. cv.[7]

The information available at present gives no indication of the concentrations of free monoterpenes relative to monoterpene β-D-glucosides, nor of their metabolic interrelationships. Similarly, although the development of the essential oil content of *R. damascena* Mill. cv. flowers has been studied as a function of flower maturation,[8] no corresponding information is available regarding the accumulation of individual free and bound monoterpenes during similar periods.

This paper reports studies on the accumulation of free and bound monoterpenes during maturation of the flowers of the Hybrid Tea rose "Lady Seton", and describes the detailed chemical characterization of the major monoterpenoids.

RESULTS

Free Monoterpenes

Extracts containing free monoterpenes were obtained from petals of fully opened flowers of the rose "Lady Seton", both by direct steam distillation of the petals or by solvent extrac-

[1] H. SCHMID, H. BICKEL and TH. M. MEIJER, *Helv. Chim. Acta* **35**, 415 (1952).
[2] K. SHETH and E. RAMSTAD, *Tetrahedron Letters* 321 (1961).
[3] J. GRIMSHAW, *Chem. & Ind.* 403 (1961).
[4] H. INOUYE, S. INOUYE, N. SHIMOKOVA and M. OKOGAWA, *Tetrahedron Letters* 683 (1968).
[5] D. A. YEOWELL and H. SCHMID, *Experientia* **20**, 250 (1964).
[6] E. BOURQUELOT and M. BORDEL, *C.R. Acad. Sci., Paris* **157**, 72 (1913).
[7] V. M. STAIKOV and G. D. ZOLOTOVITCH, *C.R. Acad. Bulg. Sci.* **13**, 735 (1960).
[8] G. D. ZOLOTOVITCH, M. NIKOLOVA and M. ZOLOTOVITCH, *C.R. Acad. Bulg. Sci.* **14**, 839 (1961).

tion (see Experimental). Gas–liquid chromatography (GLC) and combined gas–liquid chromatography–mass spectrometry (GC–MS) of these two extracts showed that they possessed a very similar qualitative and quantitative monoterpene composition, although the material extracted with solvent contained numerous higher terpenes and non-terpenoid substances not observed in the steam-volatile fraction. The major component (Peak F, see Fig. 1A) coincided in relative retention volume with added authentic geraniol and Peaks B, C and D with citral, citronellol and nerol respectively. Comparative GC–MS and TLC analyses with known standards confirmed these identifications.

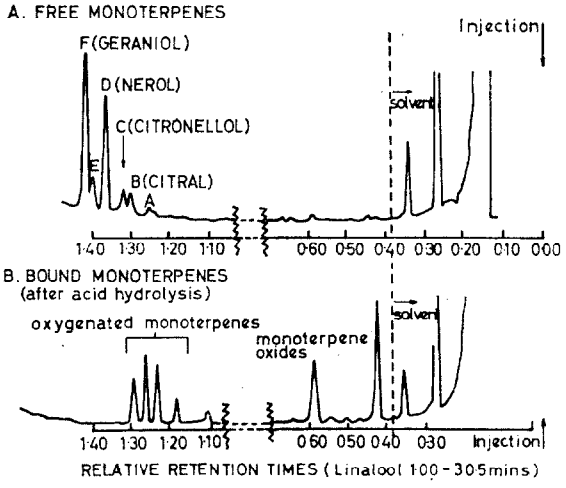

FIG. 1. GLC ANALYSIS OF PETAL MONOTERPENES.

Petals were steam distilled before and after addition of 11 N HCl to pH 0·6. Monoterpenes were extracted from the steam distillates with 0·50 ml petroleum ether. Analyses for monoterpenes in the petroleum ether layers were performed by GLC on 2·75 m × 4 mm helical glass columns packed with 10% "FFAP" on 100–120 mesh celite with a N_2 flow rate of 60 ml/min and temperature programmed at 3°/min from 50–220°. Quantitation was achieved by comparison with standard geraniol solutions.

Bound Monoterpenes

Acid treatment of the non-steam-volatile residues of rose petals, and of the petroleum-extracted methanol-soluble fraction from fresh petals, liberated further quantities of steam-volatile and petroleum-extractable substances. GLC, GC–MS and TLC analyses against standards indicated these substances to be monoterpenes. These observations suggested the presence of acid labile, polar monoterpene derivatives in rose petals. Such derivatives were subsequently isolated from an aqueous extract of petals by a combination of chromatographic and electrophoretic techniques (see Experimental).

Acid hydrolysis of these water-soluble derivatives released monoterpenes (Fig. 1B) and sugars. TLC and paper chromatography showed D-glucose to be the only carbohydrate detectable, and it was subsequently established that these water-soluble derivatives contain monoterpene alcohols and D-glucose in equimolar proportions. Hydrolysis of the intact glucosides with β-glucosidase and GLC, GC–MS and TLC analyses of the released monoterpenes showed the major component to be geraniol (80–85 per cent) with smaller concentrations of nerol and citronellol.

Thus the major water-soluble monoterpene derivative in the flowers of the rose "Lady Seton" appeared to be geraniol-β-D-glucoside. The identity of this material was confirmed by a comparison of its behaviour during TLC (Table 1) and thin-layer electrophoresis with that of authentic geraniol β-D-glucoside. Geraniol β-D-glucoside was also shown to be present in the flowers of *Rosa damascena*, cv. *versicolora*.

TABLE 1. TLC OF ISOLATED BOUND MONOTERPENES

Compounds	R_f's		
	Development solvents		
	A EtOH–EtOAc (15:85, v/v)	B *n*-ProH–EtOAc–H_2O (6:3:1, v/v/v)	C $CHCl_3$–HOAc–MeOH (85:2:13, v/v/v)
Purified bound monoterpenes	0·47–0·53	0·73–0·77	0·33–0·39
Chemically synthesized geraniol β-D-glucoside	0·48–0·53	0·73–0·77	0·32–0·36
Enzymatically synthesized geraniol β-D-glucoside	0·49–0·53	—	0·33–0·36
Mixture of above	0·46–0·52	0·69–0·74	0·30–0·36

Kieselgel G layers of 0·25 mm thickness were used. The plates were developed for 15 cm at 2°, air dried and the bound monoterpenes visualized with the vanillin–sulphuric acid spray.[16]

Accumulation of Free Monoterpenes and of Monoterpene β-D-Glucosides During Flower Maturation

Rose petals were taken at various stages of flower maturation and the content of free monoterpenes and of monoterpene β-D-glucosides was determined by steam distillations of

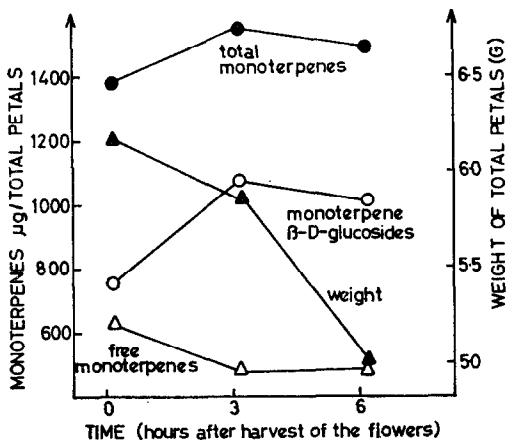

FIG. 2. CHANGES IN WEIGHT AND MONOTERPENE CONCENTRATIONS DURING STORAGE OF HARVESTED PETALS.

Petals from fully opened flowers were divided into three portions, care being taken to distribute the petals randomly within each sample. The petals were weighed and stored in air at 16° for up to 6 hr. The petals were re-weighed at known times and free monoterpene and monoterpene β-D-glucoside concentrations determined by GLC (see legend to Fig. 1 for details).

Table 2. Amounts of free monoterpenes and of monoterpene β-D-glucosides from petals at various stages of maturation

Maturation stage	Days from initial splitting of calyx			No. flowers analysed	Weight of petals		Monoterpene content (μg/total petals)					
							Free monoterpenes		Monoterpene β-D-glucosides		Total monoterpenes	
	No. estimations	Range (days)	Average (days)		Range (g)	Average (g)	Range	Average	Range	Average	Range	Average
(*a*) Buds	—	1–4	—	8	0·5–3·1	—	—	0	—	0	—	0
(*b*) Buds just opening	14	3–8	6·0	4	3·7–5·5	4·9	36–170	90	120–340	210	155–355	300
Just opening flowers	18	4–8	6·4	2	4.0–4·6	4·4	150	150	400–470	435	550–620	585
(*c*) Opening flowers	11	4–9	6·8	2	4·0–4·4	4·2	150–180	165	400–565	480	550–745	650
Opening—partly opened flowers	—	—	7·4	2	4·4	4·4	195–265	230	660 700	680	895–925	910
(*d*) Partly opened flowers	20	5–11	8·1	7	4·4–8·0	7·1	225–770	540	780–1500	1010	875–2100	1550
(*e*) Fully opened flowers												
(i) Petals	18	6–11	8·7	7	6·3–10·8	7·8	660-1240	890	550–1300	795	1450–2650	1685
(ii) Other flower parts				6	0·8–1·5	—	10–20	16	10–18	14	20–38	30
Fully opened—blown flowers	—	—	—	1	—	~10	—	~1000	—	~700	—	~1700
(*f*) Blown flowers	4	10–14	12·2	5	8·3–10·0	9·2	590–780	710	50–800	505	800–1600	1215

Details of the maturation stages are given in the Experimental. Free monoterpene and monoterpene β-D-glucoside concentrations were determined by GLC (see legend to Fig. 1 for details).

the freshly harvested petals before and after acid treatment. Significant variations in composition between petals of a single flower were noted. The concentration of free monoterpenes and of monoterpene β-D-glucosides was greater in the inner petals than in the outer petals and in the distal ends of the petals than in the proximal ends of the petals. Free monoterpene

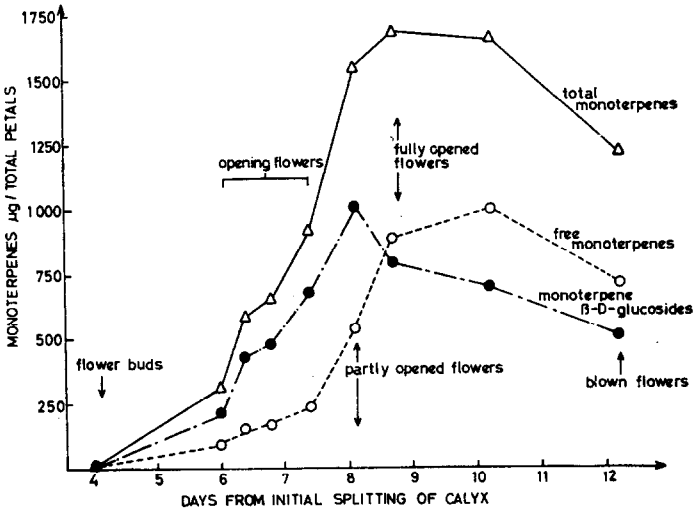

FIG. 3. AMOUNTS OF FREE MONOTERPENES AND OF MONOTERPENE β-D-GLUCOSIDES IN THE PETALS FROM FLOWERS AT VARIOUS STAGES OF MATURATION.

Petals were taken at various stages of flower maturation as measured from the time when the calyx of the flower bud had just begun to split (see Experimental). Free monoterpene and monoterpene β-D-glucoside concentrations were determined by GLC (see legend to Fig. 1 for details).

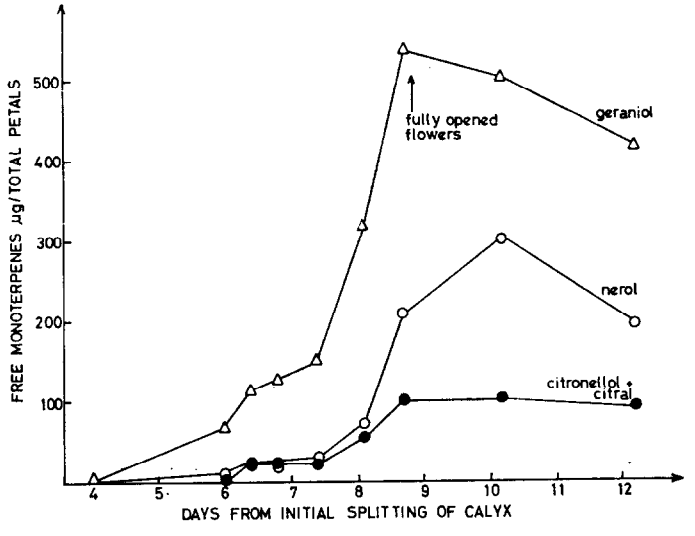

FIG. 4. AMOUNTS OF FREE GERANIOL, FREE NEROL AND OF FREE CITRONELLOL AND CITRAL IN THE PETALS FROM FLOWERS AT VARIOUS STAGES OF MATURATION.

The conditions of extraction and estimation are the same as in Fig. 3. The amounts of citronellol and citral have been combined as these monoterpenes chromatogram as a poorly resolved double peak (peaks B and C in Fig. 1A).

concentrations were greater than those of monoterpene β-D-glucosides in both the distal and proximal ends of the petals. Figure 2 illustrates the marked changes which occurred when petals were stored in air prior to steam distillation.

Table 2 and Figs. 3 and 4 indicate the changes which occurred between the amounts of free monoterpenes and of monoterpene β-D-glucosides in the petals from flowers at different stages of maturity. Considerable difficulty was found in measuring the maturation stage of each flower, since chronological and physiological ages are not necessarily comparable. The weights of the harvested flowers varied considerably (from 6–11 g for fully opened flowers) and suffered from the disadvantage that weight estimations could be obtained from each flower at only one stage of maturation. The most suitable measure of flower maturation was found to be the time each flower took to reach a particular maturation stage as measured from the time when its calyx had just begun to split; this method had the important practical advantage that the time to reach several maturation stages could be taken from each flower.

DISCUSSION

Free Monoterpenes in Rose Petals

Free monoterpenes were isolated from petals from fully opened flowers of the rose "Lady Seton" in a yield of 650–1250 μg per flower (0·01–0·02 per cent by weight). Little difference was observed in the results according to the isolation procedure adopted, and although the solvent-extraction technique isolated a larger quantity of material than did steam distillation, the difference was largely attributable to non-terpenoid substances. Both methods showed geraniol to be the major monoterpene present (50–60 per cent), along with smaller quantities of nerol and citronellol. These results are similar to those reported for other *Rosa* sp.[9,10]

Bound Monoterpenes in Rose Petals

It has been tacitly assumed by previous workers that plant monoterpenes occur primarily in the free state.[11] Our results indicate that nearly half the total monoterpenes of petals from fully opened flowers are present in the form of their β-D-glucosides. Hydrolysis of these derivatives with β-glucosidase showed that the monoterpenes released had a very similar composition to that of the free monoterpenes. Acid hydrolysis of the mixed β-D-glucosides yielded a monoterpene fraction which contained several components not observed in the corresponding fraction obtained by enzymatic hydrolysis. These differences are undoubtedly due to the well-known lability of monoterpenes towards acid.[12] Thus treatment of pure geraniol with acid under the conditions employed for the hydrolysis of the monoterpene β-D-glucosides gave a series of monoterpenes similar to those found in the monoterpene fraction liberated from the glucosides by the same reagent.

Formation of Free Monoterpenes and of Monoterpene-β-D-Glucosides During Flower Maturation

For studies of this nature, the importance of taking a uniform sample of petals from any one flower and of distilling the petals as rapidly as possible after harvesting is demonstrated

[9] E. GUENTHER, *The Essential Oils*, Vol. 5, p. 3, D. van Nostrand, London (1952).
[10] A. R. GUSEVA and V. A. PASESHNICHENKO, *Biokhimiya* **31**, 858 (1966).
[11] W. D. LOOMIS, in *Terpenoids in Plants* (Edited by J. B. PRIDHAM), p. 59, Academic Press, New York (1967).
[12] For example: H. STRICKLER and E. SZ. KOVATS, *Helv. Chim. Acta* **49**, 2055 (1966).

by the results discussed above and by the data in Fig. 2. Thus even within one flower there are considerable variations in the free monoterpene and in the monoterpene β-D-glucoside concentrations between the inner and outer petals and between the distal and proximal ends of these same petals. Figure 2 demonstrates that marked changes in the relative and absolute concentrations of free monoterpenes and of monoterpene-β-D-glucosides occurred in the petals if they were left in air before steam distillation.

Nevertheless, consideration of Table 2 and Fig. 3 and 4 shows that clear conclusions emerge about the formation of free monoterpenes and of monoterpene β-D-glucosides. Both free monoterpenes and the β-D-glucosides begin to accumulate only when the petals start to unfurl. A phase of rapid accumulation of free monoterpenes and of the β-D-glucosides follows during the next 2 or 3 days as the petals unfurl completely, the β-D-glucosides accumulating more rapidly than free monoterpenes. The maximum concentration of the β-D-glucosides is reached about a day before the maximum free monoterpene concentration which occurred when the flowers were fully open. The total concentration of monoterpenes remained relatively constant during this final period though the ratio of free monoterpenes to monoterpene β-D-glucosides increased markedly. Figure 4 shows the changes in the amounts per total petals of geraniol, nerol and (citronellol and citral) during flower maturation. The amounts of geraniol increased more rapidly than did those of nerol (and citronellol and citral) and it may be significant that nerol, citronellol and citral can be derived theoretically respectively by direct isomerization, reduction or oxidation of geraniol.

The above results can be interperted in terms of free monoterpenes being derived by hydrolysis of the monoterpene β-D-glucosides with the latter as intermediates in the biosynthesis of monoterpenes from non-terpenoid precursors. Specifically geraniol β-D-glucoside could be derived from geraniol pyrophosphate, the C_{10} intermediate in the biosynthesis of higher terpenoids and steroids.[13] Further work is in progress aimed at clarifying these biosynthetic interrelationships.

EXPERIMENTAL

Chemicals

Polyclar AT was obtained from G.A.F., Calder Street, Manchester 2, and purified by the method of Loomis and Battaile[14]. "Free fatty acid phase" (FFAP) for GLC was obtained from Varian Aerograph, Walnut Creek, California, U.S.A. Petroleum ether refers to redistilled 40–60° analytical grade.

Plant material

The Hybrid Tea rose "Lady Seton" and *Rosa damascena*, cultivar *versicolora* obtained from W. P. Vincent, Scarisbrick, Southport, were grown in a greenhouse at a constant temperature of 22° and with 16-hr days provided by unscreened high-pressure mercury vapour lamps at a distance of approximately 3 ft. Flowers were harvested at various known times measured from the stage at which the calyces were just splitting. The maturation stages were taken to be the following (see Table 2): (*a*) Buds. This included all stages during which the calyx was split but had not fully opened. (*b*) Buds just opening. These were buds which had fully opened calyces but in which the petals were only just beginning to unfurl at the top of the bud. (*c*) Flowers opening. The various opening stages represented the relatively short period during which the outer petals were unfurling. (*d*) Partly opened flowers. These were flowers with the outer but not the inner petals unfurled completely and hence with the stamens still not visible. (*e*) Fully opened flowers. These were flowers with all their petals unfurled completely and hence with the stamens visible. (*f*) Blown flowers. These were flowers with their petals falling naturally (i.e. without shaking the flowers).

[13] J. H. RICHARDS and J. B. HENDRICKSON, *The Biosynthesis of Steroids, Terpenes and Acetogenins*, W. A. Benjamin, New York (1964).
[14] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).

Isolation of Free and Bound Monoterpenes

Method 1. Petals were separated from the other flower parts, and the free monoterpenes isolated from the petals by steam distillation for 10 min using an all-glass apparatus. In certain experiments the steam distillation was repeated three times. Bound monoterpenes were isolated from the petal residues by adding 11 N HCl to pH 0·6, incubating the mixture for 18 hr at 16° and then repeating the steam distillation. Monoterpenes were extracted from all distillates with 0·5 ml petroleum ether.

Method 2. Petals were ground in solid CO_2 and extracted with redistilled methanol (9 ml/g petals) and then with a further quantity of the same solvent (1 ml/g petals). One volume of 2 M KCl was added to the combined methanol extracts and this mixture was then extracted four times with one-tenth its volume of petroleum ether. This technique yielded a "free monoterpene" fraction. The aqueous methanol fraction was subsequently taken to dryness on a rotary evaporator and resuspended in 0·6 N HCl; steam distillation and extraction of the distillates then yielded a monoterpene fraction originating from endogenous water-soluble derivatives ("bound monoterpenes").

Chromatographic Separation and Identification of Monoterpenes

GLC analyses of monoterpenes were performed on 2·75 m × 4 mm helical glass columns packed with 10% "FFAP"[15] on 100–120 mesh celite and temperature programmed from 50–220° at 3°/min with a N_2 flow rate of 60 ml/min. The equipment employed was a Pye 104 gas chromatograph equipped with hydrogen flame ionization detectors. Geraniol and other monoterpenes were identified by comparison of their retention times with that of linalool, by co-chromatography with the appropriate standards, and by using a combined gas chromatograph–mass spectrometer (AEI-MS12). Quantitation was achieved by comparison with standard geraniol solutions. TLC analyses were carried out by the methods of Battaile *et al.*[16]

Detection and Estimation of Bound Monoterpenes

Bound monoterpenes were estimated by two methods: 1. The sample in 5·0 ml 0·10 M sodium acetate buffer, pH 5·0, was incubated with 0·10 mg β-glucosidase for 3 hr at 40°. The sample was extracted with 0·50 ml petroleum ether and the monoterpenes in the petroleum ether later estimated by GLC as described above. 2. The presence of high concentrations of phenols in the petals frequently inhibited β-glucosidase activity and low values for bound monoterpenes were consequently obtained. Bound monoterpenes were then liberated from these samples by hydrolysis with 0·1 N HCl for 90 min at 80°. The monoterpenes released by this treatment were extracted with 0·50 ml of petroleum ether either directly or from steam distillates of the hydrolysed samples and the monoterpenes in the petroleum ether layer estimated by GLC.

Monoterpene Concentrations Within Single Fully Opened Flowers

The petals from fully opened flowers were divided into inner and outer petals, approximately sixteen per sample. The petals were cut in half horizontally to give in all four petal samples from each flower, inner and outer distal ends and inner and outer proximal ends. Free monoterpene and monoterpene β-D-glucoside concentrations were then determined as described above.

Estimation of Glucose

Samples of bound monoterpenes were hydrolysed in 1 N H_2SO_4 for 90 min at 80°. The liberated glucose was estimated by the glucose oxidase method.[17]

Isolation of Bound Monoterpenes

1. Rose flowers were harvested at the stage ("partly open" stage) when they contained maximum concentrations of bound monoterpenes. In a typical preparation, 200 g petals were ground in solid CO_2 and added to 800 ml of 2·5 mM NaOAc buffer, pH 5·0 at 100°. The solution was homogenized for 5 min with an Ultra-turrax disintegrator, boiled for 15 min and filtered while hot. The residues were re-extracted twice with 200 ml 2·5 mM NaOAc buffer, pH 5·0, at 100°. The aqueous extracts were combined and 4 vol. absolute ethanol added at 2°. The precipitate was allowed to settle for 18 hr and removed by centrifugation at 2° for 30 min at 1500 **g**. This residue was then washed twice with 100 ml ethanol:water (4:1, v/v), and the three aqueous ethanol supernates combined and added to 300 g Polyclar AT. The mixture was stirred for 60 min at 16°, the Polyclar AT removed by filtration, and the Polyclar AT washed twice with 1 l. of ethanol:water (4:1, v/v). The combined ethanol supernatants were taken to dryness at 50°. The resultant purple residue was extracted three times with 50 ml redistilled EtOAc saturated with water at 16°, and the extracts combined (EtOAc preparation).

[15] Wilkens Instrument and Research, Aerograph Research Notes, p. 3 (1964).
[16] J. BATTAILE, R. L. DUNNING and W. D. LOOMIS, *Biochim. biophys. Acta* **51**, 538 (1961).
[17] Glucose oxidase estimations. C. F. Boehringer und Söhne, GBMH, Mannheim, Germany.

2. *Silica gel chromatography.* The bound monoterpenes from the EtOAc preparation were purified further by silica gel chromatography. Silica gel was used as a column 63·5 × 11·3 cm. The column was developed at 16° with EtOAc saturated with water containing successively greater concentrations of methanol. The fractions containing bound monoterpenes were eluted with 1·0–3·0% methanol. Each fraction was evaporated to dryness and redissolved in 15 ml absolute ethanol.

3. *Preparative thin-layer electrophoresis.* The bound monoterpene fractions were streaked on 20 × 20 × 0·3 cm glass plates coated with 1·0 mm layers of cellulose CC-41 and sprayed with 0·05 M borate buffer, pH 9·0. The plates were subjected to electrophoresis in the same buffer for 1·5–2·0 hr at 500 V and 200 mA. After electrophoresis the plates were dried in air, the bound monoterpenes being visible as an opaque band while the plates were still damp. The bands of adsorbent containing bound monoterpenes were scraped off and extracted three times with 10 ml absolute ethanol.

4. The ethanol extracts prepared in 3 above were evaporated to 5 ml and impurities (mainly borate) precipitated at −15°. The resultant ethanol extract contained 90–95 per cent by weight of the β-D-glucosides of geraniol, nerol and citronellol and less than 0·1 per cent of D-glucose or of free monoterpenes. The overall recovery based on the water-soluble monoterpene content of the aqueous petal extracts was 40–50 per cent.

Synthesis of Geraniol β-D-Glucoside

Chemical. A method analogous to those described by Fischer and Raske[18] and by Noller and Rockwell[19] was used. The configuration of the geraniol glucoside prepared was confirmed as being β by NMR spectroscopy.

Enzymatic. The synthesis was based on the method of Bourquelot and Bordel.[6] 10 g geraniol, 2·0 g D-glucose and 0·50 g β-glucosidase in 200 ml acetone:water (4:1, v/v) were incubated for 3 months at 16° in the dark. The mixture was taken to dryness, extracted three times with EtOAc and the pooled extracts taken to dryness. The dried extract was taken up in petroleum ether:water (1:1 v/v) and the water layer separated; the water layer was washed twice with petroleum ether. The washed water-layer contained geraniol β-D-glucoside free of geraniol and glucose in an overall yield of 0·2 per cent.

Acknowledgements—We are grateful to Dr. W. Kelly for carrying out the GC–MS analysis and to Dr. R. D. Osborne for the chemical synthesis of geraniol-β-D-glucoside.

[18] E. Fischer and K. Raske, *Berichte* **42**, 1415 (1909).
[19] C. R. Noller and W. C. Rockwell, *J. Am. Chem. Soc.* **60**, 2076 (1938).