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SEED COAT COMPONENTS OF *HIBISCUS ABELMOSCHUS*

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Key Word Index—*Hibiscus abelmoschus*; Malvaceae; ambrette; seeds; lipid analysis; ambrettolide; oxacycloheptadec-8-en-2-one; oxacyclononadec-10-en-2-one; farnesyl acetate.

Abstract—The principal components of the monoester fraction of ambrette (*Hibiscus abelmoschus*) seeds are 2-*trans*,6-*trans*-farnesyl acetate (70%), 2-*cis*,6-*trans*-farnesyl acetate (6%) and the macrocyclic musk ambrettolide (12%). A novel homologue of ambrettolide, oxacyclononadec-10-en-2-one (4.5%), was identified. These compounds are localized in the outer layers of the seed coat, but are not epicuticular. They are deposited in the seed coat after seed coat dry weight accumulation has essentially stopped, but at the same time as embryo dry weight and neutral lipid are increasing. No apparent correlation was found between the lipid fatty acid or the cutin monomer compositions and the macrocyclic musk composition in the outer integument.

INTRODUCTION

Hibiscus abelmoschus (also *Abelmoschus moschatus*, or ambrette, of the family Malvaceae) is cultivated in tropical regions as an oilseed. The plant has a variety of medicinal uses, whilst the seeds are valued for their musk-like odour. The aromatic absolute derived from these seeds has long been used in the perfume industry [1]. Ambrettolide (oxacycloheptadec-*cis*-8-en-2-one, or 16-hexadec-*cis*-7-enolide) has long been known as the major musk component of the aromatic absolute. It was the first macrocyclic lactone to be isolated and identified [2]. More recently, Maurer and Grieder analysed the volatile fractions from the aromatic absolute and identified oxacyclopentadec-*cis*-6-en-2-one, and decyl-, dodecyl-, dodec-*cis*-5-enyl- and tetradec-*cis*-5-enyl-acetates [3]. These authors also reported the presence of 2-*cis*,6-*trans*- and 2-*trans*,6-*trans*-farnesyl acetates in these more volatile distillates. However, the aromatic absolute from ambrette seeds is generally reported to be rich in 2-*trans*,6-*trans*-farnesol [4, 5]. The leaves, petals and fruit husk of the ambrette plant do not appear to contain the same aromatic constituents as the seed [6].

We wished to examine the biosynthesis of macrocyclic lactone musks in plants, a topic which appears to have received no study [7]. However, the analytical and chemical data on which to base such a study is largely absent from the literature. We were unable to find published data on the quantitative composition of fragrance compounds in ambrette seeds, on the localization of the musks within the seed, on the relationship between the musks and other lipids derived from fatty acids, and on the time of biosynthesis of the musks. These questions are addressed in the present paper.

RESULTS AND DISCUSSION

The composition of the mature seed and parts of the seed 12 hr after imbibition are given in Table 1. This imbibition period is required to soften the seed for dissection into an outer seed coat (translucent, pale brown, ribbed), an inner seed coat integument (opaque, dark brown), an endosperm and an embryo. It should be noted that the fragrance components described in detail below were not appreciably hydrolysed

Table 1. Quantities of some major components in ambrette seeds

	μg/seed in				
Component	Whole seed	Embryo	Endosperm	Seedcoat Inner	Seedcoat Outer
Dry wt	14800	6400	1700	1600	4600
Farnesyl acetate	33	<0.1	<0.05	0.2	39.5
Ambrettolide	5.6	<0.02	<0.02	0.03	6.1
Total 16-hydroxyhexadecenoic acid*	4.5	<0.08	<0.08	0.2	6.9
Total non-hydroxy fatty acid†	2470	2080	307	4.1	4.2

*16-Hydroxyhexadecenoic acid released by transmethylation.
†Saturated and unsaturated fatty acids with no additional oxygen-containing functional groups.

over a period of several days post imbibition, so that the 12 hr imbibition period used in seed dissection should have no effect on the compositional analysis. Seed morphology has been examined by Singh [8]. The outer integument is only 2–3 cells deep, and ribbed. The inner integument is rich in tannins and is composed of several distinct layers (the outer epidermis, or palisade cell layer, which is extensively lignified, mesophyll cells and the inner epidermis). The 'endosperm' layer also includes perisperm. The fragrance components are localized exclusively in the outer seed coat. This layer contains 31% of the seed dry weight, and is composed of the outer integument and palisade cell layer. The trace amounts of these components in the inner seed coat are presumed to arise from imperfect dissection. A more precise localization must await ultra-structural studies. However, a 30 sec chloroform washing of the mature seeds in the presence of hexadecanolide as internal standard removed less than 1.5% of the fragrance components. Since this is a standard procedure to quantitatively remove the epicuticular waxes of plant leaves we can assume that the fragrance compounds of the seed coat of ambrette are not epicuticular. Ambrette seeds also contain large amounts of triacylglycerols. These neutral lipids are localized mainly in the embryo, and to a lesser extent in the endosperm.

Table 2. Typical composition of the monoester fraction from the outer layer of the ambrette seed coat

Component	Retention time* (min)	Percentage mass composition
Decyl acetate	2.12	0.6
Dodecyl acetate	3.68	2.4
Dodecenyl acetate	4.23	0.4
Tetradecenyl acetate	5.90	0.8
2-*cis*,6-*trans*-Farnesyl acetate	6.88	5.8
2-*trans*,6-*trans*-Farnesyl acetate	7.19	68.5
Unknown	7.63	2.2
Tetradecenolide	8.14	2.8
Hexadecenolide	9.54	11.5
Octadecenolide	10.87	4.8

*GC parameters: 2 m × 2 mm 10% SP-2330 packed column, temperature programmed 1 min at 140°, then to 240° at 10°/min. Injector and detector (FID) temperatures 320°.

Table 2 gives a typical GC analysis of the hexane–*iso*-propanol extract (the fragrance or monoester fraction) from the outer layer of the seed coat. Farnesyl acetates make up 74%, macrocyclic lactone musks 17% and alcohol acetates *ca* 4% of the total. Although the tetradecenolide component is quoted as 2.8% it co-elutes with another minor component which was not identified. Analysis of the hydroxy fatty acid methyl ester fraction derived from transmethylation of the fragrance fraction indicates that the percentage of tetradecenolide is *ca* 0.5%. Saturated C_{16} and C_{18} macrocyclic musks and free farnesol were not detected (< 0.1%).

Table 3 shows the non-hydroxy fatty acid analyses for the whole seed and for seed parts. The different fatty acid compositions from different seed samples may be explicable by different growth conditions. The seeds we harvested in the field are presumably grown at cooler temperatures and thus richer in polyunsaturated fatty acids [9]. An important point to consider is that there is no appreciable build-up of shorter chain fatty acids (C_{10}–$C_{16:1}$) that correspond to the monoester fraction products. Thus the C_{10}–$C_{16:1}$ components from the fragrance fraction are present at *ca* 8 μg total per seed, whereas the corresponding fatty acids in the outer integument of the seed coat are < 0.25 μg total per seed. Another point of interest is that there is no significant build up of palmitate and depletion of oleate in the outer seed coat. This might have been expected if the monoester components were derived principally by the degradation of oleate alone.

As a further prelude to biosynthetic studies we also examined other potential locations of ω-hydroxy fatty

Table 3. Fatty acid composition of ambrette seeds

Fatty acid*	Percentage fatty acid composition (mass)					
	Whole seed†	Whole seed‡	Embryo	Endosperm	Seedcoat Inner	Seedcoat Outer
14:0	tr	tr	tr	0.5	2.5	1
14:1	0	0	0	0	0	<3
16:0	22	22	22	18	26.5	28.5
16:1	tr	1	0.5	0.5	3	2
16:2	0	tr	0	0	1	0
18:0	4	3.5	4	2	4	6.5
18:1	30.5	38	38.5	33	33.5	35.5
18:2	42.5	34.5	33.5	45	28	16.5
18:3	1	1	1.5	1	1.5	1
20:0	tr	tr	tr	0	0	2
22:0	tr	tr	tr	0	0	3

*Fatty acids with chain lengths of C_{12} or less were not detected.
†Field grown.
‡Received from International Flavors and Fragrances.
tr, Trace.

acids in the ambrette seed coat. Total 16-hydroxyhexadecenoic acid gave figures which, when taken within experimental error, were commensurate with the levels of ambrettolide (Table 1). From this we can conclude that for the lipid extractable materials ω-hydroxy fatty acids are found as the macrocyclic lactones, with minimal esterification to other acids and alcohols. The cutin layer of the seed coat also merited investigation. Cutin monomers include such fatty acids as 16-hydroxypalmitate, 18-hydroxyoleate, 9,10-epoxy-18-hydroxyoleate and 9,10,18-trihydroxyoleate [10, 11]. The structural relationship between these compounds and the macrocyclic musks under investigation is obvious, and gives rise to the possibility of a biosynthetic relationship. Delipidated inner and outer seed coats from mature ambrette seeds were subjected to cutin depolymerization by refluxing in BF_3–methanol. The resulting toluene soluble material was analysed by GC, both before and after trimethylsilylation. By quantitation against methyl 16-hydroxypalmitate, and comparison with the retention times of the standards, it was calculated that none of the cutin monomers mentioned above, nor 16-hydroxyhexadecenoate, was present in either the inner or the outer layer of the seed coat at levels much above 0.1 μg per seed. Since ambrettolide and octadecenolide are present at levels of *ca* 5.8 and 2.4 μg per seed, respectively, we feel that it is very unlikely that there is any direct connection between the synthesis of these macrolides and cutin, despite similarities in their chemical structure.

The major monoester components from the outer layer of the seed coat are listed in Table 2. With the exception of octadecenolide they all have already been identified in ambrette seeds. The structures of the decyl, dodecyl and tetradecenyl acetates were confirmed by GC retention times on polar and apolar columns, using decyl, dodecyl and tetradecyl acetate standards, by GC-MS, and in the case of dodecyl acetate by ^{1}H NMR. The structures of the tetradecenolide and ambrettolide musks are also well known [2, 3], but the C_{18} homologue of ambrettolide has not been reported in ambrette seeds. It is, however, found naturally in secretions from the Dofours gland of the bee [12, 13]. The structure of the C_{18} homologue was confirmed as oxacyclononadec-10-*cis*-en-2-one by GC-MS, ^{1}H and ^{13}C NMR, IR and reductive ozonolysis. ^{13}C NMR (75 MHz, $CDCl_3$) gave 16 resolvable signals, but two had double intensity when the spectrum was obtained under quantitative conditions. Thus the molecule contains 18 carbon atoms. Only five resonances could be assigned with any confidence: δ171.2 (*s*, C-2), 130.35 (*d*, C-11), 130.2 (*d*, C-10), 64.3 (*t*, C-19) and 35.0 (*t*, C-3). The remaining resonances (δ25.4–29.45) were all identified as methylene carbons by the Advanced Proton Test [14]. However, the rules for assigning methylene resonances for fatty acids and esters will break down in a macrocycle due to the restriction of conformation imposed by the ring, so further assignments were not possible. Because all the sp^3 carbons were shown to be methylenes by the APT method the macrocyclic nature of the molecule could be deduced directly from its ^{13}C NMR spectrum.

Although the stereochemistry of the isolated double bond in the macrocyclic musks can readily be determined by IR spectroscopy by measuring the *trans*-double bond C–H deformation frequency at 970 cm^{-1}, this analysis can also be performed by GC on a polar packed column. ECL values measured for hexadecanolide, *trans*- and *cis*-ambrettolide on a 10% SP-2330 column were 19.08, 19.65 and 20.38, respectively. The large increase in ECL value (+4.08) in going from methyl pentadecanoate ($C_{16}H_{32}O_2$) to hexadecanolide ($C_{16}H_{30}O_2$) is due to the macrocyclic ring. The unexpectedly large separation of the *cis*- and *trans*-monounsaturated isomers is therefore likely due to different conformations of the ring induced by the different double bonds.

Figure 1 shows developmental parameters for ambrette seeds. Each point is based on a total of 50 seeds from two pods. Flowers fully opened for one day only, and were tagged on this day. It can be seen that seed coat plus endosperm dry wt accumulates well before embryo dry wt. Facile separation of the 'seed coat plus endosperm' into inner and outer integuments, perisperm and true endosperm was not possible in developing seeds. The accumulation of embryo neutral lipid (triacylglycerol) parallels embryo dry wt increase. However, the monoester accumulation in the seed coat does not parallel seed coat dry wt accumulation, but is coincident with triacylglycerol deposition. Whether this coincidence has any common determinant, such as a hormonal control, remains to be seen. During the deposition phase we were unable to detect any significant changes in the composition of either the seed coat fragrance fraction or of the embryo fatty acids (data not shown). This developmental study defines the time period for undertaking biosynthetic experiments.

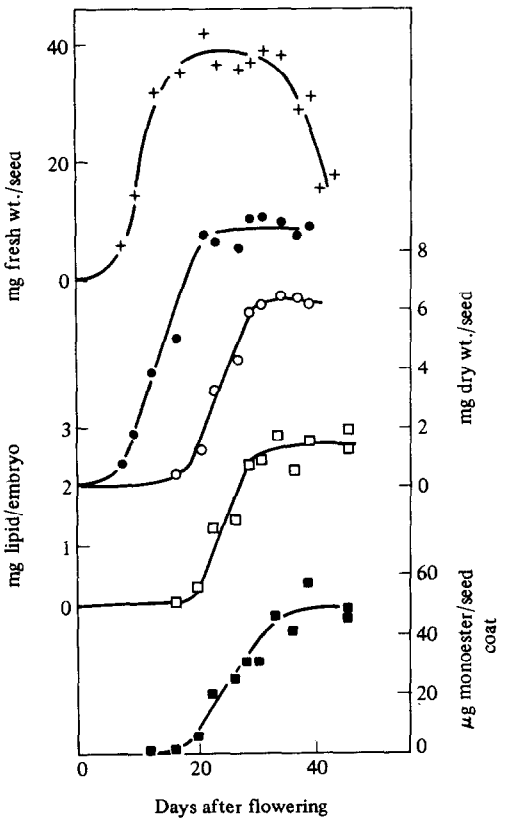

Fig. 1. Developmental parameters for ambrette seeds. Total seed fresh wt (+ —— +), seed coat plus endosperm dry wt (●——●), seed embryo dry wt (⊙——⊙), embryo neutral lipid wt (⊡——⊡) and seed coat monoester wt (■——■) are shown for maturing seeds, measured as days after flowering.

EXPERIMENTAL

Ambrette seeds were the generous gift of International Flavors and Fragrances Inc., NY. Plants were grown in the field in Pleasanton, CA. Harvesting of developing and mature seeds was made in September and October.

Lipid analysis. Lipids were extracted with hexane–*iso*-PrOH (3:2) using the method of ref. [15]. An int. standard was added to quantitate the monoester fraction (hexadecanolide) and the acyl lipids (tripentadecanoyl glycerol). Total neutral lipids were assayed by weighing the hexane soluble material. The monoester fraction was analysed directly by GC (conditions are given in Table 2). Fatty acids were analysed after transmethylation of the lipid extract by refluxing in MeOH–C_6H_6–conc H_2SO_4 (20:10:1) for 3 hr. The Me esters were extracted into hexane and analysed by temp. prog. GC using both polar (10% SP 2330) and apolar (1% Dexisil 300) stationary phases. Total hydroxy fatty acids were determined by transmethylation as described above, but GC was performed on TMSi ethers. Either the total transmethylation mixture, or the hydroxy fatty acid Me ester fraction from TLC (silica gel) was co-injected with BSTFA containing 1% TMCS.

Cutin analysis. Hydroxy fatty acid standards were prepared by exhaustive delipidation of lemon peel and spinach leaves with $CHCl_3$–MeOH (2:1) and depolymerization of the residue by refluxing in BF_3–MeOH (14% by wt) for 12 hr. TLC on silica gel furnished 10,16-dihydroxypalmitic acid (lemon), and 9(10),18-dihydroxy-10(9)-methoxystearic acid and 9,10,18-trihydroxystearic acid (spinach). These compounds were identified as their TMSi ethers by GC–MS. Ambrette seed coats (100) were extracted twice with hexane–*iso*-PrOH (3:2) to ensure delipidation prior to refluxing in BF_3–MeOH (14% by wt) for 12 hr to depolymerise the cutin. After addition of saline and extraction with Et_2O the fatty acid Me esters were analysed by GC, both as the hydroxy esters and after trimethylsilylation by heating at 110° for 2 hr with BSTFA containing 1% TMCS and 5% Et_3N. GC conditions for the analysis were 1.3 m × 2 mm column packed with 1% Dexisil-300, He at 20 ml/min, FID detector, injector and detector temps 350°, temp. prog. 180–320° at 10°/min.

Large scale purification. Whole ambrette seeds (450 g) were stirred for 2 days in EtOH (1 l.), and for a further 2 days in a fresh vol. of the same solvent. After filtration and evapn of the EtOH the residue was taken up in hexane, then re-evapd. The residue (2.7 g) was applied to $AgNO_3$–silica gel (1:19) column equilibrated in hexane and the components of the mixture eluted with a gradient of EtOAc in hexane (0:100 to 1:4). The first fractions were enriched in macrolides and alkyl acetates (0.7 g pooled, A), while subsequent fractions of almost pure farnesyl acetate were obtained (0.9 g total). Pooled fraction A was further purified by prep. C_{18}-reversed phase HPLC using a Whatman ODS-2 column (2.2 × 50 cm) and isocratic elution with MeCN (4 ml/min). Elution was monitored by UV at 210 nm and by GC. The order of elution was 2-*cis*,6-*trans*-farnesyl acetate, 2-*trans*,6-*trans*-farnesyl acetate, decyl acetate, ambrettolide, tetradecenyl acetate, dodecyl acetate and finally oxacyclononadec-10-en-2-one. Fractions could be collected that were highly enriched (> 95%) in each of the above except tetradecenyl acetate, despite the fact that in many instances baseline separation of the components was not achieved.

Identification of oxacyclononadec-10-en-2-one. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.28 (18H, *m*, bulk methylenes), 1.61 (4H, *m*, H-4 and H-18), 2.04 (4H, *m*, H-9 and H-12), 2.31 (2H, *t*, J = 7 Hz, H-3), 4.10 (2H, dist. *t*, H-19), and 5.31 (2H, *m*, H-10 and H-11). GC-MS (EI) 70 eV *m/z* (rel. int.): 280 $[M]^+$ (4), 137 (5), 123 (11), 109 (20), 96 (45), 95 (44), 82 (72), 81 (66), 68 (52), 67 (74), and 55 (100). Double bond position was determined as follows. The macrocycle was ring-opened by transmethylation to produce the corresponding ω-hydroxy Me ester. This was cleaved by reductive ozonolysis using triphenylphosphine using the method of ref. [16]. The products, Me 9-oxononanoate and 9-oxononan-1-ol, were identified by GC on a SP-2330 column using standards. Double bond configuration was determined by IR spectroscopy, by monitoring the absorption at 970 cm^{-1} (in CCl_4) characteristic of a *trans*-double bond. Using Me elaidate as calibrant a *trans*-content of less than 5% was established. IR spectroscopy also showed absorptions at 3020 cm^{-1} and 1740 cm^{-1} corresponding to olefinic C–H stretch and ester carbonyl stretch, respectively.

Stereomutation of ambrettolide. *cis*-Ambrettolide was converted to a mixture of *cis*- and *trans*-ambrettolides by the use of nitrogen oxides, a procedure which does not cause double bond migration [17, 18]. *cis*-Ambrettolide (100 mg) was heated at 60° for 2 hr in dioxan (5 ml) containing 0.1 ml 6 M aq. HNO_3 and 0.15 ml of 2 M aq. $NaNO_2$. After extraction the isomers were separated by $AgNO_3$–silica gel (1:19) TLC, and examined by GC and IR spectroscopy.

Identification of farnesyl acetate isomers. In the absence of authentic standards the identity of the farnesyl acetate isomers was examined by ^{1}H and ^{13}C NMR. These isomers were isolated by $AgNO_3$–silica gel CC in high purity (> 90%). The NMR spectra obtained were compared with published data for all the isomers of farnesol [19–21]. The 2-*trans*,6-*trans*-isomer was identifiable by both its ^{13}C and ^{1}H NMR spectra. However, the ^{13}C NMR spectra of the 2-*cis*,6-*trans*- and 2-*trans*,6-*cis*-isomers are very similar [19, 20] so it was necessary to use the ^{1}H methyl resonances to show that the minor farnesyl acetate isomer in ambrette is 2-*cis*,6-*trans*-isomer [21].

3,7,11-*Trimethyldodeca*-2-trans,6-trans,10-*trien*-1-*ol*, *acetate*. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.6 (6H, *s*, Me-7 and Me-12), 1.68 (3H, *s*, H-12), 1.71 (3H, *s*, 3-Me), 2.0–2.1 (8H, *m*, H-4, H-5, H-7 and H-8), 2.05 (3H, *s*, acetate Me), 4.59 (2H, *d*, J = 7 Hz, H-1), 5.1 (2H, *m*, H-6 and H-10), and 5.35 (1H, *t*, H-2). ^{13}C NMR (75 MHz, $CDCl_3$): δ16.1 (Me-7), 16.5 (Me-3), 17.75 (Me-12), 21.15 (acetate Me), 25.55 (C-12), 26.25 (C-5), 26.75 (C-9), 39.6 (C-4), 39.75 (C-8), 61.45 (C-1), 118.3 (C-2), 123.65 (C-6), 124.35 (C-10), 131.35 (C-11), 135.5 (C-7), 142.35 (C-3) and 171.2 (acetate carbonyl).

3,7,11-*Trimethyldodeca*-2-cis,6-trans,10-*trien*-1-*ol*, *acetate*. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.61 (6H, *s*, Me-7 and Me-12), 1.68 (3H, *s*, H-12), 1.78 (3H, *s*, Me-3), 2.0–2.1 (8H, *m*, H-4, H-5, H-7 and H-8), 2.05 (3H, *s*, acetate Me), 4.57 (2H, *d*, J = 7 Hz, H-1), 5.1 (2H, *m*, H-6 and H-10), and 5.35 (1H, *t*, H-2). ^{13}C NMR (75 MHz, $CDCl_3$): δ16.05 (Me-7), 17.75 (Me-12), 21.15 (acetate Me), 23.6 (Me-3), 25.75 (C-12), 26.7 (C-5 and C-9), 32.2 (C-4), 39.75 (C-8), 61.2 (C-1), 119.1 (C-2), 123.4 (C-6), 124.3 (C-10), 131.45 (C-11), 135.85 (C-7), 142.8 (C-3) and 171.2 (acetate carbonyl).

REFERENCES

1. Misra, G. and Mitra, C. R. (1964) *Res. Ind.* **9**, 325.
2. Kerschbaum, M. (1927) *Ber. Dtsch. Chem. Ges.* **60**, 902.
3. Mauer, B. and Grieder, A. (1977) *Helv. Chim. Acta* **60**, 1155.
4. Glasby, J. (1982) in *Encyclopedia of the Terpenoids*, p. 973. Wiley, New York.
5. Herout, V. (1982) in *Fragrance Chemistry. The Science of the Sense of Smell* (Theimer, E. T., ed.) p. 222. Academic Press, New York.
6. Misra, G., Nigam, S. K. and Mitra, C. R. (1971) *Acta Phytother.* **18**, 134.
7. Mookerjee, B. D. and Wilson, R. A. (1982) in *Fragrance Chemistry. The Science of the Sense of Smell* (Theimer, E. T., ed.) p. 433. Academic Press, New York.
8. Singh, B. (1968) *Phytomorphology* **17**, 282.
9. Slack, C. R. and Browse, J. A. (1984) in *Seed Physiology*

(Murray, D. R., ed.) Vol. 1, p. 209. Academic Press, Australia.
10. Holloway, P. J. (1982) in *The Plant Cuticle* (Cutler, D. F., Alvin, K. L. and Price, C. E., eds) p. 45. Academic Press, London.
11. Kolattukudy, P. E. (1980) in *The Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds) Vol. 4, p. 571. Academic Press, New York.
12. Bergstrom, B. (1974) *Chem. Scr.* **5**, 39.
13. Hefetz, A., Blum, M. S., Eickwort, G. C. and Wheeler, J. W. (1978) *Comp. Biochem. Physiol. B* **61**, 129.
14. Shoolery, J. N. (1984) *J. Nat. Prod.* **47**, 226.
15. Hara, A. and Radin, N. S. (1978) *Analyt. Biochem.* **90**, 420.
16. Stein, R. A. and Nicolaides, N. (1962) *J. Lipid Res.* **3**, 476.
17. Harlow, R. D., Litchfield, C. and Reiser, R. (1963) *J. Am. Oil Chem. Soc.* **40**, 505.
18. Gunstone, F. D. and Lie Ken Jie, M. (1970) *Chem. Phys. Lipids* **4**, 1.
19. Shaskov, A. S., Grigor'eva, N. Y., Avrutov, I. M., Semenovsku, A. V., Odinokov, V. N., Ignatyuk, V. K. and Tolstikov, G. A. (1979) *Izv. Akad. Nauk SSSR, Ser. Khim.* 388.
20. Tanaka, Y., Sato, H. and Kageyu, A. (1982) *Polymer* **23**, 1087.
21. Bates, R. B., Gale, D. M. and Gruner, B. J. (1963) *J. Org. Chem.* **28**, 1086.