Analysis of Lipids Containing Epoxy Groups: The Epoxy Glycerides of *Vernonia Anthelmintica* Seed Oil

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Abstract

Picration-TLC analysis of *Vernonia anthelmintica* seed oil shows that besides the known triglyceride of vernolic acid, it also contains two additional epoxy compounds. These were isolated by column chromatography and have been identified as the monoepoxy and the diepoxy triglycerides of vernolic acid by a rapid transmethylation-GLC micromethod. Lipase hydrolysis of these glycerides has shown that in the monoepoxy triglycerides the vernolic acid has marked preference for the 1,3-positions, while in the diepoxy triglycerides the 2-position is preferred.

Introduction

In connection with a rat feeding study (1) a concentrate of the triglyceride of vernolic (*cis*-12,13-epoxy-*cis*-9 octadecenoic) acid was sought. *Vernonia anthelmintica* seed oil is known to contain large amounts of this epoxy acid and the literature affirms that virtually all of it is found in the triglyceride (trivernolin) form (2,3,13). It seemed relatively simple, therefore, to prepare a 90% trivernolin-rich oil by a solvent recrystallization technique such as the one followed by Krewson et al. (2). The project proved more difficult than anticipated due to the presence of a considerable (>15%) amount of the epoxy acid in the form of divernolyl and monovernolyl triglycerides. These triglycerides tend to co-precipitate with the trivernolin making the purification of the latter more difficult. Since the monovernolyl triglycerides and the divernolyl triglycerides of *Vernonia* oil seem to have, thus far, escaped attention, we thought it useful to isolate them and to investigate their properties, particularly the distribution of the vernolic acid in these triglycerides.

During this work, and because of the limited amount of samples available, particular attention was paid to finding and applying rapid and efficient micromethods of lipase hydrolysis, base-catalyzed transmethylation and GLC and TLC analyses of these epoxide-containing moieties. Neither of the transesterification micromethods recently reported (5,10) has been applied to epoxy glycerides. The difficulties of quantitative GLC determination of epoxy acids have been discussed (6,7); the latest publications on the hydrolysis of epoxide-containing triglycerides (11,18) have not given a clear picture of the resulting products. Some of these problems were resolved during the course of this work. The techniques developed here were found useful in detecting the level and distribution of vernolic acid in the lipids of rats which had been fed a trivernolin-rich oil for 90 days (1).

Experimental Procedures

General

As received, *Vernonia anthelmintica* seed oil had a peroxide value of 4.4 meq/kg (8), an oxirane content of 3.41% (9) and contained 6.6% of unsaponifiable matter (17). A trivernolin-rich oil was prepared by overnight crystallization at −10 C from four volumes of petroleum ether (30–60 C). After the supernatant had been decanted, the solid was melted and freed of most of the solvent on the steam bath under nitrogen. The last traces of solvent were removed by vacuum, steam distillation at 50 C for 30 min. The oil was processed in 1–2 kg batches which were combined to give the final product. The recovery averaged 56%. This single recrystallization lowered the unsaponifiables level to 2.5% and raised the oxirane content to 4.3% for a computed "trivernolin" content of 83.9%. Handling caused the peroxide value to increase to 11.5. The level of free fatty acids, computed as epoxyoleic, was 2.0% (16).

A purified trivernolin sample was prepared from the trivernolin-rich oil by recrystallizing three more times from petroleum ether to remove the divernolyl triglycerides (13) and residual unsaponifiables, and by column chromatography on silica gel to eliminate the free vernolic acid and other polar components. The purity of the sample was estimated at >97% by TLC.

Transmethylation Experiments

The transmethylation of trivernolin was also carried out following a procedure similar to that of Luddy et al. (10). In this case 5–40 mg of triglyceride were placed in a vial along with 0.50 ml of 0.4N $KOCH_3$ in methanol. Complete transmethylation could be achieved by either allowing this mixture to stand at room temperature for 15 min with occasional shaking or, as recommended for common triglycerides (10), by shaking at 60 C for 2–3 min. Addition of 3.0 ml CS_2 containing 0.1% squalane (internal standard) was followed by addition of 5.0 ml saturated sodium chloride solution. Vigorous shaking for 2 min gave an emulsion which was broken by a 3–5 min centrifugation. The lower layer was then ready to be analyzed by TLC and GLC.

Column Chromatography

Column chromatography was used to separate the common triglycerides from the mono-, di- and triepoxy triglycerides. The column (1.5 × 75 cm) was prepared by slurrying 100 g of Davison 923 100/120 mesh silica gel (Grace Davison Chemicals) in about 300 ml 20–40 C petroleum ether. The silica gel had been previously treated by consecutive washings with ether, chloroform and methanol, and had then been dried at 110 C overnight prior to use. The column, which was attached to a 500 ml separatory funnel by means of a ₸ 18/9 ball joint, was rinsed with 200 ml petroleum ether. The sample, 1.021 g of filtrate from the crystallization of *Vernonia* seed oil described above containing 1.84% oxirane, was dissolved in 10 ml petroleum ether and applied to the column by means of a pipette. The column was eluted consecutively with 500 ml each of 4%, 10%, 25% and 50% ethyl ether in petroleum ether. The flow rate was 2–3 ml/min; 25 ml fractions were collected and evaporated to 1.0 ml under nitrogen. Twenty-five to 50 μliter aliquots were spotted on TLC plates which

were then developed and visualized as explained below. Similar fractions were then combined, and when necessary, subjected to further separation by preparative TLC, using 1 mm thick silica gel G plates and a petroleum ether-diethyl ether (75:25) solvent.

Lipolysis

Five to 20 mg of triglyceride were weighed into a 10 ml pear-shaped flask. Into it 1.0 ml M tris-(hydroxymethyl)-aminomethane buffer adjusted to pH 8, 0.1 ml 22% calcium chloride and 0.25 ml 0.1% oxgall (Baltimore Biological Laboratory, Inc.) were pipetted, and the flask was immersed in a small water bath regulated at 40 C. After 2 min 1.8 mg of Pancreatic Lipase 250 (Miles Laboratories, Inc.) in 0.2 ml of buffer were added, and the mixture was stirred vigorously for 15 min. The digestion was terminated by adding 0.1 ml 6N hydrochloric acid, while mixing vigorously, and immediately decanting into a separatory funnel containing 5 ml diethyl ether and shaking for 1 min. Two more 5 ml ether extractions were carried out in the same manner. The pooled extracts were water washed until free of acid (2–3 times), dried over magnesium sulfate, filtered, evaporated at room temperature under a stream of nitrogen and made to 5 ml with ether. Half of this volume was usually taken and evaporated for the preparation of trimethylsilyl ether (TMS) derivatives, while 25–100 μliter aliquots were spotted on thin-layer plates.

Gas-Liquid Chromatography

The GLC of transmethylated trivernolin or vernolic acid containing triglycerides was done using an F&M Model 810 gas chromatograph equipped with dual flame ionization detectors. In most instances the column used was 6 ft long, 1/8 in. OD stainless steel tubing packed with 5.3% Carbowax 20M on 100/120 mesh Gas Chrom Q. Use of 3% HIEFF-8BP (Applied Science Laboratories, Inc.) as the packing material also gave satisfactory results. The column was operated isothermally at 210 C and the helium flow was approximately 75 ml/min. The injection port and detector temperatures were 260 and 270 C, respectively.

The general procedure followed in analyzing the products of lipolyses was essentially that described by Tallent et al. (11,18). Two to 20 mg of material were placed in a small vial to which 0.5 ml bis-(trimethylsilyl)-acetamide (BSA—Aldrich Chemical Co., Inc.) was added. After 60 min, 1–5 μliter of this solution was injected into a 55 cm long, 1/8 in. OD stainless steel column packed with 3% OV-1 on 60/80 mesh Gas Chrom Q (Applied Science Laboratories, Inc.). The oven was held at 145 C for 2 min after which it was programmed to 375 C at 10°/min. The injection port temperature fluctuated between 320–350 C during the programming while the detector temperature was stable at 380 C. The carrier gas flow rate was again 75 ml/min.

TABLE I

Transmethylation of Trivernolin[a]

Experiment No.	% Recovery[b]	
	3 min. at 60 C	30 min. at RT
1	95.7	100.0
2	98.6	104.5
3	93.5	91.8
4	97.4	97.6
Average	96.3 ± 1.7	98.5 ± 3.8

[a] 10.1 mg trivernolin of 98.2% purity by GLC. See Experimental Procedures for details.
[b] GLC on Carbowax 20M at 210 C using 0.112% squalane as the internal standard. Peak areas obtained by triangulation.

Thin-Layer Chromatography

Commercially available (Analtech, Inc.) thin-layer silica gel G plates were used throughout this study. The plates, used without prior activation, were developed with petroleum ether (20–40 C) ethyl ether-acetic acid (60:40:1 v/v/v). When a clear distinction between trivernolin and vernolic acid had to be made, the acetic acid was omitted from the developing solvent, and the vernolic acid appeared as a streak beginning at the origin. In the lipolysis experiments, where the differentiation between the two was important, best results were obtained by placing the

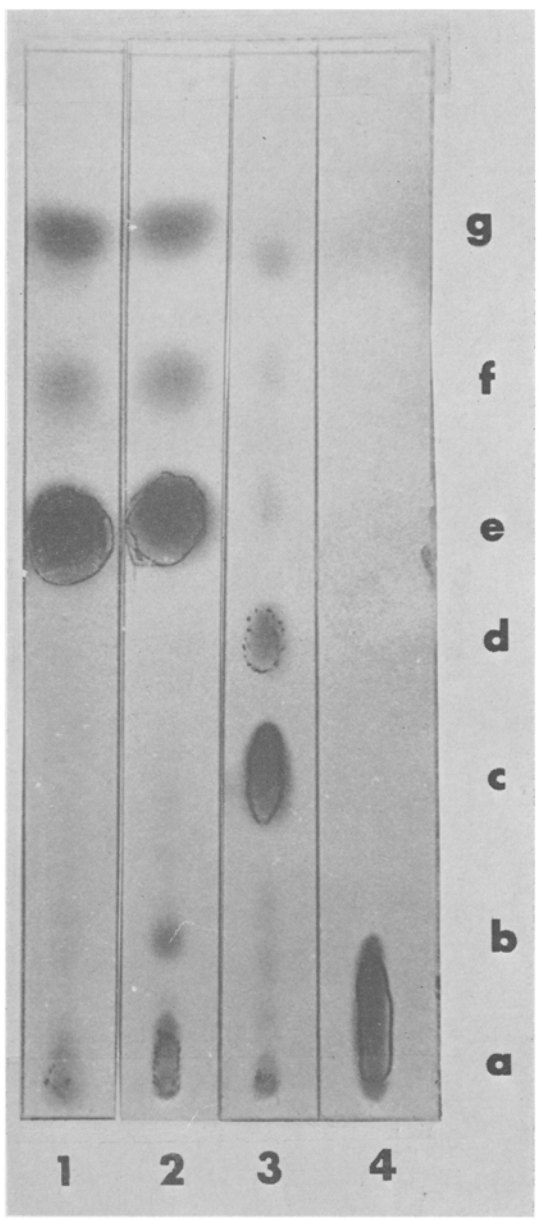

FIG. 1. Transmethylation of trivernolin-rich oil. The circled spots represent epoxide-containing moieties. (a) represents vernolic acid, (b) hydroxy-containing by-products, (c) trivernolin, (d) divernolyl triglycerides, (e) methyl vernolate, (f) common acid methyl esters, (g) squalane (internal standard) or unsaponifiables in the oil. Lanes 1 and 2 contain 500 μg of oil transmethylated for 15 min and 4 hr at room temperature, respectively. Lane 3 contains 500 μg of the original trivernolin-rich oil and lane 4 contains 100 μg of vernolic acid.

TABLE II
GLC of Transmethylated Monovernolyl Triglycerides From Vernonia Oil[a]

Injection volume[b] (μliters)	% Fatty acids in the sample[c]				
	C_{14}	C_{16}	C_{18} $C_{18:1}$	$C_{18:2}$	Vernolic
1	0.3	11.1	23.7	32.6	32.6
2	0.4	11.0	21.7	33.3	34.7
2	0.2	11.2	22.2	37.6	29.4
2	0.2	9.7	22.9	38.0	29.5
4	.....	11.4	19.8	37.6	31.3
Average	0.3 ± 0.1	10.9 ± 0.5	21.9 ± 1.2	35.8 ± 2.3	31.4 ± 1.9

[a] GLC on Carbowax 20M at 210 C; see text for details.
[b] The solution contained approximately 2 mg monovernolyl triglycerides/ml.
[c] Computed from total peak area obtained by triangulation.

plate perfectly vertical in the developing tank. After development, the plates were sprayed heavily with picric acid in 95% ethanol and allowed to stand in an atmosphere of diethyl ether-ethanol-acetic acid (80:20:1) for 30 min. Exposing the plates briefly to ammonia fumes revealed the epoxide-containing moieties as reddish-orange spots on a yellow background. The picration technique for detection of epoxides on TLC plates has been reported in greater detail elsewhere (14). After the picrate spots had been marked by dotting or circling them with a pointed microspatula, the plates were charred by spraying with chromic-sulfuric acid solution (12) and heating at 180 C for 30 min.

Results and Discussion

As lane 3 in Figure 1 illustrates, the trivernolin-rich oil obtained by solvent recrystallization contains a considerable amount of divernolyl triglycerides (d). This was somewhat surprising since the vernolic acid moiety was thought to be present almost exclusively as trivernolin (13,15). TLC shows that the original oil before crystallization also contains a significant quantity of monovernolyl triglycerides. One solvent recrystallization eliminated the monovernolyl triglycerides (as filtrate) and raised the oxirane oxygen content from 3.41% to 4.34%. It also lowered the unsaponifiables from 6.6% to 2.5%. Figure 1 also

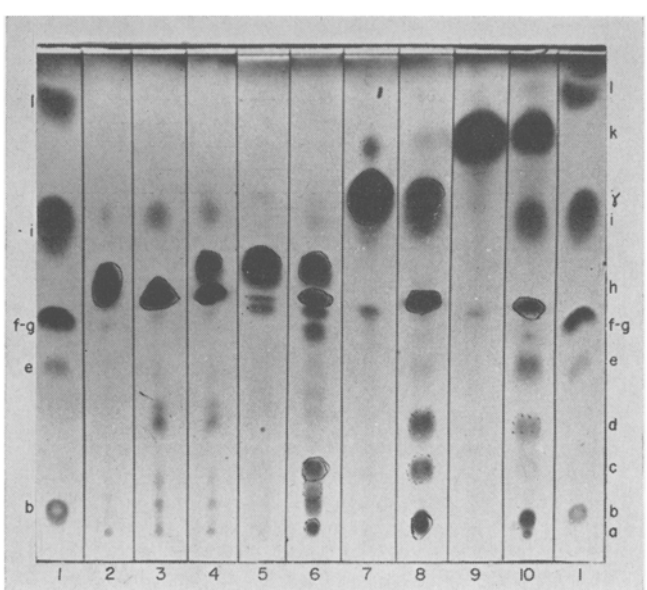

FIG. 2. TLC of epoxide-containing triglycerides from *Vernonia* seed oil before and after lipase hydrolysis. (a) is monovernolin, (b) common monoglycerides, (c) divernolin, (d) vernolic-common acid diglycerides, (e) common 1,2 diglycerides, (f) common 1,3 diglycerides, (g) vernolic acid, (h) trivernolin, (i) common fatty acids, (j) divernolyl triglycerides (k) monovernolyl triglycerides, and (l) common triglycerides. See text for description of various lanes.

TABLE III
Distribution of Vernolyl Groups in *Vernonia* Oil

Sample	Monovernolin Mole % in monoglycerides	*Proportion* of vernolyl groups in 2-position[a]	Vernolic acid mole % in total free acids	
			Calculated[b]	Found
Trivernolin[c]	100	33	100	98
Divernolyl triglycerides	72	36	64	60
Monovernolyl triglycerides	8	8	46	44

[a] Proportion in 2-position $= \frac{\text{Mole \% in monoglycerides} \times 100}{3 \times \text{mole \% in sample}}$ (15).
[b] Calculated from the mole % in the monoglycerides assuming that VVC, VCV, VCC and CVC triglycerides yield 50%, 100%, 50% and 0% vernolic acid respectively, when hydrolyzed to monoglycerides.
[c] Trivernolin obtained from *Vernonia* oil; purity = to 98.2% by GLC.

illustrates the results obtained when this trivernolin-rich oil was transmethylated at room temperature. The reaction is rapid and complete in 30 min. GLC established that the recovery of methyl vernolate was 99%; it drops to 94% after 4 hr. A 3 min transmethylation at 60 C gave a recovery of 96%. Table I shows the results obtained with pure trivernolin. Results from the 60 C reaction, as recommended by Luddy et al. (10), and at room temperature are about the same. In another series of experiments at 60 C, the sample size was varied from 5 to 40 mg trivernolin; the recovery was 100 ± 5% which is the estimated accuracy of the method. Since overall precision and accuracy of the two methods were roughly equal, the one chosen was often dictated by the time available.

After the above described micromethod had been established as a reliable tool for the analysis of epoxide-containing triglycerides, a method was sought to separate the three picrate-positive moieties in crude *Vernonia* oil. The filtrate from the oil after crystallization of the trivernolin-rich fraction contained a substantial amount of each fraction and was chosen for further work. TLC of this sample shows it to have a triglyceride distribution similar to that of *Euphorbia lagascae* (15).

Column chromatography on silica gel was carried

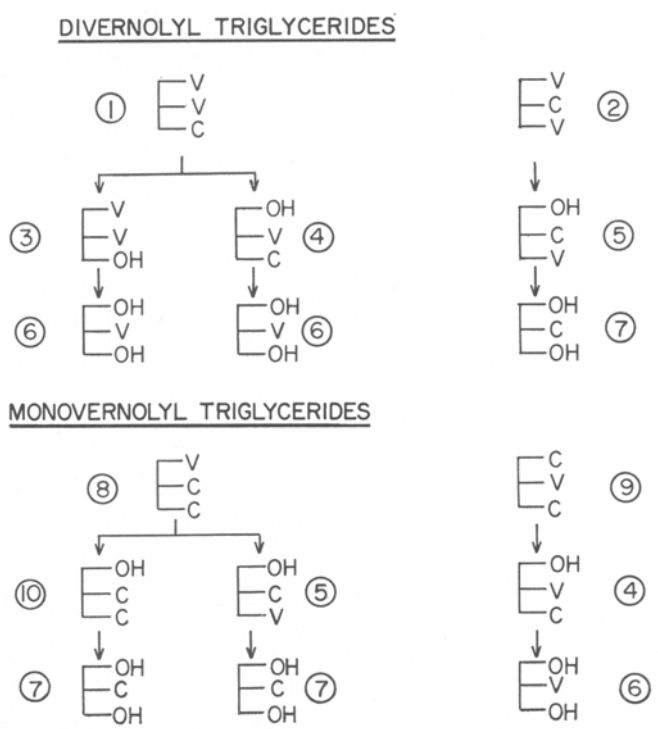

FIG. 3. Lipase hydrolysis of epoxy glycerides (assumes complete specificity for the 1,3 positions).

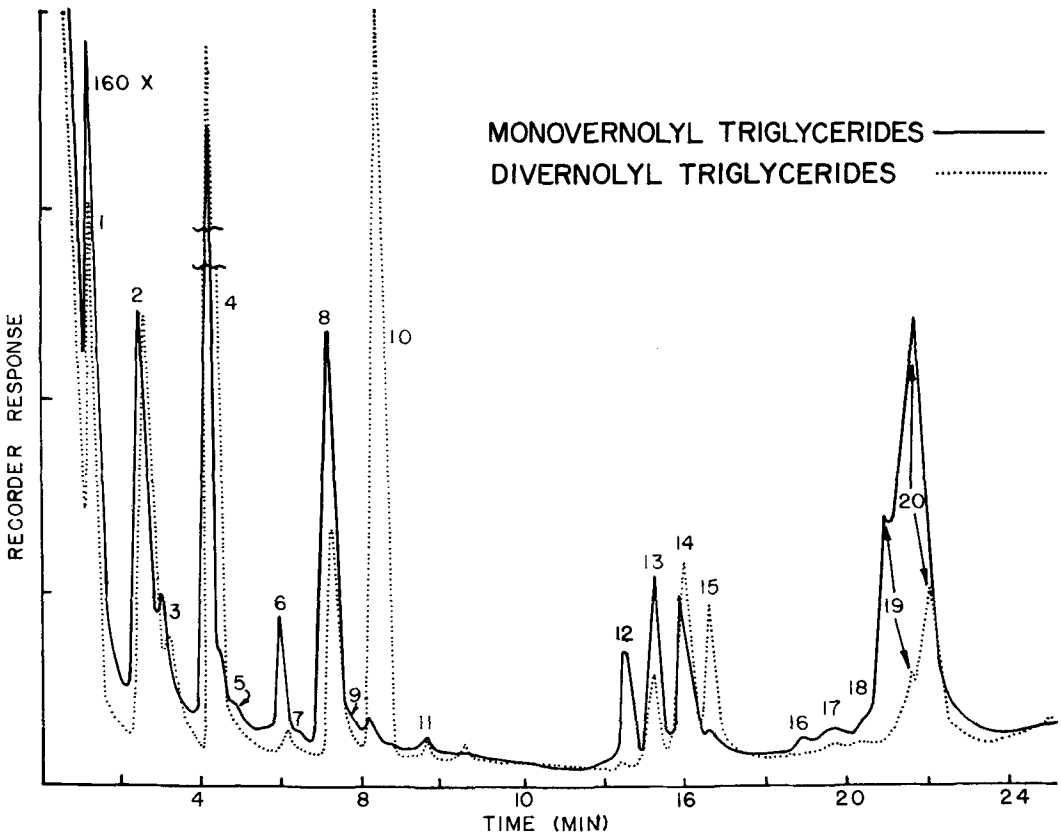

FIG. 4. GLC of lipase hydrolyzates of the mono- and diepoxy glycerides of *Vernonia* seed oil. Hydroxyl- and carboxyl-containing compounds run as the TMS derivatives. Peak (1) is palmitic acid, (2) linoleic acid, (3) oleic plus stearic acid, (4) vernolic acid, (5) and (6) chlorohydrins (?) of (4), (7) monopalmitin, (8) monolinolein, (9) monostearin and monoolein, (10) monovernolin, (11) chlorohydrin (?) of (10). Peaks 12–15 represent the diglycerides LP, LL, LV and VV, respectively. Peaks 16–20 are non-hydrolyzed monovernolyl and divernolyl triglycerides.

out using a system similar to that of Tallent et al. (15). In this case, experience indicated that a better separation could be obtained, especially between the non-epoxy triglycerides and the monovernolyl triglycerides, if an eluting solvent of increasing polarity was used. Thus, 4% ether in petroleum ether elutes the unsaponifiable fraction; 10% ether then elutes common triglycerides; 25% ether elutes the mono- and divernolyl triglycerides, and 50% ether elutes the trivernolin, vernolic acid and other polar components. Figure 2 (lanes 7 and 9) shows that monovernolyl and the divernolyl triglycerides eluted by the 25% ether fraction were resolved quite well. This gradient elution is especially important where the lipid under study contains low amounts (10% or less) of epoxy acid. Lipids from the epididymal fat pads of rats fed trivernolin-rich oil are such a case (1). Here, even the change in solvent polarity gives only a partial separation and the common triglycerides must be removed from the monovernolyl triglyceride fraction by preparative TLC.

Table II illustrates the GLC analysis of the monovernolyl triglycerides obtained by the column fractionation of *Vernonia* oil filtrate. The analyses of the methyl esters obtained by base-catalyzed transmethylation were performed on Carbowax 20 M at 210 C. The relative peak area of each component was obtained by comparison to the total area obtained by triangulation. Within the range tested (1–10 μg) the relative flame response of each component is independent of sample size. The 6% precision obtained for the major components could probably be improved by mechanical integration. Here some precision was sacrificed for speed. Under these conditions the common acid methyl esters elute in about 3 min resulting in sharp, difficult to measure peaks; methyl vernolate appears in about 9 min which makes possible the analysis of at least four samples an hour. The value of 31% vernolic acid in *Vernonia* monovernolyl triglycerides and of 32% and 65% for rat lipid monovernolyl and divernolyl triglycerides respectively (1), proves that the method is accurate.

Lipase hydrolysis of the epoxy glycerides isolated above was carried out to ascertain the distribution of vernolic acid. The micro lipase hydrolysis of triglycerides used was patterned after that described by Luddy et al. (19). The method is simple and rapid. When applied to 17.5 mg of triolein, GLC analysis of the digest obtained showed 56% oleic acid, 19% mono-olein, 13% diolein and 12% intact triglyceride. Lipase digestions of trivernolin (21) and mono- and divernolyl triglycerides (11,15,18) have been carried out in the past. They indicate that the enzyme attacks these epoxy triglycerides in the conventional manner by preferential (but not exclusive) splitting off the acid esterified in the 1,3-positions. In these publications, no mention is made about the extreme lability of the oxirane moiety to acid. Even though only one fifth of the hydrochloric acid normally stipulated was introduced (20,21), and although the addition was accompanied by mixing and followed by immediate extraction and water washing, chlorohydrin formation could not be avoided completely. This is evidenced by the presence of the non-epoxide containing spots found in the trivernolin digest (Fig. 2, lane 6). Peaks 5, 6 and 11 in Figure 4 are also thought to be due to chlorohydrin formation. For the most part however, these peaks were small when compared to their parent peak—vernolic acid and monovernolin—and were dis-

regarded when computing the data which appears in Table III.

Figure 2 shows the TLC analysis of the products from enzymatic digestion of epoxy glycerides. The resolution of trivernolin (h) and vernolic acid (g) was achieved by developing in a vertical rather than an inclined position. Lane 1 contains about 70 μg each of mono-, di- and tripalmitin plus 100 μg palmitic acid; lane 2, trivernolin; lane 3 vernolic acid (impure); lane 4 contains both vernolic acid and trivernolin; lane 5 is a trivernolin sample taken through the hydrolysis procedure but in absence of lipase; lane 6 is the trivernolin digest; lanes 7–10 show the divernolyl and the monovernolyl triglycerides from *Vernonia* oil before and after hydrolysis. The results of the enzyme hydrolysis, as shown in lanes 8 and 10 of this figure, especially a close inspection of the diglyceride spots (c-e) reveals that most of the divernolyl triglycerides are found in the VVC form, while most of the mono-vernolyl triglycerides are in the VCC form (V here represents vernolic and C a common acid). This is based on the reasoning that the symmetrical triglycerides—VCV and CVC—would only yield one diglyceride whereas two are found in all cases. Furthermore, as Figure 3 illustrates, a VVC distribution (triglyceride 1) would yield monovernolin (6) and a VCC distribution (triglyceride 8) would yield common monoglycerides (7). The presence of an epoxide-containing monoglyceride in lane 8 and a common monoglyceride in lane 10 confirm the fact that a VVC and a VCC distribution is predominant.

The scheme in Figure 3 presupposes specificity of the pancreatic lipase for the 1,3-positions which is only partly true (4,21). This is illustrated in Figure 4 by peaks 7–10. In the case of the divernolyl triglyceride hydrolyzate, although the monovernolin peak is dominant, the common monoglycerides peaks are also present; the converse is true of the monovernolyl triglyceride hydrolyzate. Aside from these considerations, however, GLC corroborates the fatty acid distribution proposed above.

In the divernolyl triglyceride sample (Figure 4), the linoleic-vernolic (LV) peak and the VV (peak 15) diglycerides are readily apparent in both samples. In the case of monovernolyl triglycerides, the fact that the monovernolin peak (peak 10) is much larger than the monolinolein one (peak 9) is also indicative of the preponderance of the VVC structure. The triglyceride peaks in Figure 4 may contain traces of C_{50}–C_{54} common acid triglycerides but are composed mostly of unhydrolyzed VPP, VPL, VLL, VVP, VVL and VLP. The peaks have not been assigned because of the poor resolution and the inability to differentiate between monovernolyl triglycerides and divernolyl triglycerides at this time. The short GC column used cannot differentiate unsaturated from saturated di- and triglycerides so that the Ls in the above discussion represent stearic and oleic as well as linoleic acid.

A quantitative expression of the assigned distribution is found in Table III. A look at this table reveals that in *Vernonia* oil divernolyl triglycerides, the vernolic acid shows a slight preference for the 2-position, while in monovernolyl triglycerides the 1,3-positions are perferred overwhelmingly. The preference for the 1,3-positions shown by vernolic acid in the monoepoxy triglycerides of *Vernonia* oil exceeds that found in *Euphorbia lagascae,* which is itself unusual when compared to other natural epoxy oils (15). As already mentioned, the very presence of these mono- and diepoxy glycerides in this particular *Vernonia* oil is surprising (15,13), and no explanation of the vernolic acid distribution found is possible at this time.

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REFERENCES

1. Fioriti, J. A., R. J. Sims and N. Buide, Lipids, in press.
2. Krewson, C. F., J. S. Ard and R. J. Riemenschneider, JAOCS *39*, 334–340 (1962).
3. Krewson, C. F., and F. E. Luddy, Ibid. *41*, 134–136 (1964).
4. Borgström, B., J. Lipid Res. *5*, 522–531 (1964).
5. Oette, K., and M. Doss, J. Chromatog. *32*, 439–450 (1968).
6. Herb, S. F., P. Magidman and R. A. Barford, JAOCS *41*, 222–224 (1964).
7. Krewson, C. F., Ibid. *45*, 250–256 (1968).
8. AOCS Official Method Cd 8–53.
9. AOCS Tentative Method Cd 9–57.
10. Luddy, F. E., R. A. Barford, S. F. Herb and P. Magidman, JAOCS *45*, 549–552 (1968).
11. Tallent, W. H., R. Kleiman and D. G. Cope, J. Lipid Res. *7*, 531–535 (1966).
12. Rouser, G., C. Galli, E. Lieber, M. L. Blank and O. S. Privett, JAOCS *41*, 836 (1964).
13. Kleiman, R., C. Smith, Jr., S. G. Yates and A. Jones, Ibid *42*, 169–172 (1965).
14. Fioriti, J. A., and R. J. Sims, J. Chromatog. *32*, 761–763 (1968).
15. Tallent, W. H., D. G. Cope, J. W. Hagemann, F. R. Earle and I. A. Wolff, Lipids *1*, 335–340 (1966).
16. AOCS Official Method Ca-5a-40
17. AOCS Official Method Ca-6a-40.
18. Tallent, W. H., and R. Kleiman, J. Lipid Res. *9*, 146–148 (1968).
19. Luddy, F. E., R. A. Barford, S. F. Herb, P. Magidman and R. W. Riemenschneider, JAOCS *41*, 693–696 (1964).
20. Mattson, F. H., and R. A. Volpenhein, J. Lipid Res. *2*, 58 (1961).
21. Sampugna, J., R. G. Jensen, R. M. Parry, Jr. and C. F. Krewson, JAOCS *41*, 132–133 (1964).

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