Identification of Radiation-Induced Triglyceride Adducts by Mass Spectrometry[1]

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ABSTRACT

Mass spectrometric evidence is given to show the formation of 4 types of adduct compounds induced by radiolysis of triglycerides. These are the triglyceride 2,2' dimer, the propanedioldiester dimer (i.e., a 1,2,5,6-hexanetetraol tetraester), a 2-propanedioldiester-triglyceride adduct and an α branched alkyl substituted triglyceride. Analogous compounds have been adduced for both tributyrin and tripalmitin. Adduct formation was observed at a position α to the carbonyl group.

INTRODUCTION

The occurrence of oligomeric or adduct compounds induced in triglycerides by radiolysis has been known or suspected for several years (1-6). Only a few, if any, have been well characterized because unequivocal separation and identification methods have been unavailable. Recent advances in size exclusion chromatography and in mass spectrometric (MS) ionization methods for low volatility, high mass compounds have now provided the means to identify such compounds.

In this study, the radiation-induced adduct compounds of tributyrin and tripalmitin have been characterized by field desorption (FD) and fast atom bombardment (FAB) MS. The selection of the triglycerides was made to compare the behavior of low and high molecular weight saturated compounds. Accordingly, the results reported here provide insight concerning the nature and mechanism of formation of the adducts and demonstrate the efficacy of the technique for the identification of adduct radiolysis products in fats derived from natural substances.

EXPERIMENTAL

The tributyrin (TB) and tripalmitin (TP) used in this study were purchased from ICN Pharmaceuticals, Inc., Plainview, NY. The purity of each compound was found to exceed 99% by GC/MS analysis. An 8 ft × 1/8 in. od GC column packed with 3% dexil on 80/100 Chromasorb W programmed at 10 C/min from 80 C to 340 C using a helium flow rate of 30 mL/min was used to check reagent purity. The mass spectrometer was a CEC/Dupont Model 21-491 coupled to the gas chromatograph by means of a jet-type molecular separator and used a Hewlett Packard Model 2116B computer interfaced with a Digital Equipment Corp. Model PDP 15/76 computer as a data system. Data previously reported (7) on tributyrin radiolysis products and discussed further below were also obtained on the same GC/MS/DS analysis system.

One-gram samples of tributyrin and tripalmitin were taken for analysis after irradiation in a ^{60}Co source at 25 Mrads at 25 C under vacuum.

Separation of fractions containing the adducts was achieved by means of a size exclusion liquid chromatography (SEC) column employing Styragel 60 Å (Waters Associates, Inc.) as the stationary phase. This procedure has been reported in part previously (8-11). In the present study, a system of columns was used in series for preparative separation of the adduct fractions from the bulk triglycerides. Details are given in Figure 1. For the separation of tripalmitin adducts, the wide bore section at the front end of the column was used to accomodate the injection of the 1-g samples. The liquid chromatograph used was a Waters Associates Model ALC-201 equipped with a refractive index detector and a 2-mL loop injector. The samples were dissolved in chloroform and fractionation was done using chloroform as the mobile phase at a flow rate of 2 mL/min and an inlet pressure of 800-900 psi. The fraction eluting prior to the triglycerides contains the adduct radiolysis products which was collected and evaporated to dryness for further analysis by MS. The chromatograms depicting the separations achieved are shown in Figure 1, A and B.

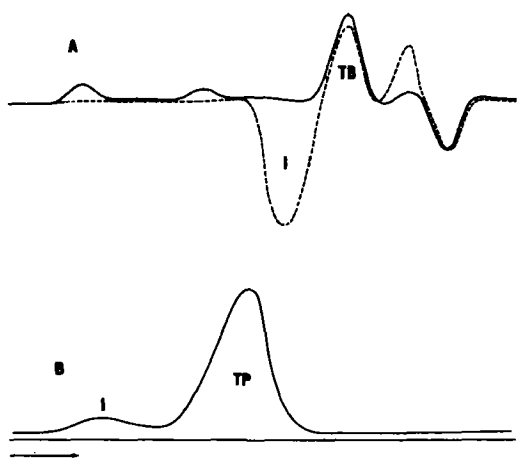

FIG. 1. Size exclusion chromatograms showing separation of triglyceride adduct fractions. A: Irradiated, dashed line, and unirradiated, solid line, tributyrin. Column: 24 ft × 3/8 in., Styragel 60 Å, $CHCl_3$, 2 mL/min; B: Irradiated tripalmitin. Column: 4 ft × 1 in. + 56 ft × 3/8 in. Styragel 60 Å, $CHCl_3$, 2 mL/min recycle. Adduct fractions designated 1.

Mass spectra of the adduct fraction from irradiated tributyrin were obtained by field desorption (FD) ionization and so-called (12) electron ionization desorption (EI/D) techniques on an appropriately equipped MAT Model CH-5 mass spectrometer (Michigan State University, NIH Biochemical Resource Facility, E. Lansing, MI). The FD and EI/D spectra were obtained by heating the emitter wire to desorb the sample thus providing ionization of the desorbing components in the high energy field (10 Kv). The ion current was repetitively scanned during desorption of the components from the wire. The sample was desorbed very rapidly during heating, and useful spectra were obtained only for the first few scans. Subsequent spectra revealed that extensive pyrolysis was occurring during desorption.

GC/MS data on tributyrin adducts from a prior study (7)

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were also considered and new GC/MS data were also acquired by means of a Finnigan OWA GC/MS system (University of Houston, Department of Biochemistry, Houston, TX) and a Finnigan 4000 GC/MS system (US Army Natick R&D Laboratories, Natick, MA). The GC column employed in the Finnigan systems was a 0.32 mm x 65 m fused silica bonded DB-1 phase using helium carrier gas at 1 mL/min and programmed at 4 C/min from 100 to 340 C. These latter data were obtained to see if improved GC separation on the fused silica column, viz, packed and glass capillary column data previously acquired (7), would provide better spectra. Unfortunately, molecular ions were not observed. However, ions corresponding to loss of acyloxy fragments and McLafferty rearrangement ions were found to agree with previously acquired spectra.

Mass spectra of the adduct fraction from tripalmitin were obtained by means of a fast atom bombardment ionization technique (13) on a VG Instruments, Ltd (VG Instruments, Ltd., Manchester, England). Model ZAB-2F double focusing high resolution mass spectrometer. The sample was placed on a target in the mass spectrometer ion source without the aid of a mulling agent and bombarded with 5 Kev argon atoms. The spectra were recorded by means of an oscillograph and the spectrum shown in Figure 4 has been reconstructed from the original data.

RESULTS AND DISCUSSION

Tributyrin

Initially, tributyrin was chosen as a model compound to study adduct formation in irradiated triglycerides, because of the expected volatility of the postulated products thus presenting the possibility of analysis by GC/MS. It was found, however, that although GC separation could be achieved and mass spectra obtained (7), the relative abundance of molecular and high mass ions in the spectra was so low (or nonexistent) that definitive structural assignments from the spectra could not be made. Accordingly, a means to enhance the molecular ion abundance was required. Although this is afforded by FD, only molecular ions are observed. A combination with EI/D provides the information necessary concerning the nature of fragment ions so that structural assignments can be made.

Although the components of the tributyrin adduct fraction can be separated by GC, collection of the fractions or direct introduction of the GC eluate in FD-EI/D mode is not feasible. The sample must be placed on the emitter wire in toto and the spectrum obtained of the mixture of components. Examination of such a spectrum, nevertheless, permits the identification of the components in a mixture. Although, in the case of irradiated tributyrin adduct compounds, several components are known from GC data to be present, the FD-EI/D spectra have thus far provided the identification of only 2 of the components and suggested the presence of a third component.

The spectra are seen in Figures 2 and 3. In Figure 2, the FD spectrum of the irradiation-induced adduct fraction shows 2 abundant components having molecular ions at 516 and 602. Evidence is seen for a component having a mass of 630, but no identification of this component has yet been possible.

The ion peaks of the EI/D spectrum shown in Figure 3 are designated in 3 different fonts to show the 3 compounds identified from the fragment ions observed.

Figure 3 shows the ions corresponding to the expected fragmentation of tributyrin 2,2' dimer, MW 602. The fragmentation pattern (Scheme I) is shown below.

The predominant characteristic cleavages for a polyhydric alcohol ester, and similar to triglycerides themselves (14), are all readily observed. Thus, for the postulated

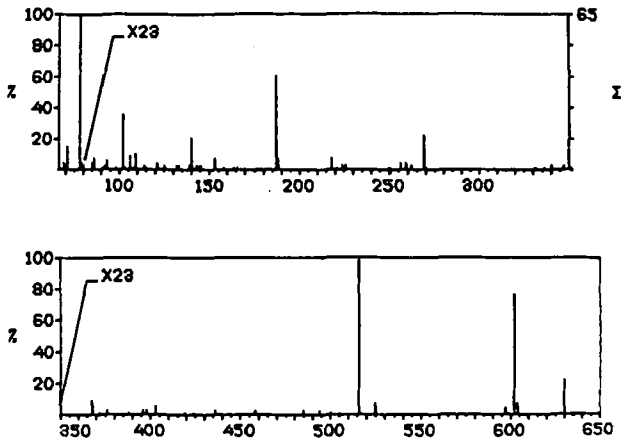


FIG. 2. Field desorption mass spectrum of irradiated tributyrin adduct fraction I. Molecular ions at m/e 602 and 516.

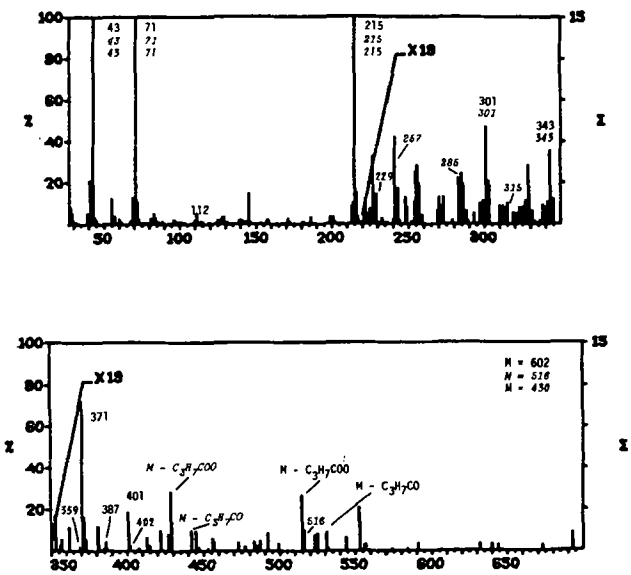
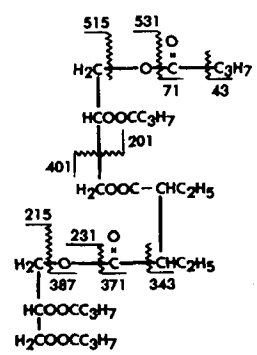


FIG. 3. Electron ionization/desorption mass spectrum of irradiated tributyrin adduct fraction I. Diagnostic ions shown for 3 components indicated in separate fonts.



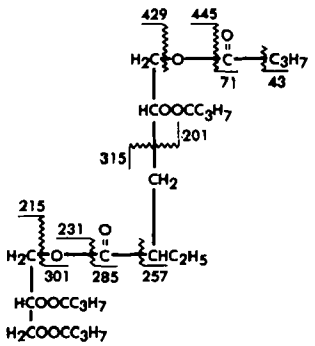
SCHEME I

dimer, loss of the acyl (C_3H_7CO; M-71) and acyloxy (C_3H_7COO; M-87) groups is seen to give rise to ions at m/e 531 and m/e 515, respectively. This is a common and very useful criterion for verification of the mass of the molecular ion (15). The low mass region of GC/MS spectra of tributyrin-derived compounds (7) all show a very abundant

(frequently the base peak) ion at m/e 71. Other spectra, e.g, of propanedioldiesters (16), similarly show an abundant ion corresponding to the acyl moiety and a corresponding, but less abundant, M-RCO ion. In contrast, the RCOO ion is frequently not seen or its abundance is very low, but the corresponding carbonium ion (M-RCOO) resulting from the cleavage is quite large. These relationships are seen to be upheld in all the spectra considered in this study.

The cleavages about the ester groupings in the vicinity of the 2,2′ dimer bond follow established principles. Thus, acyl cleavage gives a fragment of mass 371 with charge retention, whereas acyloxy cleavage gives M-387 with charge retention on m/e 215. A C-C cleavage α to the carbonyl group produces m/e 343 and a secondary cleavage of the same type at the position of dimerization leads to an abundant (RA ∿ 5%) alkyl fragmentation of high mass (relative to C_3) at m/e 112. (Note that the high mass region > m/e 220 has been enhanced by a factor of 13.) A McLafferty rearrangement ion characteristic of triglyceride type spectra is seen at m/e 574 (i.e., M-28 where 28 corresponds to the neutral loss of ethene). Other peaks in the spectrum (viz., m/e 199, 213, 227, 241, 255) are attributed to the typical series RCO + 128 + 14n characteristically found in triglyceride spectra. Likewise, m/e 145 and 186 are due to RCO + 74 and RCO + 115, respectively. The data well corroborate the identification of the postulated tributyrin 2,2′ dimer.

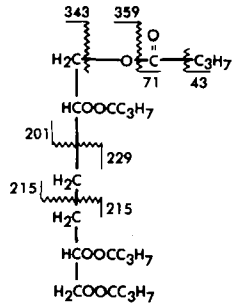
The structure of the compound having a molecular ion of 516 is similarly adduced. The fragmentation is depicted below in Scheme II and the salient features of the EI/D spectrum are shown in Figure 3.



SCHEME II

In a manner analogous to the identification of the tributyrin dimer, the propanedioldiester adduct is seen to display in its spectrum the characteristic M-RCO and M-RCOO ions at m/e 445 and 429, respectively. The cleavage vicinal to the 2 adduct position of the tributyrin shows diagnostic ions for acyl cleavage at m/e 285 and acyloxy at m/e 301. It should be noted that no ion is observed at m/e 231 for either the dimer or the propanedioldiester adduct in their spectra, but this is expected since in acyl cleavage the charge is expected to be retained by the acyl fragment. On the other hand, the acyloxy ion is found to be less abundant and the propanedioldiester moiety (i.e., m/e 215) becomes the predominant ion of the spectrum, arising in all cases regardless of whatever substituent may be introduced at the 2-position of the triglyceride fatty acid. The fragmentation ions clearly support a structure for the compound having a mass of 516 as a substituted propanedioldiester adduct at the 2-position of the butyric acid of tributyrin.

The EI/D fragmentation pattern also suggests the presence of a third adduct. Although no molecular ion is seen, the ions observed suggest a structure for a dimer of propanedioldibutyrate. The ions are indicated in Figure 3 and Scheme III is shown below.



SCHEME III

The failure to observe the molecular ion is not surprising. A weak bond between the 2 propanedioldiester moieties undoubtedly leads to easy cleavage producing m/e 215. The occurrence of this ion is not diagnostic. This ion is found also from M-RCOO cleavage in other compounds and is the largest peak in the spectrum. Stronger evidence is seen in the spectrum of irradiated tripalmitin adducts for an analogous propanedioldiester dimer (vide infra).

The parent minus acyl ion is seen to be quite small, but as is commonly observed, charge retention on the acyl ion diminishes the abundance of the M-RCO ion. Conversely, the M-RCOO, i.e., m/e 343 is quite large. Unfortunately, m/e 343 is isometric with a possible cleavage of the tributyrin dimer, and is accordingly not diagnostic. Two unique ions are seen. The cleavage at carbon number 4 on the hexanetetraol backbone (i.e., adjacent to an ester group) gives m/e 229 which is very abundant and could not be a fragment of the other compounds. Likewise, a unique McLafferty ion (M-28) is seen at m/e 402.

A mechanism for the formation of adducts in the radiolysis of triglycerides may be postulated as follows. Briefly, homolytic cleavage of the acyloxy moiety produces a propanedioldiester radical (PDDE•) and an acyloxy free radical (RCOO•). The acyloxy free radical subsequently decarboxylates to yield an alkyl free radical (R•). Either of the predominant free radicals, viz., PDDE• or R•, may abstract a hydrogen from the triglyceride, TG, to form TG•. Recombinations of TG•, PDDE• or R• form the observed compounds.

The process may be summarized as:

1) $TG \rightsquigarrow PDDE\cdot + RCOO\cdot$
$\hookrightarrow CO_2 + R\cdot$

2) $TG + \begin{matrix} R\cdot \rightarrow RH \\ PDDE\cdot \rightarrow PDDE \end{matrix} + TG\cdot$

3) $TG\cdot + TG\cdot \rightarrow (TG)_2$

4) $TG\cdot + PDDE\cdot \rightarrow TG - PDDE$

5) $PDDE\cdot + PDDE\cdot \rightarrow (PDDE)_2$

6) $TG\cdot + R\cdot \rightarrow TG - R$

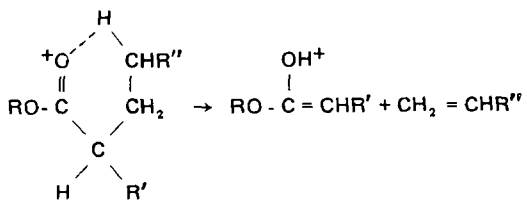
ESR data (17) have shown that the site of hydrogen abstraction in the triglyceride is probably α to the carboxyl in the fatty acid side chain. Accordingly, reaction 6 is seen to lead to the formation of a 2 alkyl substituted triglyceride. This possibility has resulted in a reexamination of the GC/MS data for tributyrin adducts previously reported (7).

Among the compounds reported by Meidani et al. (7) were the straight-chain hexanoyl and heptanoyl dibutyrates. It now appears that these compounds have branched chains and are, in fact, the 2-ethyl and 2-propyl butyryl dibutyrates. In the prior interpretation of the spectra, ions

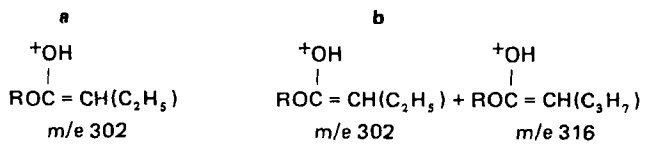
of low intensity in both the low and high mass regions, but nevertheless significant, were disregarded. The postulated structures are as follows:

where R is the propanedioldibutyrate moiety. If cleavage occurs as indicated, the ions at m/e 41 and 55 should be enhanced relative to their abundance in spectra where their formation is less favored. A comparison of the ratios of these 2 peaks in the respective spectra shows m/e 41 to be larger in **a** and m/e 55 to be larger in **b** relative to m/e 43 and m/e 57 (see Table I).

A more definitive consideration, however, is the observed mass of the McLafferty rearrangement ions. Although of low abundance, these ions appear in the spectra of the 2 compounds. Their formation may be depicted as:



If R′ is H, the resulting McLafferty ion will correspond to a loss of the entire alkyl side chain from the β carbon to the end, and in the *n*-alkyl side chains, the ion will have the same mass for any chain length. For tributyrin homologs, the ion occurs at m/e 274. When a substituent α to the carbonyl group is present, other McLafferty ions are observed corresponding to a loss of each side chain. The McLafferty ions for the respective α branched compounds postulated above are:



Where 2 ions are possible, the ion deriving from a loss of the longer chain is favored. For **b**, therefore, m/e 302 is expected to be more abundant. The high mass regions of the spectra of compounds **a** and **b** as well as that of a *n*-alkyl compound are given in Table I. Consideration of the abundant ions in the respective spectra shows that the predominant fragmentations lead to cleavages of the acyl and alkyl groups. A significant difference is observed as postulated for the low abundant McLafferty rearrangement ions. Thus, m/e 274 corresponding to a neutral loss of the entire straight-chain alkyl group is found in the spectrum of *n*-pentanoyl dibutyrin, whereas rearrangement ions corresponding to neutral losses of the side chains, i.e., m/e 302 and 316, are observed in the spectra of branched chain tributyrin homologs. It is significant that no m/e 274 ion is observed in the spectra of the branched chain compounds.

Tripalmitin

In previous studies of the radiolysis products of animal fats and model triglycerides (10,11), a partial separation of diglycerides, diol diesters, recombination products and polymeric substances was obtained from the bulk of triglycerides, indicating the presence of adducts. However, the method failed to provide adequate separation, and attempts to obtain meaningful FD-EI/D spectra were unsuccessful. Recent improvements in the size exclusion chromatographic procedure and the advent of fast atom bombardment ionization methods have now made it possible to characterize the adducts of long-chain triglyceride compounds. The data for tripalmitin are given here.

The SEC methods used in the current study provide an effective approach for the isolation of trace components from the large bulk of the lipid substrate. Separation of polymeric substances can be achieved with baseline resolution. As shown in Figure 1B, fraction I is separated effectively from 1 g of the irradiated tripalmitin. Recently, it has been possible to separate fraction I from 3 g of fat without any overlapping. This is accomplished by extending the column length of the 3/8 in. sections of 60-Å Styragel to 56 ft, incorporating a 4-foot presection of a 1-in. diameter 60-Å Styragel column, and using a recycle technique (19).

Because of the close similarity of structure and the high molecular weights of these adducts, it has not been possible to separate the individual components of fraction I. Mass spectra were obtained, therefore, of fraction I from irradiated tripalmitin without further separation. On the basis of the data obtained using FAB ionization, 4 adduct radiolysis products were identified.

TABLE I

Relative Abundance of Selected Ions from the Spectra of Tributyrin Homologs (18; S.R. Missler and C. DiPietro, unpublished data)

	n-Pentanoyl dibutyrin		2-Ethyl tributyrin		2-Propyl tributyrin	
m/e	R.A.[a]	Ion type	R.A.	Ion type	R.A.	Ion type
41	–	–	12.50	–	28.03	–
43	45.30	R	43.00	–	84.90	–
55	8.50	–	5.80	–	17.30	–
57	31.30	R′	3.30	–	8.70	–
71	100.00	RCO	100.00	R″ + RCO	100.00	RCO
85	87.94	R′CO	7.16	RCOO	55.80	R‴
99	–	–	38.50	R″CO	–	–
113	1.90	–	2.10	–	35.10	R‴CO
274 (r)	0.24	M-42	–	–	–	–
302 (r)	–	–	0.10	M-28	0.40	M-42
316 (r)		–	–	–	0.20	M-28

[a]R.A. = relative abundance.

R = C_3H_7; R′ = C_4H_9; R″ = C_5H_{11}; R‴ = C_6H_{13}.

r = McLafferty rearrangement ion.

The reconstructed mass spectrum of irradiated tripalmitin fraction I is presented in Figure 4. As described in detail later, 4 compounds were identified from the fragmentation pattern. These are the 2,2′ dimer of tripalmitin, a 2-propanedioldipalmityl tripalmitin, a propanedioldipalmitate dimer, and 2-pentadecyl tripalmitin.

The expected fragmentation scheme for the tripalmitin 2,2′ dimer, MW 1610, is given below (Scheme IV).

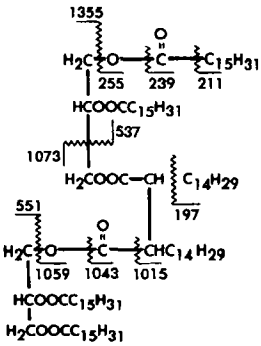
Fragmentation analogous to the tributyrin adduct spectra is observed. In the low mass region of the spectrum, ions at m/e 211, 239 and 255 are seen corresponding to cleavages resulting in the alkyl ion, $C_{15}H_{31}^+$, the acyl ion, $C_{15}H_{31}CO^+$, and the acyloxy ion $C_{15}H_{31}COO^+$. These are expected to be common to all the tripalmitin adduct spectra. An ion at m/e 197 corresponds to $C_{14}H_{29}^+$, a residual alkyl fragment indicating substitution at the position α to the carboxyl group in the palmitic acid moiety.

The most abundant ion in the spectrum, likewise common to all the compounds, is m/e 551, corresponding to the propanedioldipalmitate moiety and resulting from M-RCOO where RCOO is the substituted palmitic acid moiety. m/e 1059 is seen but has very low abundance as charge retention on the acyloxy ions is not seen to be favored in spectra of this class of compounds. Diagnostic ions for the dimer are found at m/e 1355, i.e., M-$C_{15}H_{31}COO$, and at m/e 1043 corresponding to acyl cleavage of the dimer-linked palmitic acid moiety.

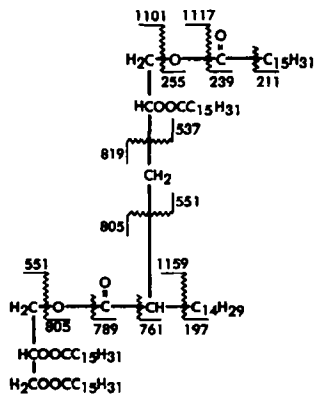
The fragmentation scheme for the propanedioldipalmitate-tripalmitin adduct, MW 1356, is given below (Scheme V).

Diagnostic ions for this compound are found in the spectrum at m/e 1117 and 1101 corresponding to palmityl and palmitoyl cleavages, respectively. The acyl and acyloxy cleavage of the palmitic acid moiety linked to the propanedioldipalmitate give rise to ions at m/e 789 and 805. The acyloxy ion at m/e 805 is isometric with an ion resulting from C-C cleavage of the adduct bond, but its relatively large abundance suggests it most likely results from acyloxy cleavage in the diol-diester-triglyceride adduct. Other ions at m/e 761 and 1159 are also indicative of the structure as shown in Scheme V.

Evidence for a dimer of a dioldipalmitate is implied by the spectrum in a manner which is closely parallel to the



SCHEME IV



SCHEME V

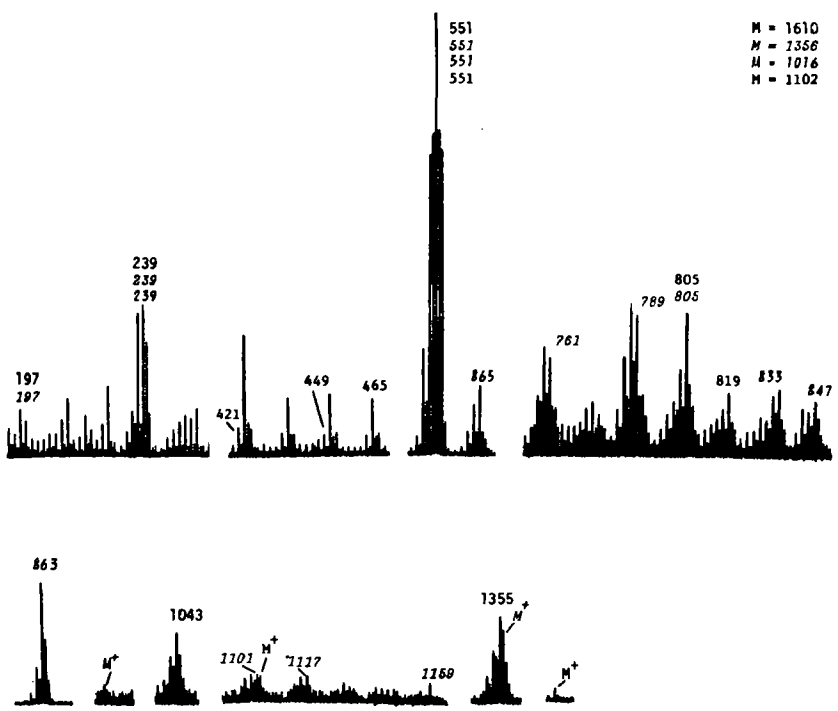


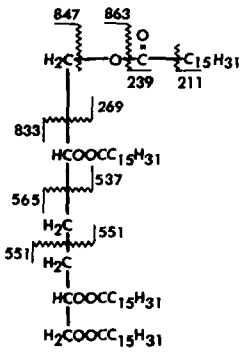
FIG. 4. Fast atom bombardment mass spectrum of irradiated tripalmitin adduct fraction I. Diagnostic ions for 4 compounds indicated in separate fonts.

data for the propanedioldibutyrin dimer. The fragmentation pattern is depicted in Scheme VI.

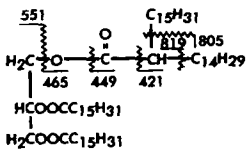
The molecular ion is seen at m/e 1102 with corresponding M-RCO and M-RCOO ions at m/e 863 and 847. The ions at m/e 565 and 833 are likewise unique.

The presence of an α-pentadecyl substituted tripalmitin is adduced from Scheme VII.

The molecular ion at m/e 1016 has a very low abundance, but the acyloxy ion at m/e 465 and the $C_{30}H_{61}^+$ at m/e 421 are both abundant. The acyl ion at m/e 449 is also clearly observed. Ions corresponding to alkyl losses of $C_{15}H_{31}$ at m/e 805 and $C_{14}H_{29}$ at 819 are both observed. Although the ion at m/e 805 is isometric with ions from the other compounds, the ion at 819 can be considered diagnostic. The structure of the pentadecyl substituted tripalmitin is further corroborated by the presence of Mc-



SCHEME VI



SCHEME VII

Lafferty rearrangement ions at m/e 806 and 820. These ions, as in the analogous tributyrin compound, provide the evidence for the 2-alkyl substituted triglyceride.

Other FAB spectra have been obtained for an irradiated, unsaturated triglyceride, trilinolein, and for an irradiated natural fat (pork). The results of these studies will be reported in a subsequent manuscript.

ACKNOWLEDGMENTS

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