Structures and stereochemistry of the very long α,ω-bifunctional alkyl species in the membrane of *Sarcina ventriculi* indicate that they are formed by tail-to-tail coupling of normal fatty acids

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Abstract In a previous study, we demonstrated that *Sarcina ventriculi* is capable of adjusting to alterations in environmental conditions (such as increase in temperature, lowering of pH, or addition of exogenous organic solvents) by the synthesis of a family of α,ω-dicarboxylic acids ranging from 28 to 36 carbons long (Jung, S., et al. 1993. *J. Biol. Chem.* **268:** 2828–2835). The chain lengths and relative abundance of the very long dicarboxylic acids found in *S. ventriculi* suggest that they may be formed after the perturbation by the (enzymatic) tail-to-tail combinations of existing regular monofunctional fatty acids and not completely de novo by direct 2-carbon addition of acetyl coenzyme A (CoA). If this were true, knowing the structures of the regular fatty acids, we can predict those of the very long chain bifunctional acids. In this work we present definitive chemical results that strongly support this mechanism. This was done by analyzing the structures and stereochemistry of the very long bifunctional species in the light of those of the regular monofunctional species. The exact structures of membrane fatty acid methyl ester derivatives components were determined by various spectroscopic and chemical methods including gas chromatographic (GC) analysis, gas chromatography–mass spectrometry (GC–MS), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, polarimetry, and reductive ozonolysis. This yielded precise structural and stereochemical information on the position of substitution of the acyl chain by methyl groups, position and configuration of double bonds, and optical activity. These results, coupled with the absence of intermediate length acyl species, indicated that the very long alkyl species (without exception) can be formed by tail-to-tail joining of existing fatty acids. The ideas of a dynamically regulated catalytic system is proposed.—**Jung, S., and R. I. Hollingsworth.** Structures and stereochemistry of the very long α,ω-bifunctional alkyl species in the membrane of *Sarcina ventriculi* indicate that they are formed by tail-to-tail coupling of normal fatty acids. *J. Lipid Res.* 1994. **35:** 1932–1945.

Supplementary key words very long chain dicarboxylic acid • bifunctional fatty acid • gas chromatography–mass spectrometry • nuclear magnetic resonance • reductive ozonolysis • fatty acid synthesis • membrane adaptation • dynamic regulation

The membrane is a universal structural feature that is responsible for signal and energy transduction, compartmentalization of biological processes, and maintenance of the structural integrity of the cell in all living systems, from microorganisms through plants and animals. Membranes play a central role in critical events such as DNA replication (1–3), cell division, protein synthesis, electron transfer, and photosynthesis.

Despite the obvious importance of lipids, it is thought that proteins are the structural entities that control membrane fluidity at elevated temperatures and that changes in external temperature are sensed directly by proteins with no stated involvement of lipids (4–6). Proteins are viewed as the universal entities that structurally preserve cell function and integrity. The model is that they sense environmental changes, activate transcription, and cause the synthesis of new proteins which then alter membrane lipid structure (7). A simpler, more desirable system would be one in which changes in membrane dynamics are transmitted through the lipids to trigger a constitutive, fluidity-regulated, dynamic (enzymatic) adaptation system. This system would chemically modify the membrane lipids to return the membrane to its original fluidity or dynamic state (at which the adaptation system is inactive). This would constitute the ultimate in adaptation systems as the entire transcription/translation machinery would not have to be set in motion. This adaptive event would, however, result in the formation of a new membrane structure.

Abbreviations: GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared.

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In the case of bacteria, a likely course would be for the organism to adopt a membrane structure that is typical of bacteria whose normal habitats are similar to the new environmental conditions. In the case of substantial temperature increases from normal ambient temperatures, the new membrane structure might resemble that found in thermophilic eubacteria (8–10) or archaebacteria (11, 12) in some important ways. Bacteria with a very dynamic range of environmental tolerance are especially suited as model systems for studying these adaptation phenomena as the chemistry of the membrane can be studied as a function of the perturbed state and mutants that are tolerant to the new conditions do not have to be obtained.

Sarcina ventriculi is a gram positive, strict anaerobic eubacterium that can adapt to environmental pH values between 3 and 9 (13–15). This organism adapts well to perturbations such as lowering pH, the addition of organic solvents, or raising the temperature. The gas chromatography profile of the methanolysate of the total cell mass after these perturbations indicated the presence of two separate domains of fatty acid chain lengths (16): an early eluting one due to regular chain length fatty acyl components and a later one due to components with lengths twice that of the regular fatty acyl components. This latter domain contained the methyl ester derivatives of very long α,ω-dicarboxylic acids. The unusual chain length distribution and the large variety of very long chain dicarboxylic species formed during this adaptive response to the various environmental perturbations suggest the possibility of an unusual, new mechanism for their formation. The typical 2-carbon addition process involving the acetyl CoA cannot be used to explain the structures (α,ω-bifunctional) and chain length distribution of the very long chain components of the membrane of this species. Here, we report chemical evidence for the mechanism of formation of very long chain bifunctional alkyl species by tail-to-tail addition of hydrocarbon chain from opposite sides of the bilayer. This would constitute an entirely new, very sophisticated mechanism for membrane adaptation. The conclusions about the mechanism are drawn from the satisfaction of certain structural and stereochemical features of the new bifunctional lipids imposed by the structures of the regular fatty acids and the nature of the mechanism. We propose that such a direct coupling mechanism could be effected by an enzymatic activity that is already resident in the membrane but whose catalytic states are accessible only after the surrounding membrane lipids are in certain (undesirable) states of motion.

MATERIALS AND METHODS

Organism and culture conditions

S. ventriculi JK was cultivated as described previously (16). For growth under pH control, 4-liter Kimax jars (Baxter Scientific Products, Romulus, MI) containing 3 liters of medium were used. The jars were equipped with a pH probe, and the culture was mixed by placing the jars on a magnetic stirrer. The organism was grown at pH 3.0. The cells were harvested at midexponential phase, washed twice with distilled water, and stored at −70°C for further analysis.

Membrane preparations

Cells were disrupted by passage through a French Pressure cell (American Instruments Co., Inc., Silver Spring, MD) at 20,000 lb/in^2. The disrupted cells were centrifuged at 20,000 *g* to remove unbroken cells, and the supernatant was centrifuged at 110,000 *g* to sediment the membranes, which were washed twice with distilled water.

Total fatty acid analyses

Fatty acid analyses were performed on whole cells or isolated membrane fractions by treatment with methanolic HCl using either of two procedures. Procedure (a) was used for whole cells and procedure (b) was used for isolated membranes. (a) Cells (1–5 mg) suspended with 0.3 ml chloroform and 1.5 ml 5% methanolic HCl solution were sealed in a Teflon-lined screw-capped vial, and heated in a water bath or oven at 72°C for 24 h. Chloroform (3 ml) was added every 8 h followed by mild sonication using a bath sonicator for 5 min. After concentration to dryness under nitrogen gas, samples were partitioned between water and chloroform and the aqueous layer was washed several times with chloroform or hexane. The combined solutions were filtered through glass wool. (b) Three ml of chloroform was added to 1 ml of membrane suspension followed by 15 ml 5% methanolic-HCl solution. The flask was sealed and heated in an oven at 72°C for 12 h. Three (3) ml of chloroform was added every 6 h followed by mild sonication using a bath sonicator for 5 min. The mixture was then concentrated on the rotary evaporator to dryness and extracted with chloroform. The combined organic fraction was redissolved in 1 ml of hexane. The fatty acid methyl esters prepared by either procedure (a) or (b) were subjected to gas chromatography (GC) analysis on a 25 M J&W Scientific DB1 capillary column using helium as the carrier gas and a temperature program of 150°C initial temperature, 0.00 min hold time and 3.0 deg/min rate, to a temperature of 200°C. A second ramp of 4.0°/min was then immediately started until the final temperature of 300°C was obtained. This temperature was held for 30 min. The relative proportions of lipid components were calculated from the integrated peak areas. The fatty acid identification and molecular weight were determined using GC–MS analysis using a Jeol JMS-AX505H spectrometer interfaced with a Hewlett-Packard 5890A gas chromatograph.

Isolation of the α,ω-dicarboxylic acid dimethyl esters

The total lipids (100 mg) extracted from the cells as described above were methanolyzed with 5% (w/v) HCl in methanol (5 ml) for 12 h at 72°C. Chloroform (1 ml) was added every 4 h followed by mild sonication using a bath sonicator for 5 min. The mixture was then concentrated on the rotary evaporator to dryness and extracted with chloroform. The combined organic fraction was redissolved in 1 ml of hexane. This fraction was applied to a silica flash chromatography column and eluted with chloroform–hexane 1:1 (by vol). Fractions were assayed by gas chromatography. Fractions containing very long chain fatty acid methyl esters were concentrated and rechromatographed on 5% $AgNO_3$-impregnated preparative thin-layer silica chromatography plates that were eluted with petroleum ether–diethyl ether–acetone 10:1:0.5 (by vol). Spots were made visible either by spraying with 50% ethanolic-sulfuric acid and heating at 250°C to char the organic components, or by spraying with a 0.1% solution of 2',7-dichlorofluorescein in aqueous ethanol (1:1) and viewing under ultraviolet light (17). Bands were scraped from the plate into a column fitted with a sintered disc and the material was eluted from the silica gel with methanol and chloroform. Each fraction was concentrated by evaporation and redissolved in chloroform for further analysis. Purity of each fraction was assayed by GC–MS.

Isotope labeling

Isotope labeling was used to aid in deducing the structures of esterified lipid components using GC–MS. Methyl esters of fatty acids obtained by acid methanolysis were further treated with 5% deuterated methanolic-HCl for 6 h at 72°C. Deuterated methyl esters of fatty acids were extracted and analyzed as described above.

^{1}H NMR and ^{13}C NMR spectroscopy

Proton NMR spectra were recorded at 300 MHz on solutions in $CDCl_3$. Fourier transform ^{13}C NMR spectra were recorded at 125 MHz on solutions in $CDCl_3$. Chemical shifts are quoted relative to the chloroform resonances taken at 7.24 ppm for proton and 77 ppm for ^{13}C measurements, respectively. Spectra were recorded on a Varian VXR 300 instrument. Typical acquisition parameters for ^{1}H spectra were as follows: SW (spectral width) 4573.2 Hz, AT (acquisition time) 3.499 s, PW (pulse width) 7.0 μs, dl(relaxation delay) 2 s, NP (number of data points) 32000, NT (number of transients) 162. Typical parameters for ^{13}C spectra were as follows: SW (spectral width) 17323.6 Hz, AT (acquisition time) 1.199 s, PW (pulse width) 8.7 μs, dl (relaxation delay) 3 s, NP (number of data points) 41536, NT (number of transients) 900.

Fourier transform infrared spectroscopy

Spectra were obtained with a Nicolet model 710 FT-IR spectrometer on a 10% (w/v) solution of dicarboxylic acid dimethyl ester in chloroform.

Optical rotation analysis (polarimetry)

Optical polarimeter (Perkin-Elmer) was used for measuring the optical activity with the mercury (Hg) light at various wavelengths (578 nm, 546 nm, and 436 nm). Measurements were performed on 20-mg samples dissolved in 1 ml chloroform solution.

Reductive ozonolysis (18, 19)

For ozonolysis of unsaturated lipids, a solution of 50 mg sample in 2 ml methylene chloride was cooled to −70°C. Ozone was passed through the solution at a rate of 60 mg/h from an ozone generator while maintaining the temperature at −70°C. The extent of the reaction was checked every 10 min by TLC. Ozonides were decomposed to aldehydes by reductive cleavage using Lindlar catalyst (20). These shorter chain aldehydes then were isolated by preparative TLC or flash column chromatography (21). The products were further analyzed by GC and GC–MS.

RESULTS AND DISCUSSION

GC analyses of fatty acids of *S. ventriculi*

Sarcina ventriculi were grown at 37°C, pH 7.0, and then shifted to 45°C at late log phase for 3 h. GC analyses of fatty acid methyl esters extracted from cells are shown in **Fig. 1.** The peaks from 1 to 4 are due to typical membrane fatty acyl components ranging from 14 to 18 carbons. Major regular fatty acids are hexadecanoic acid (peak 2, $C_{16:0}$) and *cis*-vaccenic acid (peak 3, $C_{18:1(11)}$). The peaks from A to E, however, correspond to very long bifunctional fatty acids components containing from 32 to 36 carbon atoms.

Mass spectrometric analyses of very long bifunctional fatty acids

The mass spectrum of peak B indicated that it was the previously described (16) α,ω-diacid **1.** (**Scheme 1**). The electron impact mass spectrum of peak D (**Fig. 2A**) contained major ions at *m/z* 564, 532, and 501. These corresponded to the molecular ion of a C34-α,ω-dicarboxylic dimethyl ester (M^+) with the sequential losses of methanol (CH_3OH) and a methoxy (CH_3O) group, respectively. The ion at *m/z* 514 was assigned to the structure obtained by sequential losses of methanol (CH_3OH) and water (H_2O) from the parent ion. The mass spectrum also contained

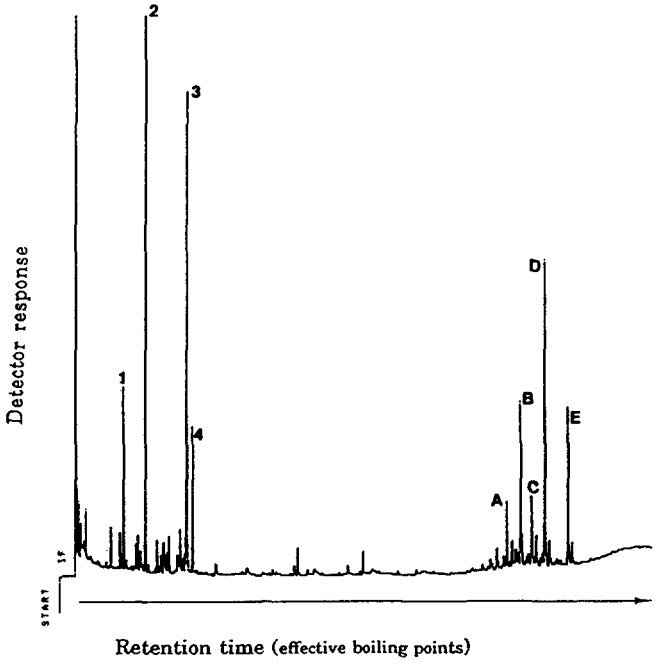


Fig. 1. Gas chromatographic analyses of the total methanolysate of the lipids in the membrane of *Sarcina ventriculi* cells grown at pH 7.0 at 37°C and then shifted to 45°C at late log phase for 3 h. The later eluting cluster of peaks is due to very long chain α,ω-bifunctional fatty acids. The major components are: 1. $C_{17:1}$-fatty aldehyde ($HCO(CH_2)_9CH{=}CH(CH_2)_4CH_3$); 2. $C_{16:0}$-carboxylic acid methyl ester ($CH_3OCO(CH_2)_{14}CH_3$); 3. $C_{18:1}$-carboxylic acid methyl ester ($CH_3OCO(CH_2)_9CH{=}CH(CH_2)_5CH_3$); 4. $C_{18:0}$-carboxylic acid methyl ester ($CH_3OCO(CH_2)_{16}CH_3$); A. $C_{33:1}$ ω-formylmethyl ester; B. $C_{32:0}$-α,ω-dicarboxylic acid dimethyl ester; C. $C_{35:1}$ ω-formylmethyl ester; D. $C_{34:1}$-α,ω-dicarboxylic acid dimethyl ester; E. $C_{36:2}$-α,ω-dicarboxylic acid dimethyl ester.

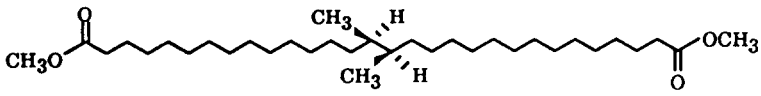
a series of ions, the general structure of which corresponded to $CH_2{=}CH\text{-}(CH_2)_n$ (n = 2,3,4..), characteristic of an alkene. It also contained a series of ions, the general structure of which corresponded to $CH_3OCO\text{-}(CH_2)_n$, beginning at *m/z* 73 (n = 1), as well as the characteristic McLafferty fragment at *m/z* 74 ($CH_3OCOH{=}CH_2$) of a saturated methyl ester. This series of ions beginning at *m/z* 73 continued up to and included two prominent ions at *m/z* 263 and 296 suggesting the presence and location of vicinal methyl groups. Scission between these methyl groups explained the primary fragments at *m/z* 269 and 295. The general structure of the molecule was consistent with the coupling between hexadecanoic and *cis*-11-octadecenoic acid at their ω-1 positions to give structure **2.** The ion at *m/z* 263 corresponded to the loss of methanol (CH_3OH) from the *m/z* 295 fragment. The ion at *m/z* 296 is due to a rearrangement resulting in elimination of a hydrogen atom from *m/z* 297 fragment or capture of a hydrogen atom by the *m/z* 295 fragment. The ion at *m/z* 237 represented the losses of methanol (CH_3OH) from the *m/z* 269. The fragments are tabulated and assigned in **Table 1.** Fig. 2B shows the electron impact mass spectrum of the deuterium-labeled molecule obtained by methanolysis with D-4 methanol/HCl solution to produce the trideutero methyl ester. The most striking change was the upward shift of the molecular ion by 6 mass units which confirmed the presence of two methoxy groups. The losses due to trideutero methanol (CD_3OH) and the sequential loss of a trideutero methoxy group (CD_3O) from the molecular ion (*m/z* 570) give ions at *m/z* 535 and 501. The deuterium-labeled McLafferty fragment (*m/z* 77) was also observed. Ions at *m/z* 272 and 299 corresponding to *m/z* 269 and *m/z* 296 in the unlabeled ester were also observed. The ion at *m/z* 263 corresponded to the loss of trideuterated methanol (CD_3OH) from the ions at *m/z* 298. Other major fragments are listed in Table 1. Although the mass spectra indicated the exact positions of vicinal methyl groups and the presence of one degree of unsaturation in the molecule, the location of the double bond could not be deduced and was proven by chemical degradation.

Figure 3A shows the electron impact mass spectrum of peak E. This spectrum contained major ions at *m/z* 590, 558, and 527. These corresponded to the molecular ion of a bis-unsaturated C36-α,ω-dicarboxylic dimethyl ester (M^+) with the sequential losses of methanol (CH_3OH) and a methoxy (CH_3O) group, respectively. This was consistent with coupling of two *cis*-11-octadecenoic acid chains to form structure **3.** The prominent ion cluster at *m/z* 293 indicated fragmentation between the vicinal methyl groups in the acyl chain followed by the elimination of hydrogen to produce two identical stabilized, bis-unsaturated acyl chain fragments. The 28 mass unit difference between the primary 293 and 321 chain cleavage fragments also indicated the presence and location of vicinal dimethyl groups.

Figure 3B shows the electron impact mass spectrum of the deuterium-labeled peak E molecule obtained by methanolysis with D-4 methanol/HCl solution to produce the tri-deuterated methyl ester. A 6 mass unit increase in the molecular ion (consistent with two methyl ester functions) was observed as well as the sequential losses of trideutero methanol (CD_3OH) and a trideutero methoxy group (CD_3O).

NMR and FTIR analyses of isolated dicarboxylic dimethyl esters

NMR (nuclear magnetic resonance) analyses of peaks D and E confirmed the presence of the double bond and vicinal methyl groups deduced from GC–MS data. In the proton spectrum of peak D (**Fig. 4A**), the methyl groups resulted in a 6H doublet at δ 0.74 (J = 7.20 Hz). A triplet at δ 5.32 (J = 6.92 Hz) in both spectra was assigned to the vinyl protons. The ^{13}C NMR spectrum of peak D (Fig. 4B) contained resonances ascribed to the ester carbonyl carbons at δ 174.5, methoxy carbon at δ 51.4, and branched methyl carbons at δ 14.5. The vinylic carbons



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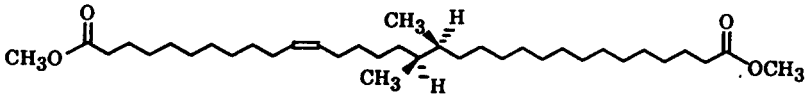

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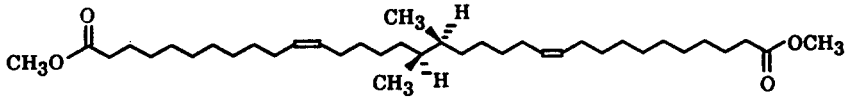

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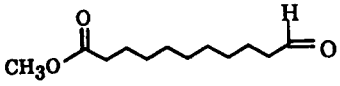

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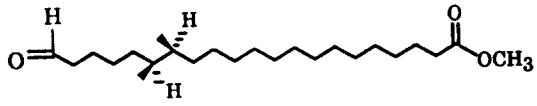

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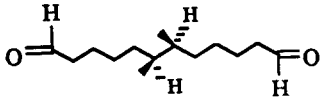

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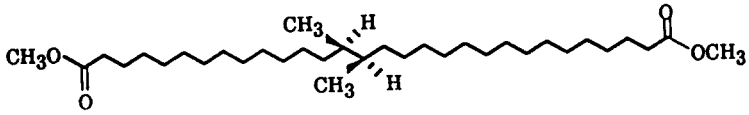

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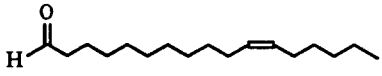

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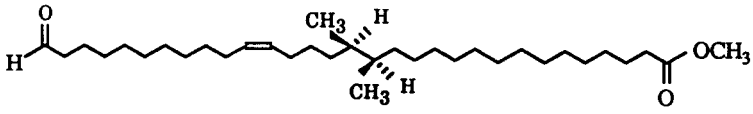

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Scheme 1.

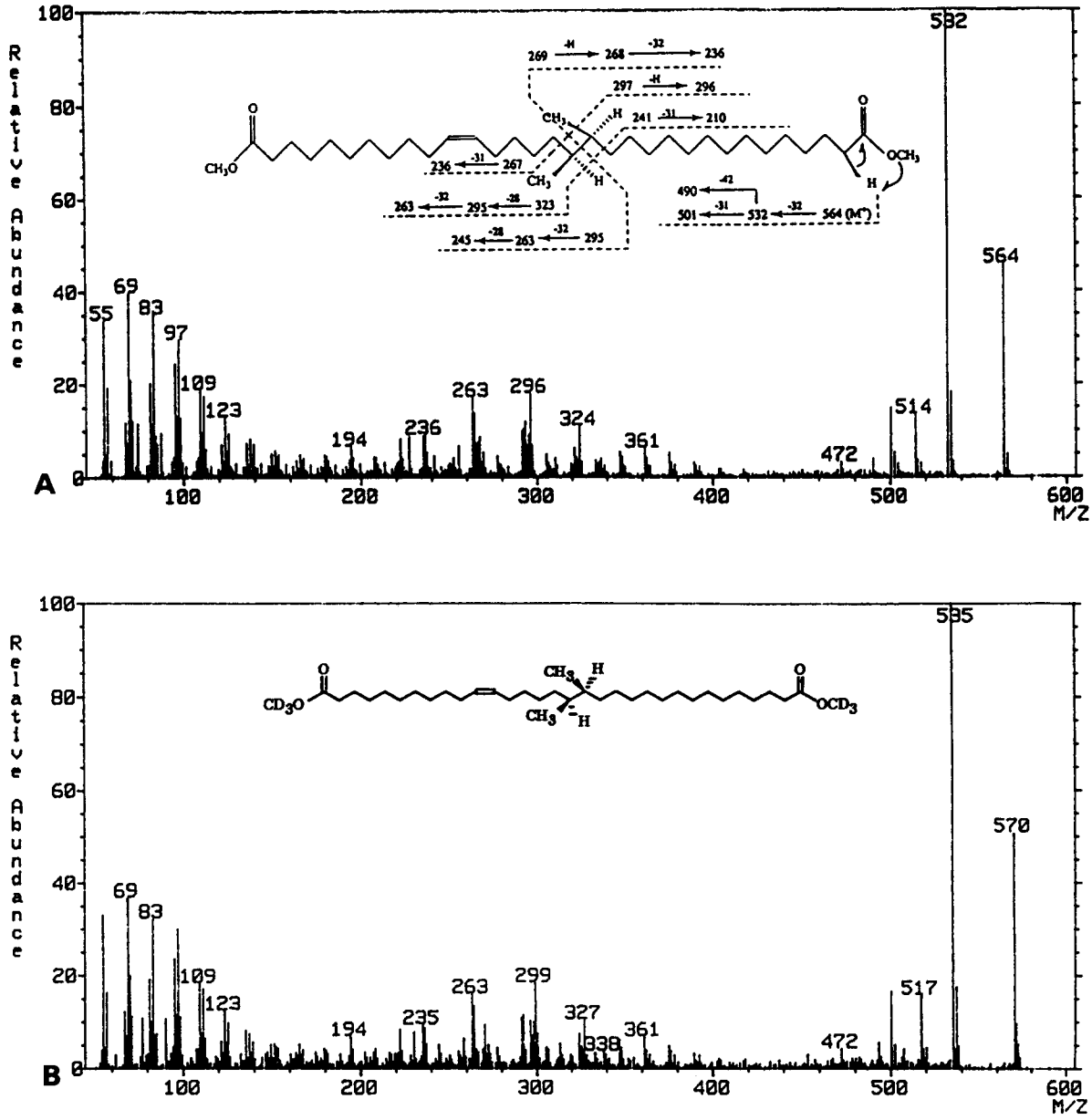

Fig. 2. (A) Electron impact mass spectrums of peak D (assigned structure **2**). Note the major ions at *m/z* 564, 532, and 510. These corresponded to molecular ion (M^+) with the sequential losses of methanol and methoxy, respectively. The cluster of ions at *m/z* 263, 296, and 324 are due to the vicinal methyl groups. (B) The electron impact mass spectrum of deuterium-labeled molecule obtained by deuteration with D-4 methanolic HCl solution. The presence of two carboxyl groups was confirmed by the shift of 6 units of the M^+ ion. Other predicted mass increases due to deuterium were easily observed.

appeared at δ 131.5. In the case of peak D, the triplet at δ 5.32 in the ^{1}H-NMR spectrum integrated for two protons. However, in the case of peak E (**Fig. 5A**) it integrated for four protons. Resonances at δ 1.27 were assigned to the methylene groups of the lipid chain. The multiplet at δ 1.58 was assigned to the methylene protons β to the carbonyl group. Resonances at δ 2.28 (t, J = 7.69 Hz) were assigned to the methylene groups α to the carbonyl function. A 6H singlet at δ 3.65 was assigned to ester methoxy group resonances. The 4H multiplet at δ 2.00 was assigned to the protons of the methylene groups α to the vinyl carbons. In the ^{13}C NMR spectrum of peak E (Fig. 5B), an increase in intensity of the signal at δ 130.8 (vinylic carbons) was observed. This spectrum only contained 19 signals confirming the proposed symmetry of the molecule. Information on the configuration

TABLE 1. Analyses of 70eV electron impact mass spectral fragments of proposed structure 2

Structure of Mass Fragments	Mass (*m/z*)	Deuterated Mass (*m/z*)
$CH_3OCO(CH_2)_9CH=CH(CH_2)_4CHCH_3CH_3CH(CH_2)_{13}OCOCH_3$	564	570
$CH_3OCO(CH_2)_9CH=CH(CH_2)_4CHCH_3CH_3CH(CH_2)_{12}CH=CO$	532	535
$OC(CH_2)_9CH=CH(CH_2)_4CHCH_3CH_3CH(CH_2)_{12}CH=CO$	501	501
532 – 18 (H_2O)	514	517
$CH_3OCO(CH_2)_9CH=CH(CH_2)_4CHCH_3CH_3CH(CH_2)_{10}CH=CH2$	490	493
$CH_3OCO(CH_2)_9CH=CH(CH_2)_4CHCH_3CH_3CH$	323	326
$CH_3OCO(CH_2)_9CH=CH(CH_2)_4CHCH_3$	295	298
$CH_3OCO(CH_2)_{13}CH(CH_3)CH(CH_3)$	297	300
297 – 1 (H)	296	299
$CH_3OCO(CH_2)_{13}CHCH_3$	269	272
$CO=CH(CH_2)_8CH=CH(CH_2)_4CHCH_3$	263	263
$CO=CH(CH_2)_{12}CHCH_3$	237	237
$CH_3OC(OH)=CH_2$	74	77
$CH_3OCO(CH_2)_n$: n = 1 ~ 13	73, 87, 101, ..	76, 90, 104, ..
$CO=CH(CH_2)_n$: n = 1 ~ 13	55, 69, 83, ..	55, 69, 83, ..
CH_3-$(CH_2)_n$: n = 3 ~ 12	57, 71, 85, ..	57, 71, 85, ..
$CH_2=CH$-$(CH_2)_n$: n = 2 ~ 11	55, 69, 83, ..	55, 69, 83, ..

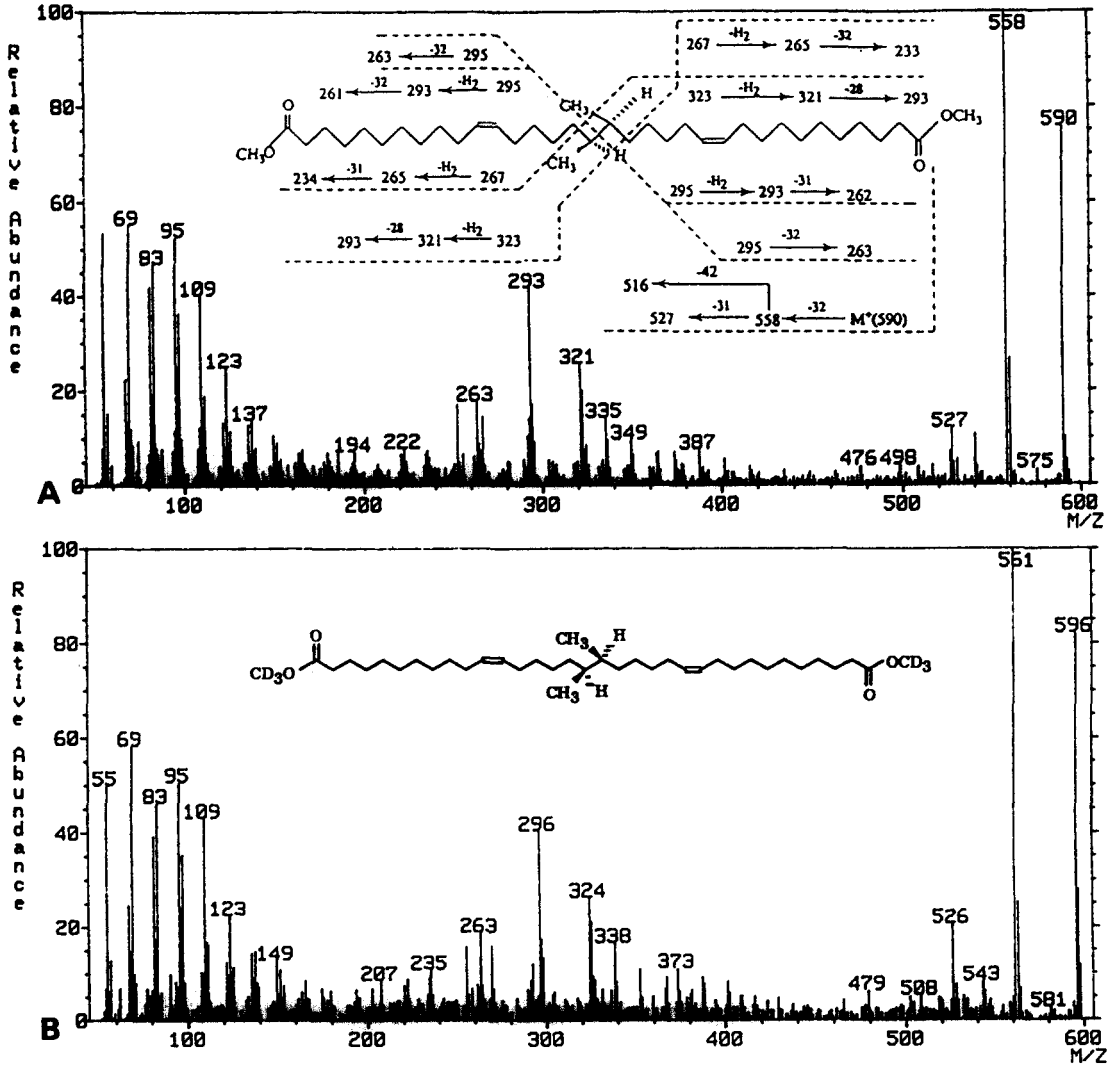

Fig. 3. (A) Electron impact mass spectrum of peak E (assigned structure 3). Major ions appear at *m/z* 564, 532, and 510. These correspond to the molecular ion (M^+) with the sequential losses of methanol and a methoxy group respectively. The cluster of ions at *m/z* 265, 293, and 321 confirm the presence and location of the vicinal methyl groups. (B) The electron impact mass spectrum of deuterium-labeled peak E obtained by deuteration with D-4 methanolic HCl solution. The presence of two carboxyl groups was confirmed by isotope labeling. Mass increases due to deuterium were easily observed. The most striking change is the increase of the molecular ion by 6 mass units.

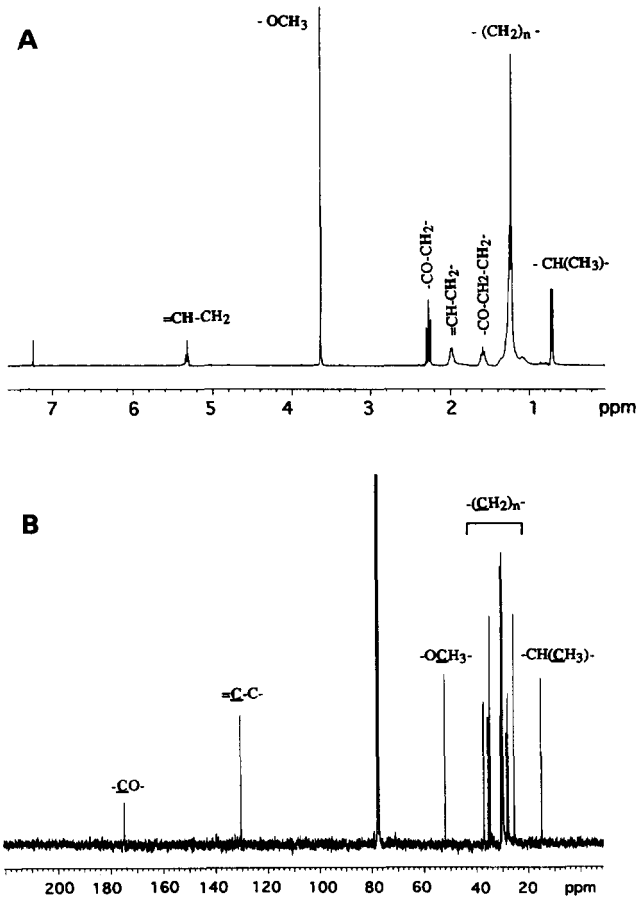

Fig. 4. (A) The ^{1}H-NMR spectrum of proposed structure **2** (peak D). The signal at δ 5.32 (t, J = 6.92Hz) represented a methine proton on an unsaturated carbon atom. The mutiplet at δ 2.00 was assigned to the protons of methylene groups adjacent to the unsaturated carbons. The peak at δ 1.27 is due to the methylene groups of the hydrocarbon chains. The signal at δ 3.65 is due to the methyl group of the methoxycarbonyl functions. The multiplet at δ 1.58 arises from the protons on the carbons β to the carbonyl function. The doublet at δ 0.74 (J = 7.20Hz) is due to the protons of the vicinal methyl groups. The signal at δ 7.24 is due to chloroform. (B) The ^{13}C-NMR spectrum of proposed structure **2.** Signals at δ 131.5 correspond to olefinic carbons. Signals at δ 174.5 and at δ 51.4 confirmed the presence of methyl ester group. The vicinal methyl group gives rise to the signal at δ 14.5. The signals at δ 77.0 are due to chloroform.

of the double bonds as well as other information confirming the specific chemical functional groups was obtained by Fourier transform infrared spectroscopy. The infrared spectrum of peak D (not shown) showed a strong aliphatic C-H asymmetric stretching absorption at 2928 cm^{-1} and symmetric stretching at 2856 cm^{-1}. The characteristic alkene stretching (=C-H) was observed at 3020 cm^{-1}. A band due to the bending vibration of aliphatic C-H bonds in the methylene groups appeared at 1464 cm^{-1} (scissoring) and one due to the twisting and wagging deformation at 1315 cm^{-1}. The characteristic C=O absorption bond of the aliphatic ester group appeared at 1732 cm^{-1} and the ester alkoxy stretch at 1213 cm^{-1}. The stereochemistry (*cis*- or *trans*-) of the unsaturated carbons was determined by the clear presence of a strong peak at 667 cm^{-1} due to the C=C-H bending deformation for *cis*-alkenes (22). The IR spectrum of peak E also indicated a *cis*-configuration for the two unsaturated double bonds in the molecule. Note that the configuration of the double bonds of the regular length $C_{18:1(11)}$ acyl chains was *cis* from *cis*-vaccenic acid (11-octadecenoic acid). With regard to the mechanism of the formation of peak D or E, one of the most important questions was the location of the unsaturation in the acyl

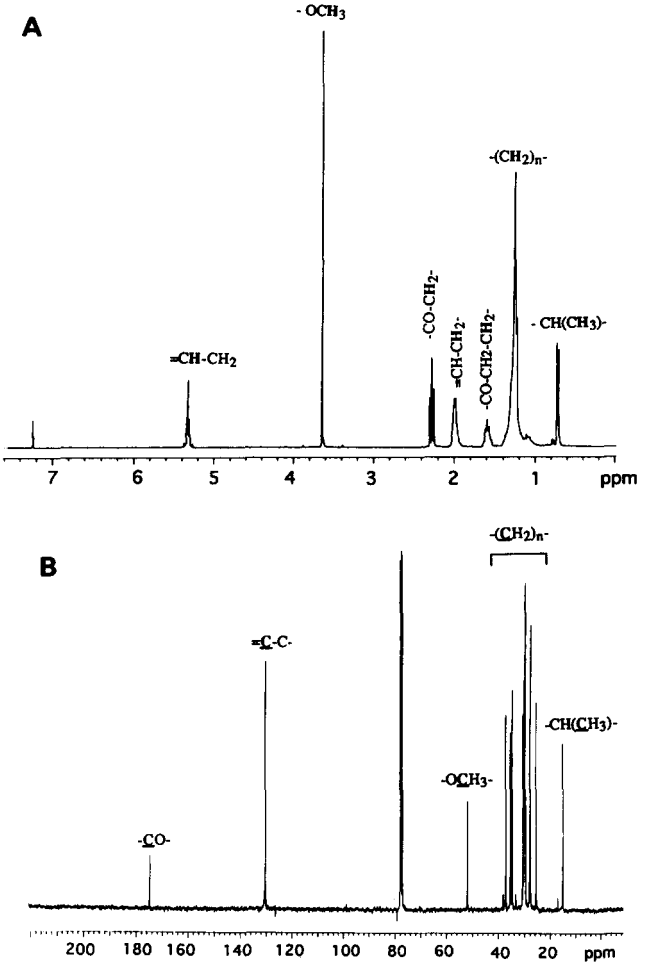

Fig. 5. (A) The ^{1}H-NMR spectrum of proposed structure **3.** The signal at δ 5.32 (t, J = 6.83Hz) is due to a methine proton on an unsaturated carbon atom. The mutiplet at δ 2.00 is assigned to the protons of the methylene groups adjacent of the unsaturated carbons. The intense peak at δ 1.27 is due to the methylene groups of the hydrocarbon chains. The signal at δ 3.65 result from methyl group of the methoxycarbonyl functions. The multiplet at δ 1.58 for the protons of the carbons β to the methylcarbonyl function. The doublet at δ 0.70 (J = 7.58Hz) arise from the protons of the vicinal methyl group. (B) The ^{13}C-NMR spectrum of proposed structure **3.** Signals at δ 130.8 correspond to olefinic carbons. Signals at δ 174.8 and at δ 51.5 confirmed the presence of methyl ester group. The vicinal methyl group gives rise to the signal at δ 14.8.

chain. The regular length, monofunctional, unsaturated acid contained the double bond at the 11 position of its carbon chain. The determination of the exact position of unsaturation of the bifunctional acyl chain was therefore very important. If it were located at any position other than the 11 position (with respect to the closest end) as we demonstrated here, our model would have been invalidated.

Reductive ozonolysis and mass spectrometry

The exact locations of the unsaturation in **2** and **3** were confirmed by ozonolysis followed by reduction of the ozonide with zinc/acetic acid. This led to several fragments that were assigned structures **4, 5,** and **6** based on their mass spectra. The structures of these fragments were consistent with the proposed locations of the double bonds. As expected, structure **2** produced two fragments on ozonolysis. In contrast, three fragments (two of which were identical) were formed from structure **3.** The mass spectrum of **4** (**Fig. 6A**) contained major ions at *m/z* 186, 183, 171, 74, and 87. These corresponded to the ions of M

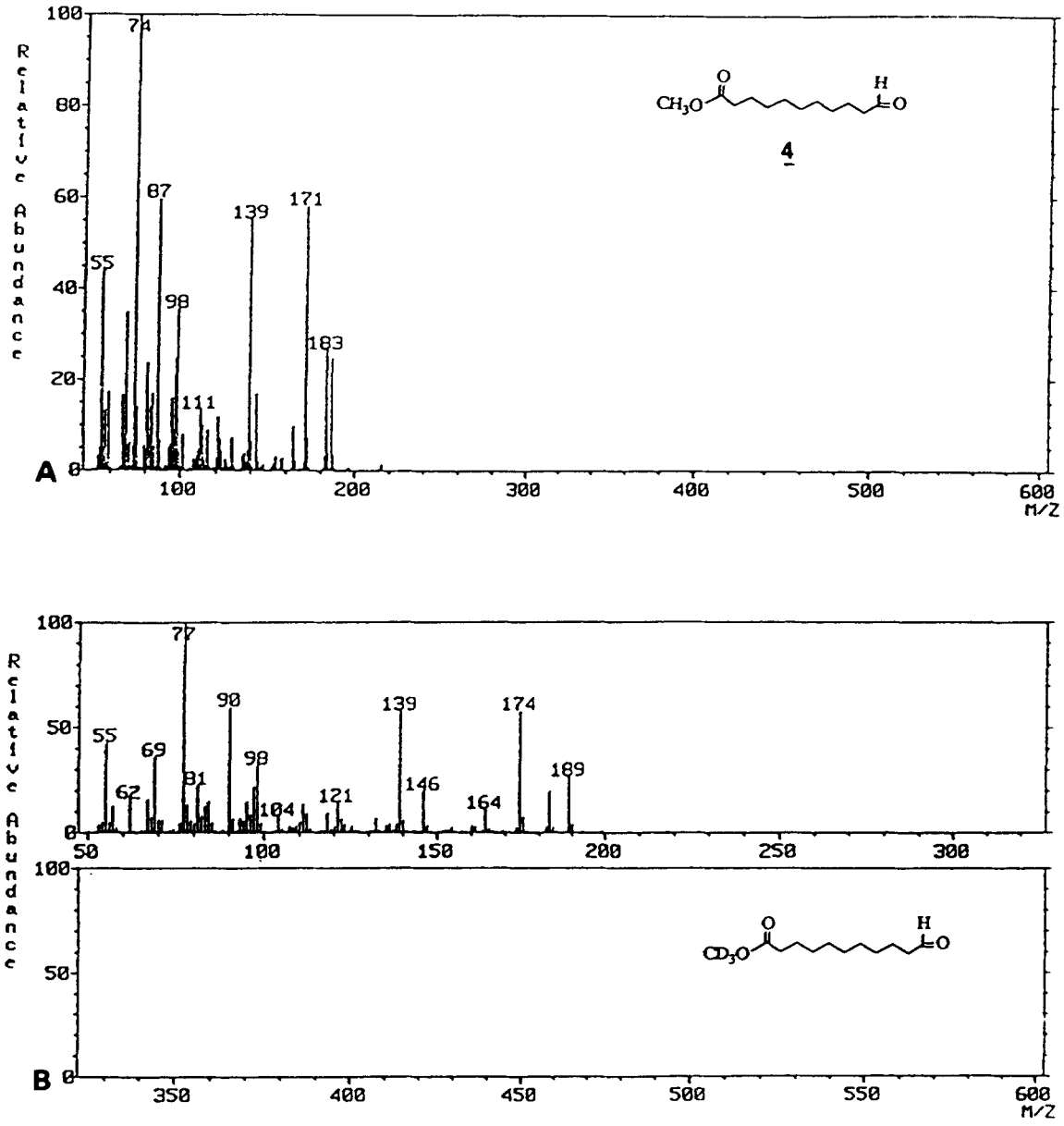

Fig. 6. (A) The electron impact mass spectrum of **4.** Note the major ions at 186, 183, 171, and 74. These correspond to loss of ethylene ($CH_2=CH_2$) from the molecular ion (M^+ = 214), loss of methoxy group from M^+, loss of $CH_2=CH-O$ from M^+ and the typical McLafferty fragment (at *m/z* 74). The ion at *m/z* 139 was assigned to the sequential losses of $CH_2=CH-O$ and a methoxy group from the molecular ion. (B) The electron impact mass spectrum of deuterium-labeled **4** obtained by deuteration with D-4 methanolic HCl solution. Note the expected mass increases because of deuterium.

(m/z 214) −28 ($CH_2=CH_2$), M −31 (CH_3O), M −43 ($CH_2=CH-O$) and the typical McLafferty fragment (m/z 74) of an aliphatic methyl ester. The ion at m/z 139 was assigned to the sequential losses of $CH_2=CH-O$ and a methoxy group from the molecular ion. Fig. 6B shows the EI mass spectrum of the deuterium-labeled molecule with structure **4** obtained by performing the methanolysis with D-4 methanol/HCl solution. The expected mass increases due to the deuterium were easily observed.

The EI mass spectrum of **5** (**Fig. 7A**) contained major ions at m/z 382, 364, 357, and 350. These were attributed to the molecular ion (M^+) and the losses of water (H_2O), ethene, and methanol, respectively. It also contained the typical ion series of the general structure ($CH_3OCO-(CH_2)_n$), beginning at m/z 73, for a saturated methyl ester. There were three prominent ions at m/z 237, 269, and 297 (296 is due to the loss of one hydrogen from m/z 297). The intense clusters of ions 28 mass units apart

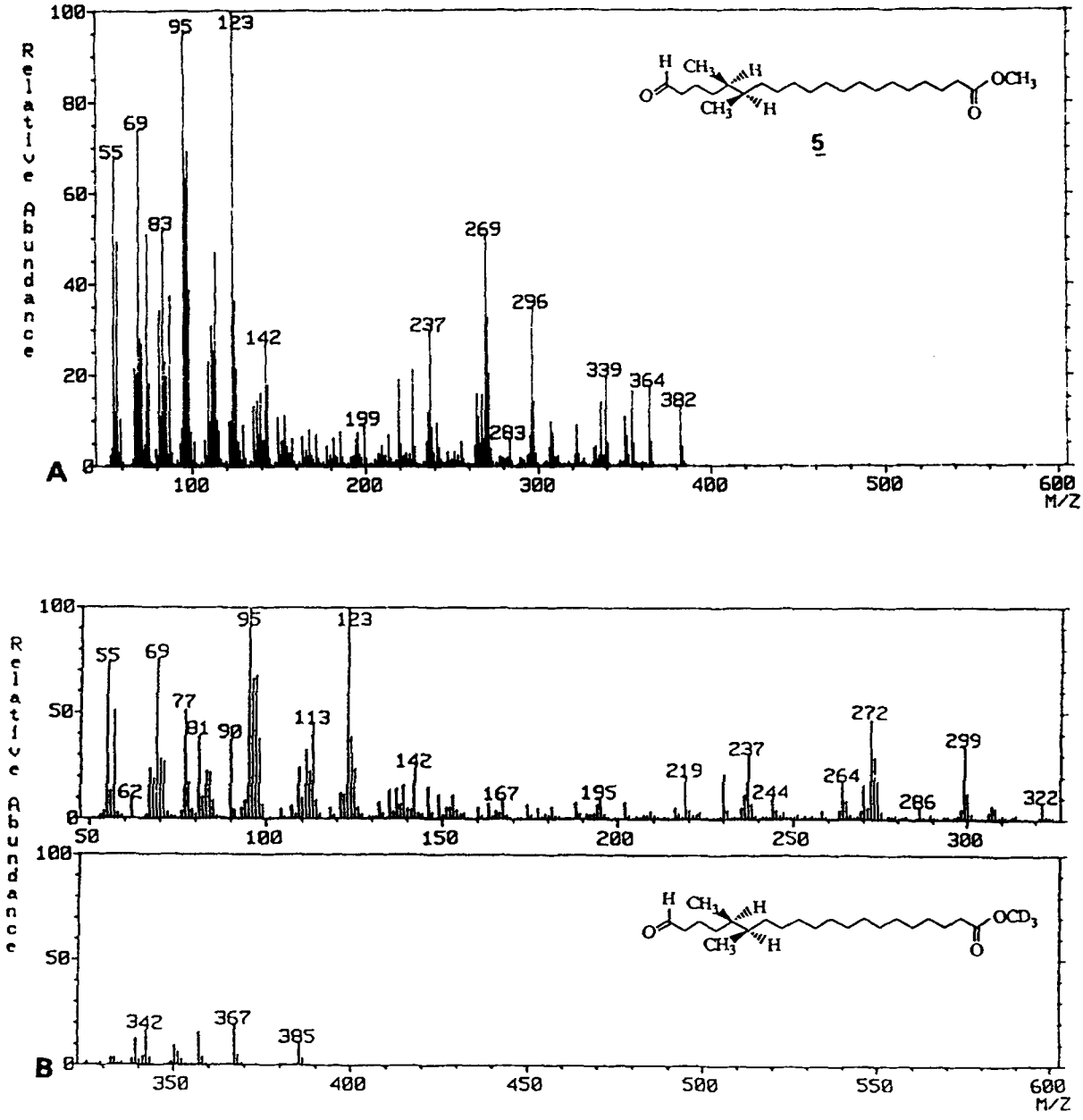

Fig. 7. (A) The electron impact mass spectrum of **5**. Note the major ions at 382, 364, 339, and 269. These can be attributed to M^+, M-18 (H_2O), M-28 ($CH_2=CH_2$), M-43 ($CH_2=CH-O$) and M-113 ($HCO(CH_2)_5CH(CH_3)$). (B) The electron impact mass spectrum of deuterium-labeled **5** obtained by deuteration with D-4 methanolic HCl solution. Mass increases because of deuterium are easily observed.

centered at *m/z* 269. And 297 indicated the presence of a vicinal dimethyl group. The ion at *m/z* 237 represented the loss of methanol (CH_3OH) from the ion at *m/z* 269. Fig. 7B shows that the EI mass spectrum of the deuterium-labeled molecule with structure **5**. The expected increases in mass units due to isotope labeling were easily observed. The electron impact mass spectrum of the dialdehyde (**6**) was also obtained (not shown). It contained major ions at *m/z* 208, 182, and 95. These were assigned to M^+-18(H_2O), M-44(CH_2=CH-OH) and *m/z* 113-18(H_2O). The ion at *m/z* 113 was designated to the product formed by the fragmentation between the two methyl branches. There was no difference in the EI mass spectrum before and after the deuterium labeling, thus indicating the absence of a methyl ester function. The dimethyl acetal that should have been formed would have been hydrolyzed back to the aldehyde during work up.

These reductive ozonolysis studies provided structures consistent for peaks D and E. They also provided conclusive proof for the proposed coupling mechanism as the exact positions of unsaturation of the bifunctional acyl chains and the position of the vicinal methyl groups were completely consistent with ω-1 coupling of two monocarboxylic acid acyl chains, one of which was *cis*-vaccenic acid. The combined spectroscopic and chemical methods confirmed the proposed structures corresponding to peaks D and E as α,ω-(17,18-dimethyl)-*cis*-11-dotriacotaenedioate dimethyl ester (**2**) and α,ω-(17,18-dimethyl)-*cis*-11-23-hentriacotadienedioate dimethyl ester (**3**), respectively. Compounds **1**, **2**, and **3** all displayed an optical rotations (**Table 2**). This precluded the possibility of any of these bifunctional acyl chain species being racemates or meso-compounds.

Chemical evidence for the mode of formation of the very long chain dicarboxylic acids

The presence of very long bifunctional lipids in the membrane of *S. ventriculi* could have arisen by coupling the tails of fatty acids of membrane lipids from the same side of the bilayer. This, incidently, was ruled out by freeze-fracture studies that demonstrated that the leaflets could not be separated and that the very long chain fatty acids were transmembrane (data not shown). Several pieces of information showed unequivocally that the formation of these very long fatty acids occurred by the chemical linkage at the ω-1 positions of the tails of fatty acids from opposite halves of the bilayer. The structures and gross relative proportions of individual very long chain fatty acids were closely correlated with the structures and the relative proportions of normal chain fatty acids in the same isolates. As $C_{16:0}$ and *cis*-$C_{18:1(11)}$ fatty acids were the major regular chain species, the predominant very long chain fatty acid species were combinations of $C_{16:0}$ plus $C_{16:0}$, $C_{16:0}$ plus *cis*-$C_{18:1(11)}$ and *cis*-$C_{18:1(11)}$ plus *cis*-$C_{18:1(11)}$. In this case the $C_{18:1}$ species was *cis*-11-octadecenoic acid (*cis*-vaccenic acid) thus explaining the positions of the double bonds of the unsaturated α,ω-dicarboxylic acids. When an odd-numbered carbon alcohol was added to the growth medium, the regular chain fatty acids were all of odd carbon number (mostly $C_{17:0}$, *cis*-$C_{17:1}$, and $C_{15:0}$). This is consistent with propanol being converted to propionyl CoA which was used to initiate fatty acid synthesis with a C-3 unit leading to odd-numbered fatty acids. However, in this case, the new very long fatty acids were all of even chain length and predominantly saturated C-30 (strictly speaking dimethyl C-28), monounsaturated C-32 (dimethyl C-30), and diunsaturated C-34 (dimethyl C-32) dicarboxylic acids. Further proof of the ω-1 coupling mechanism was obtained by the structural comparison of the two different C-32 (or vicinal methyl branched C-30) α,ω-dicarboxylic fatty acid esters obtained from the two different induction conditions: propanol induction and temperature induction. They are both vicinal methyl-branched C-30 α,ω-dicarboxylic acids. However, careful analyses of the mass spectra clearly indicated that the one formed on propanol induction (**Fig. 8**) bore the vicinal methyl group in a position consistent with the combination of $C_{15:0}$ plus $C_{17:0}$ to give structure **7**. The mass spectrum contained major ions at *m/z* 538, 506, and 475. These corresponded to the molecular ions and sequential losses of methanol (CH_3OH) and a methoxy (CH_3O) group, respectively. Important fragments giving information on the position of the vicinal methyl groups appeared at *m/z* 255 and 283. These prominent peaks corresponded to the fragmentation products formed by cleavage between the two methyl branches. In contrast, the mass spectrum of the acid with the symmetrically placed vicinal methyl group (consistent with a combination of $C_{16:0}$ plus $C_{16:0}$) obtained from acidic or high temperature culture conditions showed the predominent alkyl chain cleavage ion *m/z* 269 (16). Another proof of the proposed mechanism came from the isolation of a very long bifunctional species formed by the combination of a fatty acid and a fatty aldehyde. Using GC-MS (**Fig. 9A**), it was possible to demonstrate the presence of normal chain fatty aldehydes such as **8** indicating the presence of plasmalogens. Corresponding ω-formylmethyl esters with structures such as **9** (structure **8** plus $C_{16:0}$) were identified (Fig. 9B) by mass spectrometry.

TABLE 2. Optical rotation analyses of **1**, **2**, and **3** indicating that all of bifunctional molecules are chiral

	Structures		
Hg Light	1	2	3
nm			
$[\alpha]_{578}$	0.51	5.44	5.76
$[\alpha]_{546}$	0.89	6.30	6.46
$[\alpha]_{436}$	5.49	11.39	11.49

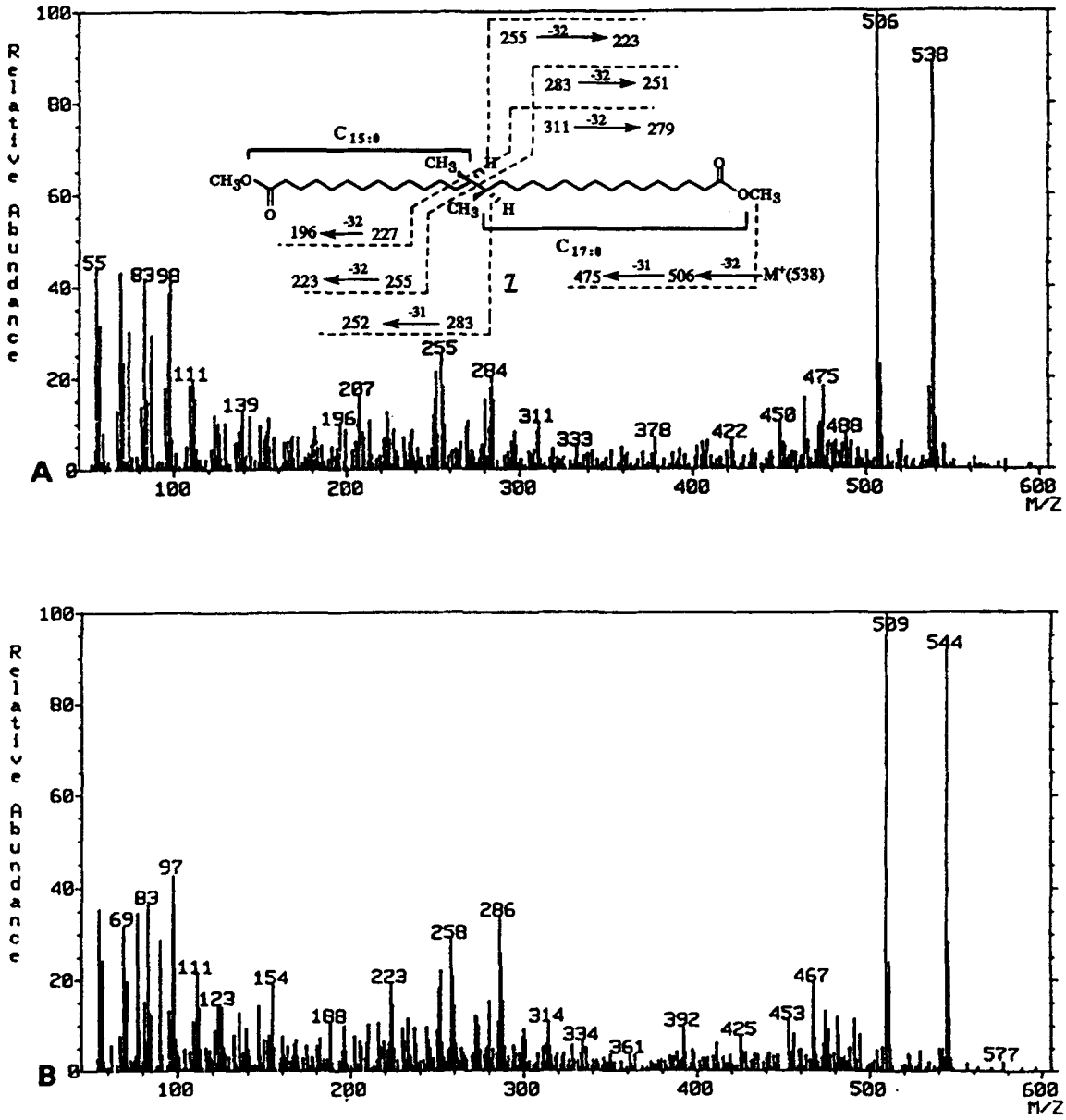

Fig. 8. Mass spectral fragments of $C_{32.0}$ α,ω-dicarboxylic very long chain acyl species (**7**) in the case of propanol (0.15 M) induction. The location of the vicinal methyl groups was determined by GC-MS analyses. Fig. 8A shows the EI mass spectrum. Fig. 8B shows the EI mass spectrum of deuterium-labeled **7**. The ions at *m/z* 258 and 286 corresponded to the trideutrated $C_{15:0}$ fatty acid methyl ester and trideuterated $C_{17:0}$ fatty acid methyl ester.

The optical rotations of the dicarboxylic acids clearly showed them to be chiral. This is consistent with a transition state that gives rise to the stereochemistry having two centers of asymmetry. In an enzymatic process leading to coupling of the alkyl chains by the proposed mechanism, the methyl groups would be expected to be directed away from the face of the enzyme and would therefore appear on the same side of the Neuman projection. A possible mechanism would be abstraction of a hydrogen radical from each of the two opposing fatty acids from the same side followed by coupling of the radical species. The secondary radical is more stable than a primary radical hence coupling at the ω-1 instead of ω-positions. The resulting molecule would have the (*R, R*) or (*S, S*) stereochemistry and would be chiral even if the two chains are identical as is the case in structures **1** and **3**. If coupling of two identical chains occurs on the same side of the bilayer by the same mechanism, the resulting product would be *meso* and have the (*R, S*) or (*S, R*) stereochemistry.

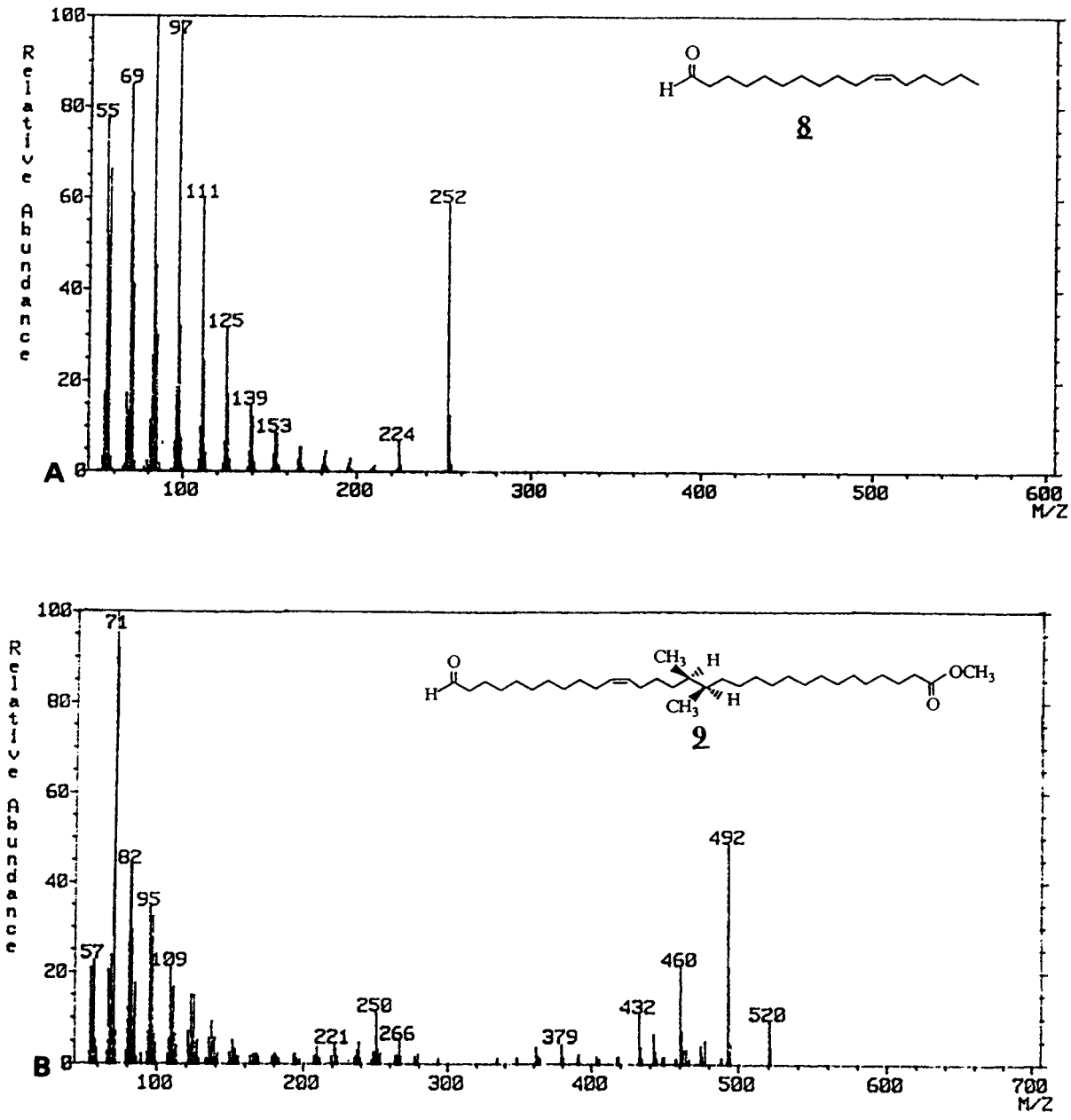

Fig. 9. (A) Electron impact mass spectrums of **8**. Note the major ions at *m/z* 252, 224, and 55 + 14n (n = 1,2..). These corresponded to molecular ion (M^+), M^+-28 (CO), and a long chain alkene series, respectively. (B) Electron impact mass spectrums of **9** contained major ions at *m/z* 520, 492, and 460. These corresponded to molecular ion (M^+), with the sequential losses of carbon monoxide (CO), and methanol (CH_3OH), respectively.

General significance of the chemical coupling mechanism

The unusual mechanism has very important implications for adaptive processes in bacteria. The tail of the fatty acids of a lipid can be expected to have the highest freedom of motion in the entire molecule as the other end is firmly attached to the head group. It is, therefore, desirable for the organisms to develop methods or mechanisms to restrict this motion especially under conditions (such as increased temperature or the presence of organic solvents) that will tend to increase it. One very important aspect of the adaptive response that we propose here is that it accomplishes this motional restriction by the most direct mechanism one could imagine: the direct coupling of fatty acid tails across the bilayer. Another striking aspect of this proposed adaptive response is that it utilizes pre-existing fatty acids rather than initiating the complete biosynthesis of the new species in a de novo fashion. It also points to an entirely new mode of enzyme catalytic control that can be termed "dynamic regulation." Both the

swiftness and the site of coupling appear to preclude the possibility of the synthesis of a new activity in response to the perturbation. The indication is that an existing, latent activity becomes activated in response to the new membrane dynamic state brought about by the perturbation. Experiments are in progress to test this theory and the generality of this phenomenon.

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