Occurrence and Chemical Structure of Nonmethylene-Interrupted Dienoic Fatty Acids in American Oyster *Crassostrea virginica*

M. PARADIS[1] and **R.G. ACKMAN**[2], Environment Canada, Fisheries and Marine Service, Halifax Laboratory, Halifax, Nova Scotia B3J 2R3

ABSTRACT

The American oyster, *Crassostrea virginica*, was found to contain structurally homologous nonmethylene-interrupted dienoic (NMID) fatty acids. The major C_{20} and C_{22} nonmethylene-interrupted dienoic fatty acid isomers were shown to occur as two pairs of homologues 5,13-20:2 with 7,15-22:2 and 5,11-20:2 with 7,13-22:2. A combination of analytical procedures was required for conclusive structure determination.

INTRODUCTION

Interpretation of open tubular gas chromatograms of the methyl esters of fatty acids from marine molluscs in the region of 20:1 and 22:1 has been complicated by the presence of unidentified C_{20} and C_{22} fatty acids (1-5). These details are not apparent in conventional gas liquid chromatography (GLC) with packed columns (1,6-8). These components now have been identified in oysters as basically two homologous series of C_{20} and C_{22} nonmethylene-interrupted (NMID) fatty acids. They have been purified partially by a combination of silver nitrate-thin layer chromatography ($AgNO_3$-TLC) and preparative GLC. Structures were determined by oxidative and reductive ozonolyses combined with GLC and confirmed spectroscopically. The major isomers were found to be 5,13-20:2 and 7,15-22:2 along with lesser amounts of 5,11-20:2 and 7,13-22:2. There were many other minor isomers.

EXPERIMENTAL PROCEDURES

Oysters, *Crassostrea virginica*, were collected on several occasions between 1966 and 1970 near Ellerslie, Prince Edward Island, and transported to holding tanks at the Halifax Laboratory. Extraction of animals was carried out on total wet organic tissue with chloroform-methanol (9). Lipids were saponified by AOCS Method Ca-6b-53 (10). The fatty acids were recovered and converted to methyl esters by refluxing with 7% BF_3-MeOH.

Initial enrichment of NMID fatty acids (Scheme 1) was achieved by $AgNO_3$-TLC on Prekotes (Adsorbosil 5, from Applied Sciences Laboratories, State College, Pa.) previously immersed horizontally in a 10% solution of $AgNO_3$ in acetonitrile and dried. Development was with chloroform. Purification of chain lengths was achieved by preparative GLC using a packed stainless steel column (5% SE-30, 6 ft x 1/4 in. inside diameter, carrier gas He, column temperature 190 C), a heated collection unit (250 C), and glass collection tubes with local reheating to reduce losses from "fogging." Concentrates of fatty acid methyl esters then were identified structurally by GLC analysis of both oxidative (11) and reductive (12,13) ozonolysis degradation products. GLC of oxidative ozonolysis products was carried out on packed (10% EGSS-X on Gas Chrom P, Applied Science Laboratories) columns (6 ft x 1/8 in. inside diameter) at either 130 C for short chain methyl esters or at 180 C for dimethyl esters. Methyl ester preparations and reductive ozonolysis products were examined on stainless steel open tubular capillary columns (150 ft x 0.01 in. inside diameter) coated with butanediolsuccinate polyester (BDS) in Perkin-Elmer model 226 or 900 GLC apparatus. Column temperatures used were: for aldehydes, 60 C, for aldehyde-esters, 150 C; for long chain methyl esters, 170 C. Other operating conditions are described elsewhere (1,14). Acidic and aldehyde products were converted from wt percent to mole percent to facilitate comparison with the open tubular GLC peak proportions.

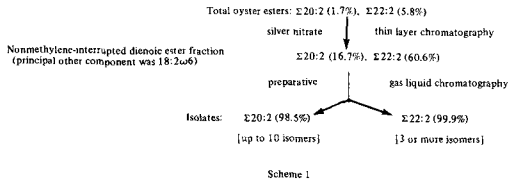
Mass spectroscopy was carried out on a DuPont CEC 21-110B double focusing mass spectrometer at 70 eV. The NMR spectra of NMID fatty acid methyl esters were obtained using a Varian A-60 spectrometer. Samples were dissolved in deuterated chloroform. IR spectra were obtained using a Perkin-Elmer model 237 spectrometer. Samples were run as a film on a salt block.

RESULTS AND DISCUSSION

The combination of $AgNO_3$-TLC and preparative GLC gave very effective separation of 20:2 and 22:2 NMID fatty acid methyl esters

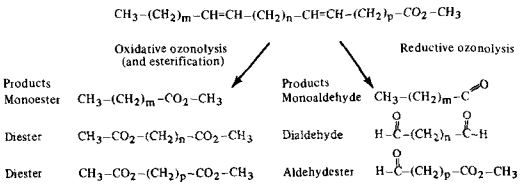
[1]Present address: Revenue Canada, Tariff Programs and Appraisal Branch, Laboratory and Scientific Services Division, Ottawa, Ontario, Canada K1A OL5.

[2]Author to whom correspondence should be addressed.



Scheme 1

Purification of Nonmethylene-Interrupted Dienoic Fatty Acid Methyl Esters of *Crassostrea virginica*



Scheme 2

Respective Products from Oxidative and Reductive Ozonolyses of Typical Nonmethylene-Interrupted Dienoic Fatty Acids

(Scheme 1). However, separation of individual isomeric species within these fractions could not be achieved. Attempts at partial purification of the isomers by low temperature $AgNO_3$-TLC (15) met with limited success. Fractions obtained from this procedure still contained a multiplicity of isomers but in proportions which differed greatly from the original "purified" sample composition. Analyses of these fractions were useful in confirming proposed structures (Table 1). Conclusions were drawn on the basis of quantitative data from degradative studies rather than from spectroscopic analyses of pure, single components or their derivatives. IR spectroscopy gave essentially identical spectra for both the C_{20} and C_{22} fractions purified by GLC. Both spectra were in all ways characteristic of fatty acid methyl esters (16) and had absorption maxima (~1700 cm^{-1}, ~2990 cm^{-1}) indicating the presence of *cis* unconjugated double bonds (17). NMR spectra confirmed IR findings. No CH_2 protons subject to shifts by more than one functional center were observed, suggesting the presence of two double bonds separated from each other and from the carboxyl function by several methylene groups.

Mass spectroscopy was used only to obtain a molecular ion for each chain length preparation (Scheme 1). These were respectively m/e 322 and m/e 350, corresponding to methyl eicosadienoate and methyl docosadienoate, respectively. An attempt was made to prepare trimethyl-silyloxy and methoxy derivatives of the

TABLE I

Mole Percentages of Oyster 20:2 and 22:2 Major Nonmethylene-Interrupted Dienoic (NMID) Isomer Component Peaks[a] as Recovered by Scheme 1 and after Additional Purification by Low Temperature Silver-Nitrate-Thin Layer Chromatography Compared to Products from Reductive Ozonolysis

Sample origin		Ozonolysis products			
A 20:2 NMID esters isolated from oyster		Aldehydes	Percent	Aldehyde esters	Percent
Isomer	Percent				
1	22.8	C_5	3.0	C_5	88.6
2	61.2	C_6	3.0	C_7	11.4
3	13.7	C_7	61.6		
4	2.3	C_8	1.6		
		C_9	24.5		
		C_{10}	6.6		
B 20:2 NMID repurified esters					
Isomer	Percent	Aldehydes	Percent	Aldehyde esters	Percent
1	53.6	C_7	49.3	C_5	86.9
2	29.7	C_9	39.5	C_7	13.1
3	16.6	C_{10}	11.3		
C 22:2 NMID esters isolated from oyster					
Isomer	Percent	Aldehydes	Percent	Aldehyde esters	Percent
1	22.6	C_7	78.5	C_7	100.0
2	75.7	C_9	19.7		
3	1.7	C_{10}	1.8		
D 22:2 NMID repurified esters					
Isomer	Percent	Aldehydes	Percent	Aldehyde esters	Percent
1	55.5	C_7	43.6	C_7	100.0
2	44.5	C_9	56.4		

[a]From open tubular gas liquid chromatographic analysis.

TABLE II

Comparison of Oxidative Fission Acidic (Methyl Ester) Fragments (Mole %) and Proportions of Theoretical Products from Two Major 20:2 and 22:2 Nonmethylene-Interrupted Dienoic (NMID) Acids Isolated from *Crassostrea virginica*

A	20:2 NMID methyl esters			Dimethyl esters		+	Methyl esters	
	Isomers	Percent		Theoretical (min)	Measured		Theoretical (min)	Measured
	5,11	22.8	C_5	42.0	28.1			
	5,13	61.2	C_6	11.4	7.1	C_6	---	14.1
	3	13.7	C_7	---	12.1	C_7	61.2	45.0
	4	2.3	C_8	30.6	40.5	C_8	---	5.9
			C_9	---	7.1	C_9	22.8	21.0
			C_{10}	---	3.0	C_{10}	---	13.3
			C_{11}	---	2.1			
B	22:2 NMID methyl esters			Dimethyl esters		+	Methyl esters	
	Isomers	Percent		Theoretical (min)	Measured		Theoretical (min)	Measured
	7,13	22.6	C_5	---	6.1			
	7,15	75.7	C_6	11.3	7.2	C_6	---	12.5
	3	1.7	C_7	49.2	45.2	C_7	75.7	46.7
			C_8	37.9	37.1	C_8	---	6.6
			C_9	---	---	C_9	22.6	25.0
			C_{10}	---	1.9	C_{10}	---	9.4
			C_{11}	---	1.2			

esters for mass spectroscopy, but the derivatives obtained were of questionable purity, gave complex mass spectra, and yielded no useful information beyond the inconclusive suggestion of a fragment of eight carbons from between ethylenic bonds.

Oxidative degradation of ozonides was somewhat more useful in defining the precise structure of the components. As Scheme 2 indicates, however, the products obtained from oxidative fission only suggest two alternative structures for each isomer, since the diesters may originate from either of two locations in the molecule.

Reductive degradation of ozonides, also outlined in Scheme 2, yields three different classes of components. These need only be matched up according to their relative abundance and the total chain length of the original esters to give all the analytical information required for complete identification. However problems can be encountered here also. The central dialdehyde fragments are subject to polymerization (18), before or after GLC, and may, for this and other reasons, give reduced yields. The aldehyde-ester fragment is more stable but potentially difficult to identify conclusively, since not all the necessary standards are readily available. Quantitation problems also arise with this method due to the multiplicity of isomers and innate differences in volatility, column loss, and differential detector sensitivity among breakdown products (13,19). For these reasons and because our starting material was a mixture, we have proposed conclusive structures for the major isomers and for the present prefer to only record the occurrence of the minor isomers and note possible degradation products.

Table I shows results from reductive ozonolysis. In the 20:2 NMID, representing four distinct isomeric esters, C_7 and C_9 dominate the product aldehydes, and the major aldehyde ester was C_5. There is insufficient C_7 aldehyde ester to combine with more than a fraction of the two major aldehydes. The total of the C_7 and C_9 aldehydes is very similar to the total amount of C_5 aldehyde ester. The mole percentage of C_7 aldehyde is the same as that of the second 20:2 NMID isomer, while the percentage of C_9 aldehyde is very similar to that of the first NMID isomer. The major 20:2 NMID isomer must, therefore, be 5,13-20:2, while the next most common isomer must be 5,11-20:2. These correspond to two (respectively b and a) of the three NIMD peaks (a,b, and c) observed primarily in gastropods, such as the moon snail *Lunata triseriata* or periwinkle *Littorina littorea* (1). The third unusual dienoic peak (c) observed in the gastropods probably corresponds to isomer 3, Table I. It may consist basically of the normal methylene interrupted 7,10-20:2 structure proposed previously on the basis of calculation of GLC retention time (1), as this accounts for the C_7 aldehyde ester and the unexpected C_{10} aldehyde, with perhaps minor amounts of other isomers. Isomer 4 perhaps could have an $\omega 5$ (for the C_5 aldehyde) and an $\omega 7$ (or $\omega 9$) combination, these or similar positional details accounting for the later elution relative to isomer 3 for reasons elaborated elsewhere (20,21). The ozonolysis products from the repurified low temperature $AgNO_3$-TLC fraction (Table I) provides further

quantitative support for the three major isomers.

Analogous reasoning with the 22:2 NMID isomers indicates that 7,15-22:2 is the most common and 7,13-22:2 the next most common isomer in the oyster lipids, although this may not be the case in other molluscs (1) or oyster samples (3). The fewer isomers permit a better fit for the fragments, and the homologues are in keeping with an origin in the two 20:2 NMID and simple chain extension by two carbon atoms.

The oxidative ozonolysis process applied to the same two mixtures of oyster isolates used for the reductive studies yielded dicarboxylic acid products (Table II), such as C_6 and C_8, from between the ethylenic unsaturations which could be combined with the C_5 diacid from the 20:2 NMID and C_7 from the 22:2 NMID (both identifiable as having original carboxylic acid functions from the C_5 and C_7 aldehyde ester fragments, Table I) in ca. the original isomer proportions discussed above for the C_{20} and C_{22} chain lengths. Losses of shorter chain products seem to have occurred, but it is not known if this occurred in recovery or is a GLC problem (19). The presence of ca. equal molar proportions of pimelic (di-C_7) and capric (mono-C_{10}) acids in the acidic 20:2 NMID products supported the normal methylene-interruped 7,10 structure for the third 20:2 isomer.

Open tubular GLC also reveals that there are relatively minor proportions of one or more dienoic components superimposed on the 18:1 complex (ω9,ω7, and ω5) from mollusc lipids (2). These have not been characterized as NMID acids, and a normal methylene-interrupted 5,8-18:1 homologue of the proposed 7,10-20:2 appears to be one possibility. The 20:2 and 22:2 NMID occur in both polar and nonpolar lipids of another oyster sample in varying proportions of 0.5-6.5% of total fatty acids in lipid fractions but ca. 2% for NMID 20:2 and 2-4% for 22:2 (3) or ca. the same proportions as in the sample examined in this study (Scheme 1).

Nonmethylene-interrupted unsaturation rarely occurs in fatty acids obtained directly from natural sources and is more often encountered as an intermediate stage in the catalytic hydrogenation of oils (22). Relatively large amounts of NMID fatty acids previously have been observed in a few plant species (23-26). In terrestrial animals, NMID fatty acids generally occur as trace components (27,28) often as the result of dietary factors (29,30). Novel 26:2 and 26:3 NMID recently have been found in marine sponges (31). The occurrence of somewhat similar components in shellfish need not indicate a close trophic relationship to the marine algae in their diet, even though some species of algae contain NMID fatty acids (23). An animal desaturase mechanism may be responsible for the production of these unusual compounds. Potential metabolic precursors for such compounds are likely to be the corresponding shorter chain monoethylenic ω9 (9-18:1, 11-20:1) and ω7 (11-18:1, 13-20:1) acids which are present in large amounts in the oyster and other molluscs (1-3,5). A discussion of the metabolic and taxonomic significance of these unusual components has been given previously by Ackman and Hooper (1).

ACKNOWLEDGMENTS

NMR analyses were performed by D. Hooper, Department of Chemistry, Dalhousie University, Halifax, Nova Scotia. Mass spectroscopy was performed by S. Safe, Atlantic Regional Laboratory, National Research Council of Canada, Halifax, Nova Scotia.

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[Received July 16, 1974]