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The Lipids of *Ruvettus pretiosus* Muscle and Liver*

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ABSTRACT: The muscle of the gempylid fish, *Ruvettus pretiosus*, contains 14.7% (wet wt) of lipid, which is predominantly wax esters of 34 and 36 carbon atoms with one and two double bonds. The liver lipids contain only about 2% wax esters.

Contrary to a previous report, the muscle lipid does not contain hydroxy fatty acids. Gas-liquid chromatographic analyses are reported for the fatty acids of several lipid fractions, including the muscle wax esters, for the long-chain alcohols of the muscle wax esters, and for the unhydrolyzed wax esters.

The flesh of *Ruvettus pretiosus*, a fish of the family Gempylidae, was reported (see Gudger, 1925) to have strong purgative properties, hence the trivial name "castor oil fish." Cox and Reid (1932) analyzed samples of oil from specimens caught in the Ellice Islands of the western Pacific Ocean and reported (a) that the oil consisted mostly of the wax esters cetyl oleate and oleyl oleate, and (b) that hydroxyoleic acid constituted approximately 13% of the total fatty acids. They also found the oil to be a mild laxative for rats, although ingestion of an amount of the crude *Ruvettus* oil which was within the range of dosage levels usually prescribed for castor oil produced no effect in the junior author. Our purpose in reexamining the lipids of *R. pretiosus* was to confirm the presence and establish the structures of the hydroxy fatty acids present, and to investigate the wax esters for comparison with unpublished data previously obtained for a second gempylid fish, *Lepidocybium flavo-brunneum*.

Experimental Methods and Results

Chromatographic Techniques. Thin-layer chromatography on silica gel G (E. Merck A. G., Darmstadt, Germany; available in the U.S.A. from Brinkmann Instruments, Inc., Great Neck, N.Y.) or Anasil S (Analytical Engineering Laboratories Inc., Hamden, Conn.) was used for the qualitative assay of the fractions of

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interest (Mangold, 1962). The various lipid classes were separated quantitatively by adsorption-column chromatography on silicic acid (Fillerup and Mead, 1953) or Florisil (Carroll, 1961).

Gas-liquid chromatography was used to determine the composition of the unhydrolyzed wax esters and the derived fatty acid and alcohol moieties. The instrument used was a Barber-Colman Model 10 equipped with an argon ionization detector (radium-226 source). We used several different liquid phases coated on 70/80 mesh Anakrom ABS (Analytical Engineering Labs) and packed into U-shaped glass colums, 1-m × 4-mm id, unless indicated. The specific phases and operating conditions used are described when each type of compound analyzed is considered. The liquid-phase loadings are given as initial weight per cents. Quantitative values for each component were determined from the areas under the peaks evaluated either by triangulation (summation of the products of the peak height by the width at half height; *cf.* Rodegker and Nevenzel, 1964) or the use of a disk integrator (Disc Instruments, Inc., Santa Ana, Calif.). Quantitative results with National Heart Institute methyl ester Standard F (Horning *et al.*, 1964) on ethylene glycol succinate agreed with the stated composition data with a relative error less than 4% for major components (> 10% of total mixture) and less than 12% for minor components (<10% of total mixture).

Extractions. The portion of the fish available to us (weight 2 kg; the anterior third including the liver) was kept frozen from the time it was purchased in a Tokyo, Japan, fish market until 24 hours before use. A 276-g portion of the flesh (white muscle with only small amounts of red muscle) was ground in a Waring Blendor with 600 ml of chloroform-methanol (1:1 by volume) and then reground with 600 ml of chloroform alone. The final chloroform solution was filtered, and the extracted tissue was washed with diethyl ether. The combined extracts were washed with water and dried over anhydrous $MgSO_4$, and the solvent was removed *in vacuo* in a rotary evaporator (Rinco Instrument Co., Inc., Greenville, Ill.) to yield 40.7 g of a mobile, pale-yellow oil. The extracted tissue after drying *in vacuo* weighed 37 g. The oil, then, was 14.7% of the wet tissue or approximately 52.5% on a dry weight basis.

The liver (30.9 g fresh weight) was ground in 2-propanol in the Waring Blendor, and the resulting slurry was heated for 5–10 minutes on the steam bath before the supernatant liquid was decanted. The tissue was resuspended once in cold 2-propanol and three times in diethyl ether. The solvent was evaporated from the combined extracts in a rotary evaporator below 40°, and the total crude lipid was obtained by dissolving in petroleum ether (bp 60–70°), filtering, and evaporating off the petroleum ether under a stream of nitrogen on the steam bath; weight 8.06 g or 26.1% of the wet weight of the liver. Wherever possible all manipulations of the lipids were carried out in an atmosphere of nitrogen to minimize autoxidation.

Isolation of Lipid Classes. The separation of the lipid classes was achieved by chromatography on columns of silicic acid, eluting the wax esters with 1% (by volume) diethyl ether in petroleum ether (bp 60–70°; redistilled), the triglycerides with 5% ether, and more polar lipids with 100% ether, before collection as phospholipid of the material eluted by absolute methanol. The fraction eluted by 100% ether was a mixture of free acids, alcohols, and sterols with probably some monoglycerides. The identities assigned to these fractions were substantiated by their behavior on thin-layer chromatography. In routine analyses we did not detect as much as 4% of triglycerides in the wax esters, an amount easily separated and detected by column chromatography of a 300-mg sample. It is recognized that the phospholipid fractions contained polar materials other than organic phosphate esters. However, specific color reagents were used to confirm the presence of phosphate (Dittmer and Lester, 1964) and free amino groups (with ninhydrin). From the thin-layer chromatography results we concluded that both the muscle and liver contained principally lecithin. In Table I are given the compositions of the muscle and liver lipids. The gas-liquid chromatographic analysis on 3.0 wt % SE-30 at 244° and 13 psi inlet pressure of the unhydrolyzed wax esters from the muscle is reported in Table II. The component identifications given in Table II were arrived at by (a) indirect comparison with the retention times for saturated C_{33} and C_{35} wax esters, (b) the known straight-line relationship between the logarithms of the retention times and the number of carbon atoms for members of a homologous series, and (c) the known properties of the nonpolar SE-30 phase, where at constant chain length the most unsaturated members emerge first and the saturated compound last. As indicated in the table, components having the same chain length but differing by one double bond gave single broad peaks; their areas were determined by the disk integrator.

TABLE I: Composition (weight %) of *Ruvettus pretiosus* Liver and Muscle Lipids.

Fraction	Muscle	Liver
Wax esters	91.5	3.9[a]
Triglycerides	3.3	43.1
Polar lipids[b]	3.4	37.8
Phospholipids	1.8	15.0

[a] Includes sterol esters. [b] Includes free fatty acids, alcohols, and lower glycerides.

Preparation of Methyl Esters. The fatty acids of the triglyceride and phospholipid fractions were converted to their methyl esters by sodium methoxide–catalyzed methanolysis (see Rodegker and Nevenzel, 1964), and the esters were purified by silicic acid chromatography. It was thought necessary to avoid the possibility that

TABLE II: Composition (mole %) of the Wax Esters of *Ruvettus pretiosus* Muscle.

Retention[a] Time (min)	Component	Observed	Calculated[b]
8.32	30:1	0.1	Trace
[13.6 est.	32:0	3.6	0.6
[12.70	32:1		2.7
16.21	33:1	0.6	0.6
	34:0		0.6
20.60	34:1	57.4	46.6
	34:2		5.9
26.4 est.	35:1	1.0	0.4
	35:2		0.6
[32.9 est.	36:1	31.1	8.4
[31.9 est.	36:2		22.3
[53.7 est.	38:1	6.2	2.1
[51.3 est.	38:2		3.6
	40:1		0.6
	40:2		1.0

[a] SE-30 column at 244° and 13 psi. Methyl ester 30:0 had a retention time of 16.44 minutes. The bracketted peaks were not resolved. [b] From the data of Table III recalculated to mole %, assuming random combination of the alcohol and acid moieties. Components of less than 0.5% are omitted to conserve space.

methanolysis of the wax esters might be incomplete, since we had no simple technique for detecting wax esters in methyl esters, either by column or thin-layer chromatography. Neither would our routine gas-liquid chromatographic methods for the methyl esters show the presence of wax esters because of the very long retention times of the latter.

Therefore, the wax esters were hydrolyzed completely to the free acids and long-chain alcohols, which by chromatography on a Florisil column were readily separable from each other and from any unhydrolyzed wax esters remaining. The wax esters (276 mg) from *Ruvettus* muscle were refluxed for 1 hour with *ca.* 50 mg of sodium metal in 50 ml of absolute methanol; then 2.5 ml of 45% aqueous KOH was added, and heating on the steam bath was continued for 30 minutes. The resulting mixture of acids and alcohols was fractionated by chromatography on a Florisil column, eluting the alcohols (146.9 mg) with 5 column volumes of 10% ether in petroleum ether, and the acids (162.4 mg) with 4% glacial acetic acid in ether. The fatty acids were methylated by refluxing for 5 minutes in 25 ml of absolute methanol containing 4 ml of BF_3-methanol reagent (Metcalfe and Schmitz, 1961), and the resulting methyl esters were purified on a silicic acid column; the purified esters weighed 126.7 mg.

The free fatty acids present in the liver lipids were isolated by rechromatography of the polar lipid fraction on Florisil and methylated with BF_3-methanol, as described for the hydrolyzed wax esters. From about 500 mg of crude lipid 69.6 mg of chromatographically purified methyl esters was obtained. The crude liver lipids therefore contained a minimum of 13% free fatty acids.

Results are presented in Table III for the gas-liquid chromatographic analyses of the various methyl ester fractions done on 16.9% ethylene glycol succinate polyester coated on 80/100 mesh Gas-Chrom P (Applied Science Laboratories, Inc., State College, Pa.) at 150–88° and 8–11 psi; the identification of some components was checked by runs on a 2.97% Apiezon L column at 180° and 31 ml/min gas flow (9 psi).

Preparation of Trifluoroacetates of the Alcohols. The alcohol fraction obtained by Florisil chromatography of the hydrolyzed wax ester was dissolved in 3 ml trifluoroacetic anhydride and 1 ml of benzene; 300 mg of silver trifluoroacetate was added, and the slurry was heated on a steam bath for 30 minutes (Bourne *et al.*, 1950). The excess anhydride was evaporated with a stream of nitrogen on the steam bath, and the last traces were removed by addition and evaporation of two portions of CCl_4. The derivative was extracted from the residual salts with petroleum ether and purified on a column of silicic acid, collecting as trifluoroacetates the material eluted with petroleum ether; weight 137.9 mg. On the basis of the average molecular weights for the alcohols (254) and wax esters (517) calculated from the gas-liquid chromatographic analyses, it is estimated that the yield of alcohol trifluoroacetates from the wax esters was 74% of the theoretical. The composition of the alcohols from the wax esters of the muscle is given in Table III, as obtained from gas-liquid chromatographic analyses of their trifluoroacetate derivatives (Van den Heuvel *et al.*, 1961) on the ethylene glycol succinate column at 142–148° and 10 psi; the presence of a C_{22} component was confirmed on 3.2% QF-1 at 140° and a gas flow of approximately 55 ml/min (10 psi).

Attempted Isolation of Hydroxy Fatty Acids. By thin-layer or gas-liquid chromatography of the methyl esters obtained as described above we could not detect any hydroxy acids. The infrared spectrum of the total crude muscle lipid was not conclusive because of the presence of other lipid types with free hydroxyl groups, e.g., free alcohols, free fatty acids, and phospholipids; nevertheless it was possible to say that no more than 1.7–3.6 wt % might be hydroxy acid. Of course, if the hydroxyl groups of the hydroxy acid moieties were not free, but acylated (perhaps as an estolide; see Morris and Hall, 1965), they would not absorb in the 2.7 to 2.9-μ region and would not be detected by our infrared technique. Several attempts specifically designed to isolate hydroxy acids were therefore made, involving adsorption-column chromatography before and after hydrolysis to separate fractions having the properties expected for the hydroxy acids or esters. These fractions were further characterized by thin-layer or gas-liquid chromatography and infrared spectroscopy. No hydroxy acids were identified, but neither was it possible to unequivocally rule out the presence of as much as 0.05% of hydroxyoleic acid in the total lipid.

TABLE III: Composition (weight %) of the Fatty Acid and Alcohol Moieties of *Ruvettus pretiosus* Lipids.

Carbon Numbers of Components[a]	Fatty Acids				Alcohols
	Liver			Muscle	
	Triglycerides	Free Fatty Acids	Phospholipids	Wax Esters	Muscle Wax Esters
14:0	0.5	0.3	0.5	0.1	2.0
16:0	20.0	12.2	46.6	0.9	57.6
16:1	3.9	2.6	0.8	1.4	5.9
17:1	0.1	0.3	Trace	0.3	0.5
18:0	3.7	2.0	5.6	0.9	2.9
18:1	62.6	72.9	34.1	76.7	29.0
18:2	0.3	0.4		1.1	
20:1	5.9	3.7	3.1	11.0	0.9
20:4		1.6	1.6		
20:5	0.2	0.6	0.6		
22:1	1.2			3.6	0.1
22:6	0.5	2.0	4.1		
24:1	0.2		0.4	1.0	
24:3			0.7	0.3	
26:1				0.8	

[a] To conserve space some minor constituents were omitted from this table. None were, individually, more than 0.5%; for all lipid fractions the constituents listed total at least 98.4%.

Finally we turned to demonstrating that our techniques were adequate to detect and identify a few per cent of hydroxy fatty acids when they were added to the muscle lipid. To 433.7 mg of crude lipid were added 2.0 mg of methyl α-hydroxyoctadecanoate[1] and 6.3 mg of methyl ricinoleate (isolated from the methanolysis products of the seed oil of *Ricinus communis* by chromatography on silicic acid). This mixture was saponified as described for the wax esters, and the crude products were chromatographed in Florisil, collecting alcohols and other neutral lipids (205.7 mg) by elution with 100% diethyl ether; nonhydroxylated fatty acids plus ricinoleic acid (total 245.9 mg; this is fraction A), eluted with 4% acetic acid in diethyl ether, and α-hydroxy fatty acids (7.3 mg) eluted with 1% trifluoroacetic acid plus 4% acetic acid in ether[2] (this is fraction B).

Fraction A was methylated with BF_3-methanol and separated by silicic acid chromatography into: 1.7 mg of material eluted with 1% ether; 227.4 mg of nonhydroxylated esters eluted with 2% ether; 3.4 mg of mixture of two-thirds nonhydroxylated esters plus one-third long-chain alcohols, eluted with 20% ether; fraction C, 6.5 mg of hydroxy esters plus a little alcohol, eluted with 30% ether; and 4.8 mg of polar material eluted with absolute methanol. Hydroxy esters were detected only in fraction C by use of thin-layer chromatography on Anasil S plates developed with 30% diisopropyl ether in petroleum ether and visualized by spraying with a solution of 5 g K_2CrO_7 in 100 ml of 40% by weight H_2SO_4 followed by heating to about 120° on a hot plate; this produced blue spots on a yellow background. In this system methyl α-hydroxyoctadecanoate and methyl ricinoleate were indistinguishable, with R_F 0.2; long chain alcohols had R_F 0.5, and the unsubstituted esters R_F 0.8–0.9.

Gas-liquid chromatographic analyses of fraction C (on a 183-cm × 6-mm id glass column packed with 12.0% stabilized diethylene glycol succinate polyester (DEGS; Analytical Engineering Labs) operated at 210° and 50 ml/min gas flow [11 psi]) showed it to consist of at least 86.7% methyl ricinoleate of retention time 21.8 minutes, with oleate, stearate, and eicosaenoate together making up another 12.7%.

[1] The α-hydroxyoctadecanoic acid was a gift from Dr. A. J. Fulco; it was methylated by the use of BF_3-methanol.

[2] J. C. Nevenzel, unpublished observations. Presumably the difference in behavior of the α- and 12-hydroxy acids is a consequence of their respective acid strengths. For the elution of a carboxylic acid adsorbed onto the active sites on the surface of the basic Florisil, displacement by a stronger acid is required. Acetic acid (pK_a 4.76) is adequate to elute stearic acid (pK_a 5.75) and other long-chain fatty acids, all of which have similar dissociation constants. But acetic acid is not capable of displacing an α-hydroxy acid of pK_a 3.9–4.2 (for the C_2-C_5 acids). Trifluoroacetic acid (pK_a 3.07) will elute α-hydroxy fatty acids but not maleic acid (pK_a^I 1.92). Based on this theory the β- and γ-hydroxy fatty acids, which have dissociation constants intermediate between those of the α-hydroxy and the unsubstituted fatty acids, are expected to be completely eluted from Florisil by trifluoroacetic acid; fatty acids with substituents farther away from the carboxyl group than the 5 position would be eluted with acetic acid alone. The dissociation constant values quoted are taken from Brown *et al.* (1955).

The presence of α-hydroxy acids in the 7.3 mg of material originally eluted from the Florisil column by trifluoroacetic acid, fraction B, was confirmed by thin-layer chromatography on Florisil plates. The plates were prepared without binder from sieved Florisil and were activated by drying at 110° for 1 hour. The finest material, through 200 mesh, gave the most uniform and adherent plates, but the 180–200 mesh fraction was usable. When developed with 4% acetic acid in isopropyl ether known acids had the following R_F values: α-hydroxyoctadecanoic acid, 0.05; ricinelaidic acid, 0.81; and unsubstituted fatty acids, 0.85–0.90; developed with 1% trifluoroacetic plus 4% acetic acids in isopropyl ether the unsubstituted acids and ricinelaidic acid had R_F values greater than 0.9, and α-hydroxyoctadecanoic acid had an R_F of 0.64–0.69, seemingly moving with a secondary solvent front of trifluoroacetic acid. Thin-layer chromatography of fraction B on Florisil developed with 4% acetic acid showed three components: nonhydroxylated acids of R_F greater than 0.9; an unknown, R_F 0.4 (which may be an impurity from the Florisil adsorbent); and the α-hydroxy acid, R_F 0.04–0.05.

In view of its heterogeneity in thin-layer chromatography, fraction B (7.3 mg) was methylated (BF_3-methanol) and the esters were chromatographed on silicic acid, collecting: 2.6 mg of nonhydroxylated esters eluted with 2% ethyl ether; 1.1 mg of fraction D, eluted with 20% ether; 1.4 mg of fraction E, eluted with 30% ether; and 1.9 mg of polar material eluted with absolute methanol. By thin-layer chromatography on Anasil plates developed with 30% isopropyl ether fraction D contained both nonhydroxylated esters (R_F 0.92) and hydroxyl esters (R_F 0.23); fraction E contained mostly hydroxy esters with a small amount of unknown, less-polar material of R_F 0.36. Gas-liquid chromatographic analysis under the same conditions as described for fraction C showed that fraction D contained 90.0% methyl α-hydroxyoctadecanoate, retention time 10.93 minutes; 10.0% of an unknown component, retention time 21.02 minutes (very similar to methyl ricinoleate, retention time 21.65 minutes; and traces of methyl oleate, eicosaenoate, and α-hydroxyhexadecanoate. The latter was present in the original α-hydroxyoctadecanoate added to the muscle lipid sample. Fraction E by gas-liquid chromatography contained 84.7% methyl α-hydroxyoctadecanoate, 3.5% of what may be methyl ricinoleate (retention time 21.86 minutes versus 22.02 minutes for an authentic sample), 10.6% methyl oleate, 1.3% methyl eicosaenoate, and a trace of the C_{16} α-hydroxy homolog. The material balance of the recovered hydroxy acids is summarized in Table IV.

TABLE IV: Recovery of the Hydroxy Acids Added to *R. pretiosus* Muscle Lipid.

Component	Methyl α-Hydroxy-octadecanoate	Methyl Ricinoleate
Added	2.0 mg	6.3 mg
Recovered		
Fraction C		5.6
D	1.0	0.1
E	1.2	0.05
Total	2.2	5.8
% recovered	110	92

Discussion

The excellent recoveries of added hydroxy acids demonstrated here give added credibility to the conclusion that there were no hydroxy fatty acids in our sample of *Ruvettus* muscle lipid. Furthermore, the recovered hydroxy acids, both α-hydroxy and 12-hydroxy, had the same composition after reisolation as when added; that is, there was no dilution of the added acids with homologs or with positional isomers from the *Ruvettus* lipid—again indicating that no hydroxy fatty acids were present in the lipid from *Ruvettus* muscle. Probably the hydroxyoleic acid reported by Cox and Reid (1932) was derived from hydroperoxides formed by extensive autoxidation during the preparation, transportation, and storage of their samples of oil and the subsequent preparation and distillation of the ethyl esters. The hydroperoxides would behave like hydroxy acids in the reactions used by Cox and Reid to characterize the material: acetylation followed by determination of the saponification and neutralization numbers of the acetylated sample. The acetyl values reported by Cox and Reid (1932) and by Tsujimoto and Koyanagi (1937)[3] for the crude lipid are equivalent to a maximum of 4% hydroxyoleic acid. Furthermore, we also found free alcohols in our sample of the total muscle lipid. Assuming that they were also components of the oils investigated by Cox and Reid and by Tsujimoto and Koyanagi, the amount of hydroxy acids which could have been present in their samples is further reduced. We believe that *in vivo* the muscle of *R. pretiosus* does not contain hydroxy fatty acids. The purgative effect of the castor oil fish, then, is not due to hydroxy fatty acids, but probably simply to the mild lubricant action of the large amount of oil in the flesh, enhanced by the relatively poor adsorption of the wax esters from the gut. Note that a half-pound portion (wet wt) of the flesh contains initially about 39 ml of oil.

Our results confirm the conclusions of Cox and Reid and of Tsujimoto and Koyanagi that the lipids of *R. pretiosus* muscle consist largely of hexadecyl oleate and oleyl oleate.[4] This conclusion is further supported by our

[3] The abstract of this paper in *Chemical Abstracts* [*32*, 1963 (1937)] uses only the Okinawan common name for the fish, inguandaramé, and no scientific name. Consequently its pertinence to *Ruvettus* is not apparent.

[4] Kimura (1926) had already reported that the oil of *Ruvettus* was very high in unsaponifiables (45.1%), of which cetyl (hexadecyl) and oleyl (octadecenyl) alcohols were the main constituents. His paper was apparently never abstracted by either *Chemical Abstracts* or *Chemisches Zentralblatt*.

isolation of the wax esters as a class (93% of the total lipids) and confirmation of their chain lengths by gas-liquid chromatographic analysis. We did not make a detailed investigation of these wax esters, such as Haahti (1961) did for human sebum, but the data of Table III clearly indicate that the principal wax ester, 34:1,[5] is largely hexadecyl octadecenoate, which must make up at least 45% of the total wax esters. The 36:2 wax ester is largely octadecenyl octadecenoate, but 36:1 is probably a mixture of hexadecyl eicosaenoate and octadecyl octadecenoate in a ratio of about 3:1. These values are calculated from the fatty acid and alcohol compositions (after converting the weight % data of Table III to mole %), assuming random combination. The theoretical values so obtained are in good agreement with the observed gas-liquid chromatographic results for the wax esters when comparing only the total esters of a given chain length (Table II). Our unpublished data on the wax esters from two other fish, *Lepidocybium flavobrunneum* and *Latimeria chalumnae*, and for spermaceti support the random combination of the acids and alcohols.

The SE-30 column used for the gas-liquid chromatographic analyses of the wax esters was not capable of resolving components differing by only one double bond. Furthermore, as discussed here, the 36:1 component, for example, is not a single compound but rather consists of two main esters differing in the position of the ester

grouping (—C—O—) with respect to the ends of the carbon chains. Such structural differences are known (Lefort *et al.*, 1961) to produce small, but definite, differences in the retention times. With the *R. pretiosus* wax esters the differences may be accentuated by the presence of isomers in which the positions of the double bonds also vary. Thus there may be some overlap of the retention times of the monoenes and dienes. The presence in the *Ruvettus* acids and alcohols of double-bond positional isomers has not been established, but is known for other fish lipids, such as tuna muscle lipids (Roubal, 1963) and menhaden body oil (Stoffel and Ahrens, 1960). Lack of suitable reference materials has precluded further investigation of the effect of these structural changes on retention times and separation factors for, say, 36:1 and 36:2 on SE-30 columns. The unusually high percentage of free fatty acids present in the liver lipids (13% minimum) and the low percentages of C_{20} and C_{22} polyunsaturated acids found (see Table III) suggest that some degradation of these lipids has occurred.

[5] A shorthand designation for a compound having a total of thirty-four carbon atoms (in unbranched chains) and containing one double bond, position not specified. This is a convenient convention for gas-liquid chromatographic results, since within a homologous series the resolutions achieved depend on the chain length and the degree of total unsaturation (see Stoffel *et al.*, 1958).

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