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Utility of Nicotinoyl Derivatives in Structural Studies of Mono- and Diacylglycerols by Gas Chromatography/Mass Spectrometry

Part 3—Application to Acylglycerols with Methyl Branchings and Epoxy and Cyclopropyl Rings

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Mono- and diacylglycerols with methyl branches and epoxy and cyclopropyl rings were synthesized and, subsequently converted into their nicotinoyl derivatives by reaction with nicotinic acid and *N,N'*-dicyclohexylcarbodiimide in the presence of *N,N*-dimethyl-4-aminopyridine. The resulting nicotinoyl derivatives were examined by gas chromatography/mass spectrometry (GC/MS). Their electron impact mass spectra reveal the structures of mono- and diacylglycerols in greater detail than the mass spectra of other acylglycerol derivatives. The positions of methyl branches and epoxy and cyclopropyl rings in mono- and diacylglycerols can be determined from characteristic features in the fragmentation patterns, which are caused by radical-induced cleavage of the alkyl chains following random hydrogen abstraction by the pyridine nucleus. These results offer a promising approach to the structural analysis of glycerophospholipids by means of GC/MS.

KEYWORDS: acylglycerols; monoacylglycerols; diacylglycerols; nicotinoyl derivative; gas chromatography/mass spectrometry

INTRODUCTION

Gas chromatography/mass spectrometry (GC/MS) is a very useful technique for analysis of a wide variety of mixtures. Glycerophospholipids occur in nature as complex mixtures, but these compounds are not amenable to GC/MS unless the polar head groups are selectively removed by phospholipase C.

Unfortunately, the resulting underivatized diacylglycerols show poor gas chromatographic and mass spectrometric behaviour.[1] To improve this situation, the formation of several derivatives of the hydroxyl groups in the glycerol moiety has been attempted. *O*-Methyl,[2] isopropylidene[3,4] and acetyl derivatives,[1,3,5] however, reveal no molecular mass information in most cases. Their mass spectra are still dominated by low-mass alkyl fragment ions and ions, which are derived from α-cleavage in the glycerol moiety or the fatty acid alkyl chains. Nevertheless, the carbon and double bond number of the fatty acid alkyl chains may be determined.

A more useful mass spectrometric behaviour was demonstrated for trimethylsilyl ethers.[6–10] These derivatives are amenable to GC and can, therefore, be used for the investigation of complex acylglycerol mixtures by GC/MS. Their mass spectra show distinct differences between mono- and diacylglycerols and their respective isomers. The molecular masses and carbon and double bond numbers of the fatty acid alkyl chains can easily be determined. Superior results have been obtained with the *tert*-butyldimethylsilyl ethers, because these derivatives permit also the distinction of the reverse isomers of mixed 1,2-diacylglycerols.[11,12]

None of the acylglycerol derivatives mentioned above, offer, however, any information about the detailed structure of the fatty acid alkyl chains, e.g. positions of double bonds or methyl branchings. Chemical ionization (CI), which is frequently used in GC/MS analysis also produces no mass spectra containing additional structural information.[13,14]

Other soft ionization techniques, such as fast atom bombardment (FAB),[15] secondary ion mass spectrometry (SIMS)[16,17] and laser desorption (LD),[18] are useful for the mass spectrometric investigation of intact glycerophospholipids without prior degradation and derivatization. Especially well investigated is the utility of FAB ionization, which allows a detailed analysis of the fatty acid alkyl chain structure when used in conjunction with collisionally activated dissociation combined with tandem mass spectrometry (FAB-MS/MS).[19–21] This is only possible, however, when pure glycerophospholipids are investigated. Otherwise, FAB-MS/MS/MS

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may be used for the mass spectrometric analysis of lipid mixtures.[20]

In the past few years, an alternative derivatization strategy has been developed for the structure elucidation of long alkyl chains based on using derivatives containing a pyridine nucleus. These derivatives have been successfully adapted to the GC/MS analysis of fatty acids (picolinyl),[22–24] fatty alcohols (nicotinoyl,[25] dimethylnicotinylsilyl[26]), long-chain 1,2-diols (bisnicotinoyl[27]), 1-mono-*O*-alkylglycerols and 1-monoacylglycerols (nicotinylidene[28]). They induce under electron impact (EI) ionization conditions random hydrogen abstraction from the alkyl chains followed by radical-induced cleavage at each carbon–carbon bond. The resulting mass spectra contain a series of ions, which permit the determination of the exact structure of the alkyl chains.

Recently, we have shown that nicotinoyl derivatives of saturated and unsaturated mono- and diacylglycerols similarly produce fragmentation patterns reflecting the structure of the fatty acid alkyl chains.[29,30] The determination of double bond positions has been shown to be easily possible.[30] Additionally, the mass spectra allow the distinction of mono- and diacylglycerols and their positional isomers. The molecular mass as well as the carbon and double bond number of the fatty acids are easily determinable.[29]

We have now extended our investigations to partially acylated acylglycerols containing methyl branchings and epoxy and cyclopropyl rings. The use of nicotinoyl derivatives was investigated for the determination of methyl branchings and epoxy and cyclopropyl rings in mono- and diacylglycerols by GC/MS. Additionally, a mild derivatization method is reported, which is suitable for the preparation of nicotinoyl derivatives of labile mono- and diacylglycerols.

EXPERIMENTAL

Micro-preparation of nicotinoyl derivatives[31]

At 0 °C 11 mg of *N,N'*-dicyclohexylcarbodiimide were added to a solution of 2 mg of acylglycerol, 6 mg of nicotinic acid and 0.5 mg of *N,N*-dimethyl-4-aminopyridine in dry dichloromethane (3 ml) under argon. After 5 min, the reaction mixture was warmed to room temperature and kept at this temperature for 14 h. Subsequently, colourless crystals of dicyclohexylurea were removed and the remaining solution was diluted with 4 ml of *n*-hexane. This solution was washed twice with each of 0.5% hydrochloric acid, saturated sodium hydrogencarbonate solution and saturated sodium chloride solution. The solvent was evaporated under a stream of nitrogen. The residue was dissolved in dichloromethane (3 ml) and aliquots of the solution were injected into the GC/MS system.

Synthesis of mono-diacylglycerols

Methyl-branched acylglycerols. A solution of 2 mg of dry glycerol in 2 ml of dry pyridine was added at room temperature to an equimolar amount of methyl-branched fatty acid chloride under argon. After 3 h the reaction mixture was heated at 60 °C for 1 h. Subsequently, the pyridine solution was diluted with 4 ml of water and extracted four times with *n*-hexane (2 ml). The hexane phases were washed twice with water and the solvent was evaporated under a stream of nitrogen. The residue was derivatized according to the above-described procedure. Separation into mono- and diacylglycerol derivatives and their positional isomers was then carried out by thin-layer chromatography (TLC) (silica gel plates with ethyl acetate–light petroleum (1 : 1) as solvent).

Freshly prepared fatty acid chlorides were obtained by reaction of the fatty acids with thionyl chloride at 60 °C. Before using them, excess thionyl chloride was completely removed in a stream of dry nitrogen.

Epoxyacylglycerols. The 9,10-epoxyacylglycerols were obtained by reaction of the mono- and di((*Z*)-9-octadecenoyl)glycerols with 3-chlorobenzoic peracid.[32]

Di(*cis*-9,10-methyleneoctadecanoyl)glycerols. These cyclopropyldiacylglycerols were synthesized as reported by Neises and Steglich[31] by reaction of glycerol with a twofold amount of *cis*-9,10-methyleneoctadecanoic acid in the presence of *N,N'*-dicyclohexylcarbodiimide and *N,N*-dimethyl-4-aminopyridine. After preparation of the nicotinoyl derivatives, the positional isomers were separated from each other by TLC (silica gel plates with ethyl acetate–light petroleum (1 : 1) as solvent).

cis-9,10-Methyleneoctadecanoic acid was obtained as reported by Simmons and Smith.[33]

Chemicals

1,2-Di((*Z*)-9-octadecenoyl)-*rac*-glycerol, 1-mono((*Z*)-9-octadecenoyl)-*rac*-glycerol and 15-methylheptadecanoic acid were purchased from Larodan (Malmö, Sweden). The 1,3-isomer of di((*Z*)-9-octadecenoyl)glycerol and 16-methylheptadecanoic acid were supplied by Sigma (Deisenhofen, Germany). Diiodomethane, nicotinic acid, *N,N'*-dicyclohexylcarbodiimide and zinc–copper couple were obtained from Merck (Darmstadt, Germany) and 3-chlorobenzoic peracid and *N,N*-dimethyl-4-aminopyridine from Fluka (Buchs, Switzerland).

Apparatus

GC/MS analyses were performed with a Fisons VG Quattro mass spectrometer coupled to a Fisons MEGA 5300 gas chromatograph and equipped with a VG Lab Base (Version 2.1) data system. The gas chromatograph was equipped with an on-column injection system. A 15 m × 0.32 mm i.d. fused-silica capillary column with DB-1 bonded phase (film thickness 0.25 μm) was used. The carrier gas was helium at a flow rate of 3 ml min^{-1} (measured in the absence of the mass spectrometer vacuum). Temperatures were as follows: injector, 40 °C; transfer line, 300 °C; ion source, 260 °C; and GC column oven, programmed at 25 °C min^{-1} from 40 to 200 °C and at 7 °C min^{-1} from 200 to 350 °C, the final temperature being maintained at 350 °C for 10 min. Every 1.6 s a mass spectrum with the range 50–800 u

was recorded at an electron energy of 25 eV and an electron current of 150 μA.

MS/MS data were recorded with a Fisons VG Quattro triple quadrupole mass spectrometer equipped with a direct inlet system and a VG Lab Base (Version 2.1) data system. Low-energy collisions (100 eV) were effected with argon in the second r.f.-only quadrupole as collision cell.

RESULTS AND DISCUSSION

The reaction conditions for preparing the nicotinoyl derivatives, which were described in a previous paper,[30] were found to be too severe for the derivatization of epoxy- and cyclopropylacylglycerols. Therefore, a mild esterification reaction with nicotinic acid and *N,N'*-dicyclohexylcarbodiimide[31] was successfully adapted to the micro-scale derivatization of such labile acylglycerols. Using this method, all the partially acylated acylglycerols investigated were converted with excellent yields into the nicotinoyl derivatives. No underivatized acylglycerols could be detected. The excess of *N,N'*-dicyclohexylcarbodiimide, which is soluble in organic solvents, could not be removed from the reaction mixture. Because of its distinctly shorter GC retention time, however, it does not disturb the mass spectrometric investigation of the nicotinoyl derivatives.

A superficial inspection of Figs 1–5 shows a similar appearance of the mass spectra of methyl-branched and epoxy- and cyclopropylacylglycerols in the low- and middle-mass regions. The low-mass regions are dominated by protonated nicotinic acid at *m/z* 124 and, in the case of the monoacylglycerols, additionally by the nicotinoyl cation at *m/z* 106 and the cyclic acetal ion at *m/z* 164.[29]

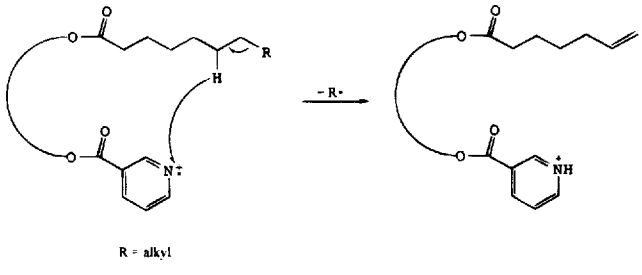
The distinction between 1- and 2-monoacylglycerols and 1,2- and 1,3-diacylglycerols is possible by characteristic differences in the abundance of several fragment ions. This was previously described for the saturated acylglycerols.[29] Not all epoxy- and cyclopropylacylglycerol isomers produce these mass spectral differences to a sufficient extent, however. Identification of positional isomers might then be possible only by the use of reference mass spectra or by comparison of GC retention times.

In the middle-mass region the determination of the masses of the fatty acid residues is easily possible by the fragment ions at *m/z* 285 (*a*) and *m/z* 303 (*b*) in the case of the monoacylglycerols and *c* and *d* in the case of diacylglycerols. These ions reflect the loss of [RCO – 2H] (*b*, *d*) and fatty acid acyloxy radicals (*a*, *c*) from the molecular ions. Their striking 18 u distance and their abundance allow easy detection in the mass spectra.[29]

The fatty acid alkyl chains are reflected by regular series of ions in the high-mass region, which result from random hydrogen abstraction by the pyridine nucleus followed by radical induced carbon–carbon bond cleavages (Scheme 1).[25] These nicotinoyl-induced fragment ion series are marked off at the alkyl end of the chain by the molecular ions and at the carboxyl ends by the ion pairs *e–f* in the case of monoacylglycerols and *g–h* in the case of diacylglycerols. These ion pairs exhibit a striking 13 u rather than 14 u distance, which results from the formation of *e* and *g* by a McLafferty rearrangement,[29] rather than formal simple cleavage.

Methyl-branched mono- and diacylglycerols

Figure 1 shows the mass spectrum of the nicotinoyl derivative of 1-mono(16-methylheptadecanoyl)glycerol (iso-methyl branching). The nicotinoyl fragmentation pattern can be observed in the high-mass region between the McLafferty ion *e* (*m/z* 344) and the molecular ion (*m/z* 568). All methylene groups of the alkyl chains are reflected by peak clusters separated by 14 u spacings (marked with asterisks). The methyl branching at position 16 of the fatty acid alkyl chain produces striking characteristics in this regular series of fragment ions. As ion formation at the branch point requires the rupture of two C—C bonds, the ion at *m/z* 539 is greatly reduced in relative abundance. The enhanced



Scheme 1.

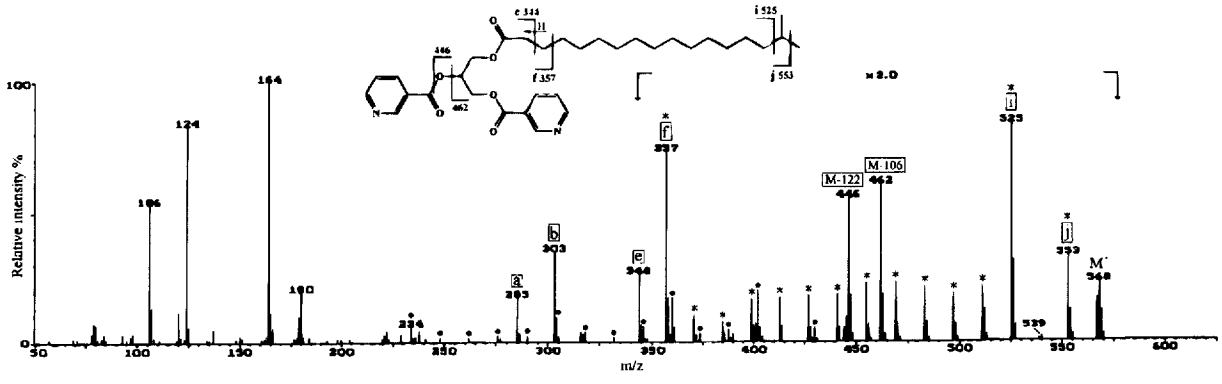


Figure 1. Mass spectrum obtained by GC/MS at 25 eV of the bisnicotinoyl derivative of 1-mono(16-methylheptadecanoyl)-*rac*-glycerol. Fragment ions of the nicotinoyl fragmentation pattern are marked with asterisks. Fragment ions resulting from the loss of nicotinic acid molecules are marked with dots.

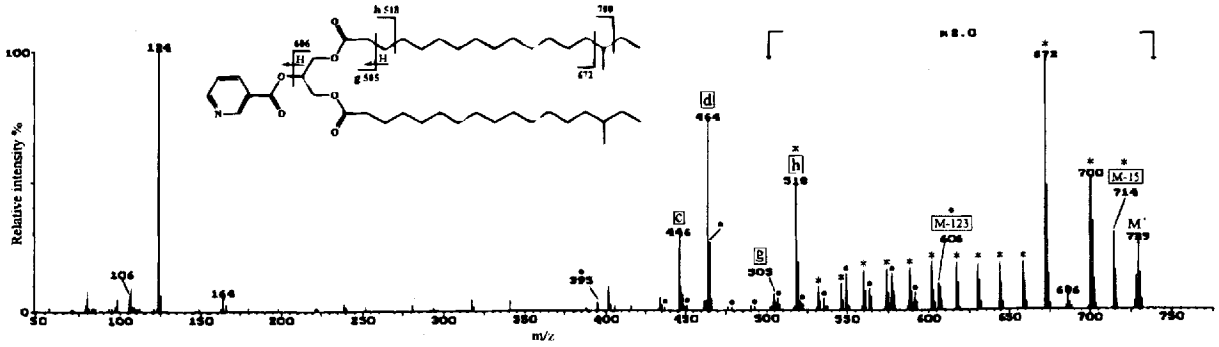


Figure 2. Mass spectrum obtained by GC/MS at 25 eV of the nicotinoyl derivative of 1,3-di(15-methylheptadecanoyl)glycerol. Fragment ions of the nicotinoyl fragmentation pattern are marked with asterisks. Fragment ions resulting from the loss of nicotinic acid molecules are marked with dots.

abundance of both neighbouring fragment ions (*i*, *m/z* 525, and *j*, *m/z* 553) is presumably due to the formation of energetically favourable radicals and fragment ions. Similar fragmentation pathways have been already proposed for the picolinyl derivatives of methyl-branched fatty acids.[22]

Besides two other abundant fragment ions, which arise from the loss of a pyridine-3-carboxy radical (*m/z* 446, [M − 122]) and a nicotinoyl radical (*m/z* 462, [M − 106]) from the $M^{+\cdot}$, a second fragmentation series (marked with dots) is present in the high-mass region between the fragment ions at *m/z* 234 and 446 ([M − 122]). By parent and fragment ion experiments (MS/MS), it could be shown, that these ions are formed by the loss of nicotinic acid molecules from the above nicotinoyl-induced fragmentation pattern. The identification of the nicotinoyl-induced fragmentation pattern is not complicated by these ions, because both fragment ion series occur at different masses.

The mass spectrometric behaviour of a diacylglycerol with an *anteiso*-methyl branching is shown in Fig. 2 (1, 3-di(15-methylheptadecanoyl)glycerol). The nicotinoyl-induced fragmentation patterns (marked with asterisks) extends from the McLafferty fragment ion at *m/z* 505 up

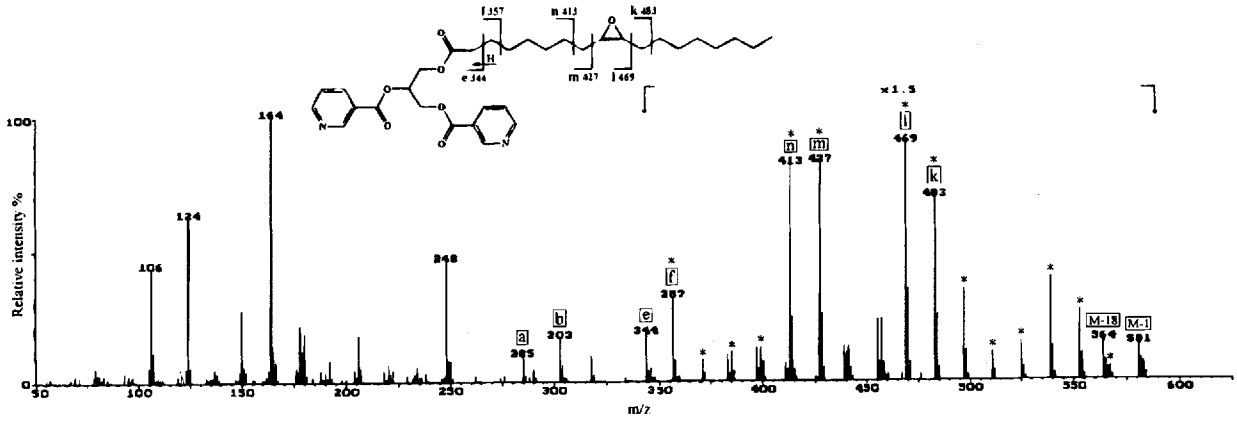


Figure 3. Mass spectrum obtained by GC/MS at 25 eV of the bisnicotinoyl derivative of 1-mono(9,10-epoxyoctadecanoyl)-*rac*-glycerol. Fragment ions of the nicotinoyl fragmentation pattern are marked with asterisks.

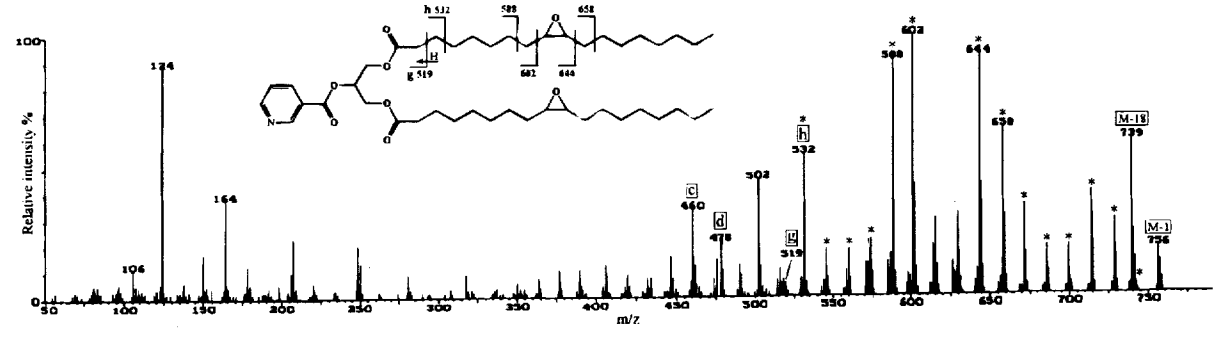
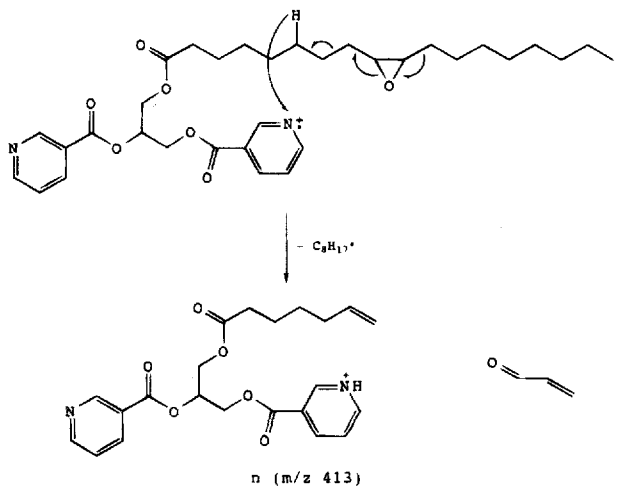


Figure 4. Mass spectrum obtained by GC/MS at 25 eV of the nicotinoyl derivative of 1,3-di(9,10-epoxyoctadecanoyl)glycerol. Fragment ions of the nicotinoyl fragmentation pattern are marked with asterisks.



Scheme 2.

to $M^{+\cdot}$ at *m/z* 729. The series of ions arising from subsequent loss of nicotinic acid extends from *m/z* 395 to *m/z* 626 (marked with dots). The position of the methyl branching can be determined by the strikingly low abundant ion at *m/z* 686, which is flanked by two prominent ions of the nicotinoyl fragmentation pattern at *m/z* 672 and 700.

A general mass spectrometric characteristic of all methyl-branched acylglycerols seems to be the relatively high abundance of the [M − 15] fragment ions (8–15% relative abundance). This is presumably due to the formation of energetically favourable ions with a doubly alkyl-substituted double bond, when methyl radicals are lost from the molecular ions. Nicotinoyl derivatives of all other acylglycerols investigated in this and previous studies produce the [M − 15] ions in only very low abundances (< 1.5% relative abundance).

Mono- and di(9,10-epoxyoctadecanoyl)glycerols

The presence of epoxy rings in the mono- and diacylglycerols is clearly indicated in the mass spectra of the nicotinoyl derivatives (Figs 3 and 4) by an abundant [M − 1] ion (loss of hydrogen atoms) and an abundant [M − 18] ion (loss of water molecules). This is typical for epoxy rings in EI-induced fragmentation.[34]

The nicotinoyl-induced fragment ion series extends in the case of the 1-mono(9,10-epoxyoctadecanoyl)glycerol from the McLafferty ion at *m/z* 344 (*e*) up to the [M − 1] ion at *m/z* 581. In the mass spectra of the di(9,10-epoxyoctadecanoyl)glycerols these series are marked off by the ion at *m/z* 532 (*h*) and the [M − 1] ion at *m/z* 756. The McLafferty ion *g* at *m/z* 519 has only low abundance.

All methylene groups of the alkyl chains are reflected by peak clusters separated by 14 u distances. In contrast to the methyl-branched acylglycerols, ions resulting from the loss of nicotinic acid molecules from the molecular ion or the nicotinoyl fragmentation pattern are not observed in these regular series.

The position of the epoxy ring can easily be determined in the mass spectrum of 1-mono(9,10-epoxyoctadecanoyl(glycerol (Fig. 3) by the strikingly high abundance of four ions at *m/z* 483 (*k*), 469 (*l*), 427 (*m*) and 413 (*n*). These ions reflect carbon–carbon bond cleavages on both sides of the epoxy ring. The high abundances are presumably caused by participation of the epoxy ring in the nicotinoyl-induced fragmentation resulting in energetically favourable ions or uncharged fragments.[34]

In Scheme 2, the fragmentation pathway leading to fragment ion *n* (*m/z* 413) is shown. Its high abundance can be rationalized by radical-induced formation of energetically favourable propenal molecules via bond cleavages in the epoxy ring and the alkyl chain. Similar fragmentation pathways are also probable for the ions *l* (*m/z* 483), *m* (*m/z* 469) and *n* (*m/z* 427).

In the mass spectra of the di(9,10-epoxyoctadecanoyl) glycerols, the epoxy rings are reflected by the same mass spectrometric characteristics (Fig. 4). The corresponding diagnostic fragment ions occur at *m/z* 658, 644, 602 and 588. Their high abundances are caused by the same fragmentation pathways as already described.

Between *l* (*m/z* 469) and *m* (*m/z* 427, Fig. 3), just as between the ions at *m/z* 644 and 602 (Fig. 4), other ions are present. They correspond to formal cleavages through the epoxy ring and can be rationalized by fragmentation pathways previously established for aliphatic epoxides.[35]

Di(*cis*-9,10-methyleneoctadecanoyl)glycerols

In the case of the (cyclopropylacyl)glycerols, only diacylglycerols have been investigated. The mass spectrum

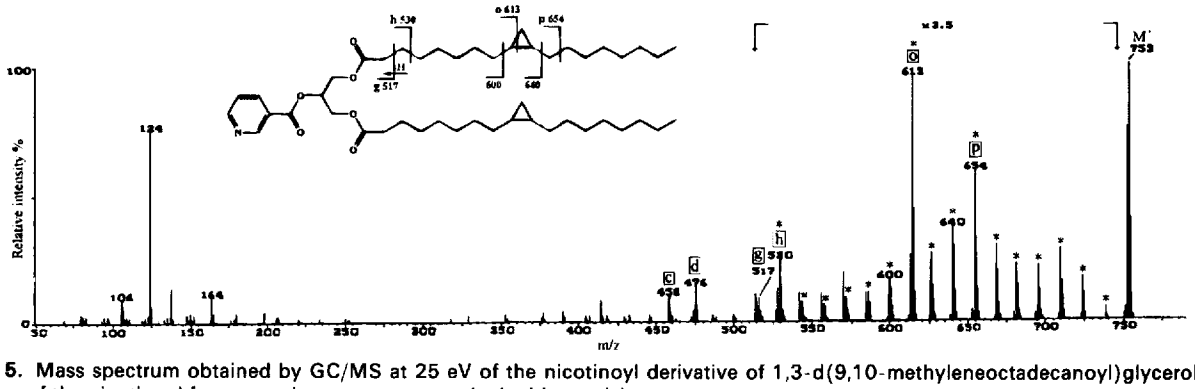
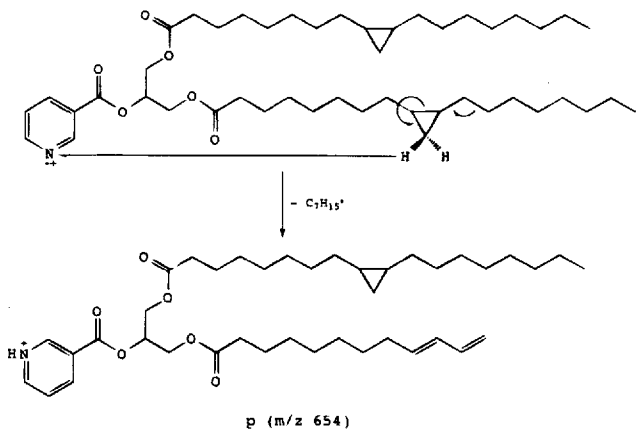


Figure 5. Mass spectrum obtained by GC/MS at 25 eV of the nicotinoyl derivative of 1,3-d(9,10-methyleneoctadecanoyl)glycerol. Fragment ions of the nicotinoyl fragmentation pattern are marked with asterisks.



Scheme 3.

of the nicotinyl derivative of 1,3-di(*cis*-9,10-methyleneoctadecanoyl)glycerol is shown in Fig. 5.

Similarly to the unsaturated acylglycerols,[30] both di(cyclopropylacyl)glycerol isomers form molecular ions with base peak intensity. The [M − 1] ions also display enhanced relative abundances.

The nicotinoyl-induced fragment ion series is marked off at the carboxyl end by the McLafferty ion *g* (*m/z* 517) and ion *h* (*m/z* 530) and at the alkyl end by the molecular ion (*m/z* 753). As described above, the methylene groups are reflected by peak clusters separated by 14 u spacings. Between the carboxyl end and the cyclopropane ring, these fragment ions are accompanied by ions 2 u lower in mass. Their structures and fragmentation pathways have not been determined. On the other hand, fragment ions reflecting the loss of nicotinic acid from $M^{+\cdot}$ or the nicotinoyl-induced fragmentation pattern are scarcely observed in this mass region.

The position of the cyclopropyl group is clearly reflected in the nicotinoyl-induced fragment ion series by a striking odd mass ion at *m/z* 613 (*o*) and an abundant ion at *m/z* 654 (*p*). A characteristic 41 u spacing between them is produced by the fragment ion *o*, which reflects a formal cleavage through the cyclopropyl ring.

The formation of ion *p* (*m/z* 654) can be rationalized by the fragmentation pathway shown in Scheme 3, which involves hydrogen abstraction by the pyridine nucleus followed by carbon–carbon bond cleavages in and next to the cyclopropane ring. The resulting energetically favourable conjugated double bond system explains the high abundance of this fragment ion. A fragmentation pathway leading to ion *o* (*m/z* 613) by formation of an alkene molecule and stabilized allylic radical cation has been proposed earlier.[23]

A second feature found to be diagnostic of the position of the cyclopropane ring is a striking 40 u (cyclopropane ring), instead of the 42 u spacing expected for three methylene groups, between the two fragment ions at *m/z* 600 and 640. These two ions reflect carbon–carbon bond cleavages directly on each side of the cyclopropane ring and additionally permit the determination of its position.

CONCLUSION

This study has shown that the positions of methyl branchings and epoxy and cyclopropyl rings in partially acylated acylglycerols can be readily determined from striking features in the mass spectra of the nicotinoyl derivatives. For their preparation, a mild esterification procedure has been established, which also allows the derivatization of labile mono- and diacylglycerols.

Considering the results of recent investigations (determination of double bond positions),[30] the nicotinoyl derivatives permit a detailed and complete structural characterization of mixtures of mono- and diacylglycerols with a variety of structural features. Compared with other derivatives, superior results can be obtained by a GC/MS method, which also offers a promising approach to the analysis of glycerophospholipids, since the polar head groups of these molecular species can be removed and the resulting diacylglycerols converted into their nicotinoyl derivatives.

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