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Synthesis and tandem mass spectrometry of chlorinated triacylglycerols

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ABSTRACT

The incorporation of 9,10-dichlorooctadecanoyl groups using enzyme-catalyzed acylation and protecting group strategies yielded specific regioisomers of di- and tetrachlorinated triacylglycerols. Hexachloro- and hexabromotriacylglycerols were synthesized by addition of chlorine or bromine to tri-(*cis*-9-octadecenoyl)glycerol. Upon electrospray ionization and tandem mass spectrometry, the sodium adduct ions of all compounds containing a 9,10-dichlorooctadecanoyl group readily lost two molecules of HCl when subjected to collision-induced dissociation. A mechanism describing sequential HCl losses and the formation of a conjugated diene is proposed for the loss of both vicinal chlorine atoms from an alkyl chain. This characteristic fragmentation behavior and the availability of characterized standards will facilitate the development of quantitative analytical methods for the determination of chlorinated triacylglycerols in lipid mixtures isolated from marine and other biological sources.

1. Introduction

The occurrence and distribution of extractable, organically bound halogens in the marine environment (Dembitsky and Srebnik, 2002), particularly in the marine vertebrate (Björn et al., 1998b; Kiceniuk et al., 1997, 1998; King et al., 2006; Lunde et al., 1976; Mu et al., 1996a,b, 1997b, 2004; Tinsley and Lowry, 1980; Wan et al., 2010; Wesén et al., 1990, 1992, 1995a,b; Zhuang et al., 2006) and shellfish (Bottaro et al., 1999; Lunde et al., 1976; Milley et al., 1997; Mu et al., 1997b; Tinsley and Lowry, 1980) constituents of the human food chain, is of ongoing ecotoxicological concern (Björn et al., 1998a; Ewald, 1999; Spickett, 2007). However, well-known, environmentally persistent, halogenated anthropogenic substances, such as polychlorinated biphenyls (PCBs), hexachlorocyclohexanes (HCHs) and certain pesticides (e.g., DDT), accounted for only a minor portion of the total halogen (Björn et al., 1998a; Ewald, 1999; Håkansson et al., 1991; Lunde et al., 1976; Milley et al., 1997; Newsome et al., 1993; Wan et al., 2010; Zhuang et al., 2006). Instead, most (up to 90%) of the bromine (Mu et al., 1997b; Tinsley and Lowry, 1980; Wan et al., 2010) and chlorine (Björn et al., 1998a; Ewald, 1999; Milley et al., 1997; Mu et al., 1996a, 1997b, 2004; Wesén et al., 1992, 1995b; Zhuang et al., 2006) was associated with storage and membrane lipids, and subsequently, several physiological effects were attributed to halogenated lipids and fatty acids (Björn et al., 1998a; Ewald, 1999; Håkansson et al., 1991; Høstmark et al., 1999; Lystad et al., 2001).

Three dichlorinated fatty acids, 9,10-dichlorooctadecanoic acid (9,10-dichlorostearic acid; $HO_2C(CH_2)_7(CHCl)_2(CH_2)_7CH_3$), 7,8-dichlorohexadecanoic acid (7,8-dichloropalmitic acid; $HO_2C(CH_2)_5(CHCl)_2(CH_2)_7CH_3$) and 5,6-dichlorotetradecanoic acid (5,6-dichloromyristic acid; $HO_2C(CH_2)_3(CHCl)_2(CH_2)_7CH_3$), were identified in several organisms by analysis of fatty acid methyl (Björn et al., 1998b; King et al., 2006; Milley et al., 1997; Mu et al., 1996a,b, 1997b; Wesén et al., 1992, 1995a,b; Zhuang et al., 2003, 2006) and pentafluorobenzyl (Zhuang et al., 2004b) esters derived from extracted lipids. The position of chlorine in 5,6-dichlorotetradecanoic acid was confirmed by interpreting the electron ionization mass spectral fragmentation patterns of 4,4-dimethyloxazoline (Zhuang et al., 2004a) and picolinyl (Åkesson-Nilsson and Wesén, 2004) derivatives.

The metabolic relationship inferred by the common structural features of the three dichloroalkanoic acids was supported by the detection of shorter-chain, halogenated fatty acids in human cell cultures and rats administered 9,10-dichlorooctadecanoic acid or 9,10-dibromooctadecanoic acid (Åkesson-Nilsson and Wesén, 2004; Conacher et al., 1984; Gustafson-Svärd et al., 2001; Lawrence et al., 1984) and by the release of radioactive carbon dioxide when [1-^{14}C]-9,10-dichlorooctadecanoic acid was fed to goldfish (Björn et al., 2004). Other experiments demonstrating the incorporation of 9,10-dichlorooctadecanoic acid into phospholipids and

Abbreviations: CID, collision-induced dissociation; DMAP, 4-dimethylaminopyridine; EDCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ESI, electrospray ionization; GC, gas chromatography; LC-MS/MS, liquid chromatography–tandem mass spectrometry.

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triacylglycerol (Björn et al., 1998a, 2004; Ewald et al., 1996; Gustafson-Svärd et al., 2001) are consistent with the proposed uptake of 9,10-dichlorooctadecanoic acid from pulp mill effluents (Björn et al., 2004; Gustafson-Svärd et al., 2001; Zhuang et al., 2003, 2006) and transfer within food chains (Mu et al., 2004; Ewald, 1999). Chlorinated fatty acids were not recognized as xenobiotic compounds by various organisms (Ewald, 1999; Björn et al., 1998a) and, when present in phospholipids, modified the properties of membranes (Ewald, 1999; Håkansson et al., 1991). Also, growth inhibitory (Björn et al., 1998a; Høstmark et al., 1999) and apoptotic effects (Lystad et al., 2001) on human cell lines have been demonstrated for 9,10-dichlorooctadecanoic acid and 5,6-dichlorotetradecanoic acid.

In previous studies (Björn et al., 1998b; King et al., 2006; Milley et al., 1997; Mu et al., 1996a, 1997a,b; Wesén et al., 1992, 1995a,b; Zhuang et al., 2003, 2004a, 2006), the presence of halogen in complex lipids was detected only after transesterification to monoesters. Additional structural information, however, can be obtained using analytical methods that determine intact neutral glycerolipids and phospholipids. Herein, regioselective syntheses of chlorinated triacylglycerols by coupling 9,10-dichlorooctadecanoic acid to mono- and didodecanoylglycerols are described to provide reference standards of defined structure for the development and validation of liquid chromatography–tandem mass spectrometry (LC-MS/MS; e.g., Herrera et al., 2010; Segall et al., 2004), or perhaps high-temperature gas chromatography (GC; Ruiz-Samblás et al., 2012), methods for the direct determination of halogenated triacylglycerols in complex mixtures. The mass spectra of the synthetic standards showed a characteristic, predominant loss of two molecules of HCl from alkyl chains containing vicinal chlorine substituents.

2. Experimental

2.1. General

Recombinant lipases from *Candida antarctica* (produced in *Aspergilis niger* and immobilized on macroporous acrylic resin) and *Rhizomucor miehei* (produced in *Aspergillus oryzae*), vinyl laurate (dodecanoate), boron trifluoride-methanol solution, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), glyceryl trioleate (tri(*cis*-9-octadecenoyl)glycerol), DL-1,2-isopropylideneglycerol, sulfuryl chloride, silica gel 60 (230–400 mesh), and analytical TLC plates were purchased from Sigma–Aldrich (Oakville, ON). Oleic acid (*cis*-9-octadecenoic acid), lauric acid (dodecanoic acid) and ion-exchange resin C-244 (strong acid, sulfonated polystyrene, 10% cross linked, 30–80 mesh) were obtained from Fisher Scientific (Ottawa, ON), Eastman Organic Chemicals (Rochester, NY) and the J.T. Baker Chemical Company (Phillipsburg, NJ), respectively.

Melting points (uncorrected) were obtained in open capillary tubes on a GallenKamp apparatus. Proton (^{1}H, 500 MHz) and carbon (^{13}C, 125 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Samples were dissolved in either $CDCl_3$ or CD_2Cl_2 (30 mg mL^{-1}). Chemical shifts (δ) in parts per million (ppm) were referenced to solvent signals ($CDCl_3$: δ_H 7.26, δ_C 77.16; CD_2Cl_2: δ_H 5.32, δ_C 54.00). Coupling constants (J) are given in Hertz (Hz); resonance multiplicities are described as: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; br s, broad singlet; and m, multiplet. The number of carbon nuclei is indicated in parenthesis when one signal corresponds to two or more carbon atoms. Electrospray ionization mass spectrometry (ESI-MS and ESI-MS/MS) was done using a Thermo-Finnigan LCQ DUO ion trap mass spectrometer running under Xcalibur software and equipped with an ESI probe set at 3.7 kV. Nitrogen was used as the source gas and the capillary temperature was maintained at 200 °C. Tandem mass spectra (MS/MS) were acquired using helium collision gas at collision-induced dissociation (CID) energies given in arbitrary units (%). Sample solutions (10 μL; 4 μM in methanol) were introduced into the ion source of the mass spectrometer by flow injection in methanol (syringe pump, 1.2 mL h^{-1}) via a Rheodyne 7725 injection valve. Accurate masses were determined using a Bruker microTOF mass spectrometer.

2.2. 9,10-Dichlorooctadecanoic acid (**2**)

Sulfuryl chloride (3 mL, 37 mmol) was added to a chloroform solution of *cis*-9-octadecenoic acid (**1**; oleic acid; 2.0 g, 7.1 mmol) in a round-bottomed flask covered with aluminum foil and immersed in an ice bath (Thurnhofer et al., 2008). After stirring for 3 h, water (5 mL) was added, and stirring was continued overnight at room temperature. The reaction mixture was washed with water (5 × 10 mL) until the aqueous wash was about pH 7. The organic layer was dried (anhydrous sodium sulfate) and rotary evaporated. Crude product was purified using column chromatography on silica gel (hexane-ethyl acetate, 2:1, v/v), yielding **2** as a yellow oil (2.3 g, 92%). ^{1}H and ^{13}C NMR spectra were consistent with published data (Denton et al., 2010); ESI(+)MS (relative intensity): *m*/*z* 375.3 (100, $[M+Na]^+$); MS/MS (33% CID) of *m*/*z* 375.3: *m*/*z* 339.0 (12), 303.1 (100).

For GC analysis, a mixture of 9,10-dichlorooctadecanoic acid (0.10 g, 0.3 mmol; in 1 mL CH_2Cl_2) and BF_3-MeOH (14%, 1 mL, 2 mmol) was heated at 80 °C for 5 min. Saturated aqueous sodium chloride solution (2 mL) was added and the mixture was extracted with hexane (2 mL). The organic layer was dried (anhydrous sodium sulfate) and a portion (1 μL) was injected onto a poly(5% diphenyl/95% dimethylsiloxane) fused silica capillary (Supleco PTE-5; 30 m × 0.025 mm id, 0.25 μm film thickness; 100:1 split injection) in an HP6890 GC connected to an HP5937 mass selective detector and an HP6890 autosampler/injector. Helium carrier gas (1 mL min^{-1}) and a temperature gradient of 10 °C min^{-1} from 100 to 320 °C with a five-min hold were employed. The injection port and transfer line were at 260 °C, while the ionization source and quadrupole were maintained at 230 and 150 °C, respectively. Mass spectra (*m*/*z* 40–550) were acquired at 2.3 scans s^{-1}.

2.3. 2-(9,10-Dichlorooctadecanoyl)-1,3-didodecanoylglycerol (**6**)

2.3.1. 1,3-Didodecanoylglycerol (**5**)

Immobilized *Candida antarctica* lipase (1.5 g) was added to a mixture of vinyl dodecanoate (**3**; 5.0 g, 22 mmol) and glycerol (**4**; 0.51 g, 5.5 mmol) in dichloromethane (2 mL) (Halldorsson et al., 2003). The suspension was allowed to stand at 4 °C for 18 h. The immobilized lipase was separated by filtration and the filtrate was rotary evaporated to afford a white crystalline product (2.4 g, 96%). mp 44–46 °C (lit (Halldorsson et al., 2003) mp 54.3–54.7 °C); ^{1}H and ^{13}C NMR spectra were consistent with published data (Halldorsson et al., 2003); ESI(+)MS (relative intensity): *m*/*z* 935.2 (82, $[2M+Na]^+$), 479.5 (100, $[M+Na]^+$); MS/MS (35% CID) of *m*/*z* 479.5: *m*/*z* 279.1 (44), 257.1 (100).

2.3.2. 2-(9,10-Dichlorooctadecanoyl)-1,3-didodecanoylglycerol (**6**)

A solution of 1,3-didodecanoylglycerol (**5**; 1.8 g, 3.9 mmol), 9,10-dichlorooctadecanoic acid (**2**; 1.43 g, 4.1 mmol), DMAP (0.164 g, 1.3 mmol) and EDCI (0.76 g, 4.0 mmol) in dichloromethane (20 mL) was stirred at room temperature for 24 h. The solvent was removed by rotary evaporation, and the crude product was purified by flash

chromatography on silica gel (ethyl acetate-hexane, 1:4, v/v). The separated product was a colorless oil (2.6 g, 83%). ^{1}H NMR (CD_2Cl_2) δ: 5.25–5.21 (1 H, m), 4.26 (2H, dd, J = 11.9 and 4.3), 4.13 (2H, dd, J = 11.9 and 6.1), 4.08–4.04 (2H, m), 2.30 (2H, t, J = 7.5), 2.29 (4H, t, J = 7.6), 1.93–1.85 (2H, m), 1.84–1.76 (2H, m), 1.63–1.56 (8H, m), 1.33–1.26 (50H, m), 0.89 (3H, t, J = 7), 0.88 (6H, t, J = 7); ^{13}C NMR (CD_2Cl_2) δ: 173.6 (2C), 173.3, 69.3, 66.4, 66.3, 62.6 (2C), 35.4, 35.3, 34.7, 34.5 (2C), 32.5 (2C), 32.4, 31.1, 30.2 (4C), 30.0 (2C), 29.9 (2C), 29.84 (2C), 29.76, 29.64 (2C), 29.61 (2C), 29.5, 29.4, 27.22, 27.17, 25.4 (2C), 25.3, 23.25 (2C), 23.21, 14.4 (3C); ESI(+)MS (relative intensity): m/z 813.6 (100, [M+Na]$^+$); MS/MS (30% CID) of m/z 813.6: m/z 777.4 (15), 741.4 (100), 613.2 (5), 595.2 (5); MS/MS (40% CID) of m/z 741.4: m/z 541.1 (100), 519.3 (50), 461.1 (20), 439.3 (14); HRMS: 813.5509 (813.5537 calculated for $C_{45}H_{84}Cl_2O_6Na$).

2.4. Chlorinated unsymmetrical triacylglycerols (*13* and *14*)

2.4.1. Monoacylisopropylidenes

A solution of 1,2-isopropylideneglycerol (**8**), fatty acid (1 equiv. of **2** or **7**), EDCI (1 equiv.) and DMAP (0.5 equiv.) in dichloromethane (20 mL) was stirred at room temperature for 24 h. The solvent was removed by rotary evaporation and crude product was purified using flash chromatography on silica gel eluted using ethyl acetate-hexane (2:1, v/v).

1,2-Isopropylidene-3-(9,10-dichlorooctadecanoyl)glycerol (**9**): coupling of 9,10-dichlorooctadecanoic acid (**2**; 2.6 g, 7.4 mmol) and **8** (0.97 g, 7.3 mmol) yielded **9** as an oil (2.9 g 85%). ^{1}H NMR (CD_2Cl_2) δ: 4.30–4.25 (1H, m), 4.14–4.02 (5H, m), 3.72 (1H, dd, J = 8.3 and 6.1), 2.35–2.30 (2H, m), 1.98–1.90 (2H, m), 1.85–1.75 (2H, m), 1.64–1.52 (4H, m), 1.40 (3H, s), 1.34 (3H, s), 1.34–1.28 (18H, m), 0.90 (3H, t, J = 7); ^{13}C NMR (CD_2Cl_2) δ: 173.9, 110.3, 74.4, 67.1, 66.49, 66.45, 65.1, 35.52, 35.49, 34.7, 32.5, 30.1, 29.9, 29.7 (2C), 29.6, 29.5, 27.35, 27.30, 27.2, 25.9, 25.5, 23.3, 14.5; ESI(+)MS (relative intensity): m/z 489.3 (100, [M+Na]$^+$): MS/MS (35% CID) of 489: m/z 452.9 (16), 416.9 (100); HRMS: 489.2498 (489.2509 calculated for $C_{24}H_{44}Cl_2O_4Na$).

rac-*1,2-Isopropylidene-3-dodecanoylglycerol* (**10**): coupling of dodecanoic acid (**7**; 2.8 g, 14 mmol) and **8** (1.9 g, 14 mmol) yielded **10** as a white crystalline solid (3.8 g, 86%). mp < 20 °C (lit (Batovska et al., 2004) mp 32.5 °C); ^{1}H and ^{13}C NMR spectra were consistent with published data (Batovska et al., 2004); ESI(+)MS: m/z 337.3 ([M+Na]$^+$); MS/MS (35% CID) of 337.3: m/z 279.1.

2.4.2. Mono-acyl glycerols

Purified 1,2-isopropylidene-3-acylglycerol (**9** or **10**) was mixed with ion-exchange resin C-244 (2 g, H^+ form) in methanol (20 mL) and dichloromethane (2 mL). The mixture was stirred at room temperature and monitored using TLC. When the starting material was consumed (4 h) the resin was filtered. The filtrate was dried (anhydrous sodium sulfate) and removed by rotary evaporation.

1-(9,10-Dichlorooctadecanoyl)glycerol (**11**): hydrolysis of 1,2-isopropylidene-3-(9,10-dichlorooctadecanoyl)glycerol (1.62 g, 3.5 mmol) yielded **11** as a colorless oil (1.35 g, 92%).^{1}H NMR (CD_2Cl_2) δ: 4.18–4.02 (4H, m), 3.91–3.87 (1H, m), 3.68–3.55 (2H, m), 2.34 (2H, t, J = 7.4), 1.99–1.88 (2H, m), 1.85–1.75 (2H, m), 1.65–1.51 (4H, m), 1.45–1.28 (18H, m), 0.90 (3H, m); ^{13}C NMR (CD_2Cl_2) δ: 174.6, 71.0, 67.1, 66.5, 65.9, 64.1, 35.5, 35.3, 34.7, 32.5, 30.3, 30.0, 29.8, 29.7, 29.6, 29.5, 27.3, 26.9, 25.5, 23.3, 14.5; ESI(+)MS (relative intensity): m/z 449.2 (100, [M+Na]$^+$); MS/MS (25% CID) of 449.2: m/z 412.9 (20), 376.9 (100); HRMS: 449.2206 (449.2196 calculated for $C_{21}H_{40}Cl_2O_4Na$).

rac-*1-Dodecanoylglycerol* (**12**): hydrolysis of 1,2-isopropylidene-3-dodecanoylglycerol (1.5 g, 4.8 mmol) yielded **12** as white crystals (1.25 g, 93%). mp 60–61 °C (lit (Rytczak et al., 2010) mp 62–63 °C); ^{1}H and ^{13}C NMR spectra were consistent with published data (Batovska et al., 2004; Rytczak et al., 2010); ESI(+)MS: m/z 297.4 ([M+Na]$^+$); MS/MS (28% CID) of 297.4: m/z 115.1.

2.4.3. Coupling of mono-acyl glycerol with two equivalent fatty acids

The coupling procedure described in Section 2.4.1 and a 33-h reaction time were used.

1-(9,10-Dichlorooctadecanoyl)-2,3-didodecanoylglycerol (**13**): coupling of 1-(9,10-dichlorooctadecanoyl)glycerol (**11**; 1.5 g, 3.5 mmol) and dodecanoic acid (**7**; 1.4 g, 7.0 mmol) yielded **13** as a waxy solid (2.4 g, 84%). mp 30–33 °C; ^{1}H NMR ($CDCl_3$) δ: 5.28–5.24 (1H, m), 4.29 (2H, dd, J = 11.9 and 4.3), 4.14 (2H, dd, J = 11.9 and 6.0), 4.04–4.01 (2H, m), 2.31 (6H, td, J = 7.5 and 3.2), 1.93–1.86 (2H, m), 1.81–1.73 (2H, m), 1.63–1.56 (8H, m), 1.32–1.24 (50H, m), 0.88 (9H, t, J = 7); ^{13}C NMR ($CDCl_3$) δ: 173.4, 173.3, 173.0, 69.0, 65.7, 65.6, 62.2 (2C), 34.53, 34.49, 34.3, 34.2, 34.1, 32.05 (2C), 31.96, 29.84, 29.75 (4C), 29.63, 29.61, 29.52, 29.48 (2C), 29.42, 29.41, 29.34, 29.25, 29.21, 29.17, 29.09, 29.0, 26.9, 26.8, 25.04, 24.99, 24.9, 22.82 (2C), 22.79, 14.3 (3C); ESI(+)MS (relative intensity): m/z 813.5 (100, [M+Na]$^+$); MS/MS (36% CID) of m/z 813.5: m/z 777.5 (20), 741.5 (100), 613.2 (5); MS/MS (33% CID) of m/z 741.5: m/z 541.3 (100), 519.3 (50), 461.2 (40), 439.1 (25); HRMS: 813.5560 (813.5537 calculated for $C_{45}H_{84}Cl_2O_6Na$).

1,2-Di-(9,10-dichlorooctadecanoyl)-3-dodecanoylglycerol (**14**): coupling of 1-dodecanoylglycerol (**12**; 1.0 g, 3.6 mmol) with 9,10-dichlorooctadecanoic acid (2.5 g, 7.2 mmol) yielded **14** as an oil (2.5 g, 73%). ^{1}H NMR ($CDCl_3$) δ: 5.26–5.21 (1H, m), 4.29–4.25 (2H, m), 4.11 (2H, dd, J = 11.9 and 6.0), 4.02–3.98 (4H, m), 2.30–2.25 (6H, m), 1.92–1.84 (4H, m), 1.79–1.70 (4H, m), 1.61–1.49 (10H, m), 1.31–1.22 (52H, m), 0.86 (3H, t, J = 7), 0.85 (6H, t, J = 7); ^{13}C NMR ($CDCl_3$) δ: 173.3, 173.2, 172.8, 69.0, 65.7 (2C), 65.6 (2C), 62.17, 62.14, 34.5 (4C), 34.2, 34.1, 34.0, 32.0, 31.9 (2C), 29.8, 29.75, 29.71 (2C), 29.6, 29.5, 29.43, 29.36, 29.30, 29.2, 29.1 (4C), 29.04, 29.01, 28.93, 28.91, 26.81 (2C), 26.76 (2C), 24.94, 24.88, 24.85, 22.8, 22.7 (2C), 14.2 (3C); ESI(+)MS (relative intensity): m/z 965. 7 (71, [M+Na]$^+$), 966.8 (44), 967.7 (100); MS/MS (30% CID) of m/z 967.7: m/z 931.4 (15), 895.5 (100), 893.5 (76), 821.6 (3); MS/MS (30% CID) of m/z 893.5: m/z 857.5 (26), 821.5 (100); MS/MS (36% CID) of m/z 821.5: m/z 621.3 (64), 599.2 (21), 541.1 (100), 519.3 (35); HRMS: 965.5726 (965.5697 calculated for $C_{51}H_{94}Cl_4O_6Na$).

2.5. Hexahalogenated triacylglycerols

2.5.1. Tri-(9,10-dichlorooctadecanoyl)glycerol (*16*)

Reaction of tri-(*cis*-9-octadecenoyl)glycerol (**15**, glyceryl trioleate; 0.52 g, 0.59 mmol) with sulfuryl chloride (3 mL, 37 mmol) as described in Section 2.2 yielded hexachlorinated triacylglycerol (**16**) as a colorless oil (0.54 g, 84%). ^{1}H NMR (CD_2Cl_2) δ: 5.25–5.21 (m, 1H), 4.26 (2H, dd, J = 12.0 and 4.3), 4.13 (2H, dd, J = 11.8 and 6.0), 4.08–4.05 (6H, m), 2.30 (6H, t, J = 7.5), 1.92–1.86 (6H, m), 1.84–1.76 (6H, m), 1.63–1.50 (12H, m), 1.42–1.26 (54H, m), 0.88 (9H, t, J = 7.0); ^{13}C NMR (CD_2Cl_2) δ: 173.6 (2C), 173.2, 69.4, 66.41 (3C), 66.37 (3C), 62.6 (2C), 35.4 (6C), 34.7, 34.5 (2C), 32.4 (3C), 30.0 (3C), 29.8 (3C), 29.6 (6C), 29.5 (3C), 29.4 (3C), 27.23 (3C), 27.19 (3C), 25.3 (3C), 23.2 (3C), 14.4 (3C); ESI(+)MS (relative intensity): m/z 1117.5 (60, [M+Na]$^+$); MS/MS (35% CID) of m/z 1119.5: m/z 1083.3 (24), 1047.4 (100), 1045.6 (40), 1009.6 (23), 973.5 (42); MS/MS (33% CID) of m/z 1047.5: m/z 1011.5 (28), 975.5 (100), 973.7 (69), 901.6 (25); MS/MS (33% CID) of m/z 973.5: m/z 937.3 (40), 901.5 (100); MS/MS (38% CID) of m/z 901.6: m/z 621.6 (100), 599.5 (38); HRMS: 1117.5812 (1117.5856 calculated for $C_{57}H_{104}Cl_6O_6Na$).

2.5.2. Tri-(9,10-dibromooctadecanoyl)glycerol (*17*)

Bromine (1.5 mL, 29 mmol) was added over 20 min to a stirred solution of tri-(*cis*-9-octadecenoyl)glycerol (**15**; 0.20 g, 0.23 mmol) in dichloromethane (5 mL) at −10 °C. After 1 h and an additional

3 h at room temperature, solvent was removed by rotary evaporation, and purification of the residue by flash chromatography on silica gel (dichloromethane) yielded hexabrominated triacylglycerol (**17**) as a yellow oil (0.16 g, 50%). ^{1}H NMR (CD_2Cl_2) δ: 5.25–5.21 (1H, m), 4.27 (2H, dd, J = 11.9 and 4.4), 4.25–4.21 (6H, m), 4.13 (2H, dd, J = 11.8 and 6.0), 2.31 (6H, t, J = 7.5), 2.06–1.89 (6H, m), 1.90–1.82 (6H, m), 1.64–1.53 (12H, m), 1.43–1.25 (54H, m), 0.88 (9H, t, J = 7.0); ^{13}C NMR (CD_2Cl_2) δ: 173.6 (2C), 173.2, 69.4, 62.6 (2C), 60.7 (3C), 60.6 (3C), 36.0 (6C), 34.6, 34.5 (2C), 32.4 (3C), 29.9 (3C), 29.8 (3C), 29.6 (3C), 29.5 (3C), 29.4 (3C), 29.2 (3C), 28.32 (3C), 28.27 (3C), 25.3 (3C), 23.2 (3C), 14.4 (3C); ESI(+)MS (relative intensity): m/z 1391.6 (17), 1390.7 (35), 1389.7 (42), 1388.7 (75), 1387.7 (59), 1386.7 (100), 1385.7 (48), 1384.7 (73), 1383.8 (23), 1382.7 (34), 1381.9 (12), 1380.7 (10, $[M+Na]^+$), 1230.0 (8), 1228.9 (16), 1227.9 (21), 1226.9 (43); 1225.9 (28), 1225.1 (50), 1224.1 (22), 1222.9 (39), 1221.9 (9), 1221.1 (14, $[M+Na-2HBr]^+$), 1067.2 (10), 1066.2 (17), 1065.2 (37), 1064.2 (23), 1063.1 (40), 1062.3 (14), 1061.2 (19, $[M+Na-4HBr]^+$); MS/MS (24% CID) of m/z 1386.7: m/z 1226.9 (41), 1224.9 (100), 1222.9 (31); MS/MS (36% CID) of m/z 901.5: m/z 621.3 (100), 599.1 (20); HRMS: 1381.2780 (1381.2825 calculated for $C_{45}H_{84}Cl_2O_6Na$).

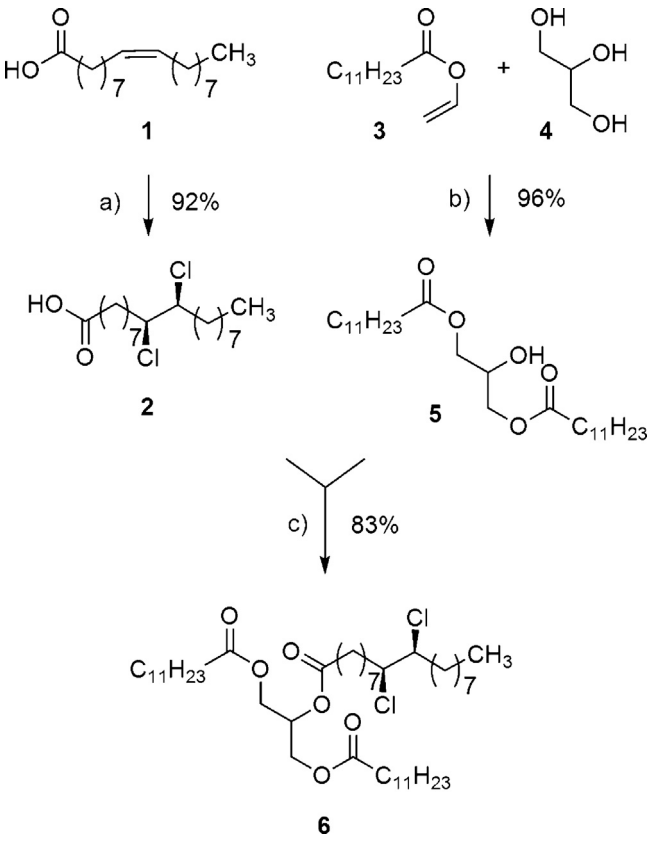
3. Results and discussion

3.1. Chlorination of oleic acid (1)

The analytical studies of halogenated lipids in marine organisms (Björn et al., 1998a; Dembitsky and Srebnik, 2002; King et al., 2006; Milley et al., 1997; Mu et al., 1997b; Wesén et al., 1992, 1995a; Zhuang et al., 2003, 2004a, 2006) most commonly detected dichlorinated fatty acids. Accordingly, 9,10-dichlorooctadecanoic acid (**2**) was prepared from *cis*-9-octadecenoic acid (**1**) and sulfuryl chloride (Bouquet and Paquot, 1946; Thurnhofer et al., 2008) (Scheme 1). By comparison of ^{1}H and ^{13}C NMR data with published values (Denton et al., 2010), the relative stereochemistry of the predominant isomer of the reaction product was assigned as *threo* (*syn*). Given the relative ^{1}H NMR chemical shifts of methyl *threo*- and *erythro*-9,10-dichlorooctadecanoate (Wesén et al., 1995a), a distinct resonance (δ 3.98–3.95) was assigned to *erythro*-9,10-dichlorooctadecanoic acid and a 7:1 *threo*:*erythro* ratio was calculated. Two closely eluting peaks (retention times of 22.4 and 22.5 min; ratio 1:5) were observed upon GC of the methyl esters in accord with the *erythro*-*threo* order noted previously (Björn et al., 1998b; Mu et al., 1996a; Wesén et al., 1995a; Zhuang et al., 2003). Consequently, the *threo* product of the sulfuryl choride reaction (Bouquet and Paquot, 1946; Thurnhofer et al., 2008) is formed by *anti*-addition of chlorine to the *cis* double bond in 9-octadecenoic acid (**1**).

3.2. Synthesis of 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoylglycerol (6)

An enzymatic approach was adopted to achieve regioselective acylation of the terminal hydroxyl groups in glycerol (Magnusson and Haraldsson, 2010; Andrews et al., 2008; Fraser et al., 2007; Halldorsson et al., 2003; Irimescu et al., 2001). Initially, vinyl dodecanoate (**3**) and glycerol (**4**) (3:1 ratio) were incubated with the 1,3-specific lipase from *Rhizomucor miehei* produced in *Aspergillus oryzae* (Halldorsson et al., 2003; Irimescu et al., 2001). ESI(+)MS analysis detected the sodium adduct ions of didodecanoylglycerol (**5**, m/z 479.6) and tridodecanoylglycerol (m/z 661.7), as well as higher mass ions (m/z 935.4, 1117.5 and 1299.6) corresponding to the sodium adduct ions of the three possible dimers of di- and tridodecanoylglycerol. The similar abundances of the five ions indicated that the *Rhizomucor miehei* lipase-catalyzed reaction yielded **5** and tridodecanoylglycerol as major products, as noted previously (Halldorsson et al., 2003).



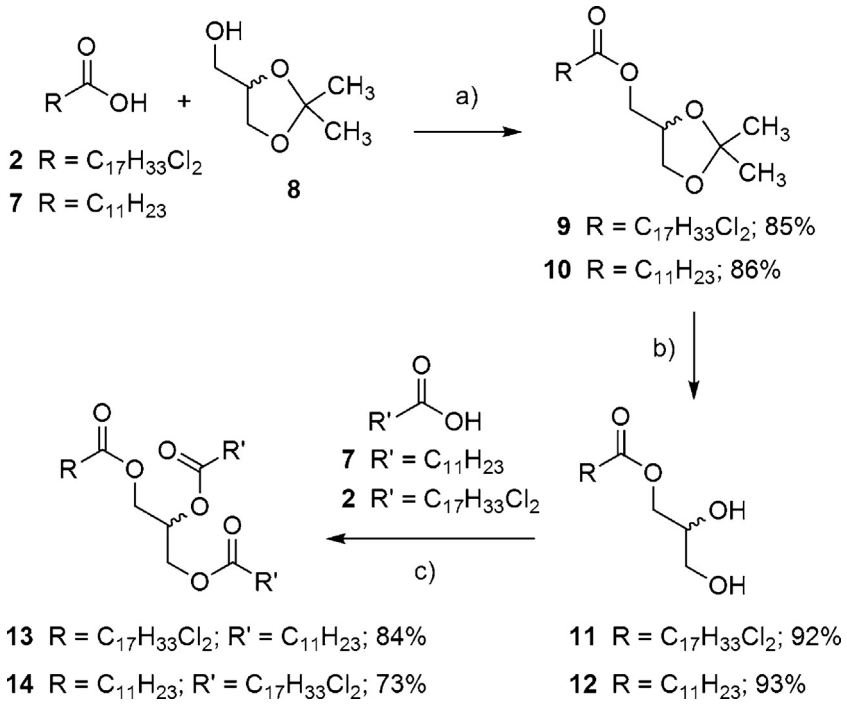
Scheme 1. Synthesis of the symmetrical triacylglycerol **6**. Reagents and conditions: (a) SO_2Cl_2, $CHCl_3$, 3 h; (b) Immobilized *C. antarctica* lipase, 4 °C, overnight; (c) EDCI, DMAP, CH_2Cl_2, 24 h.

On the other hand, incubation of glycerol (**4**) and excess vinyl dodecanoate (**3**) with immobilized *Candida antarctica* lipase at 4 °C (Magnusson and Haraldsson, 2010; Andrews et al., 2008; Halldorsson et al., 2003) gave the desired 1,3-didodecanoylglycerol (**5**) in excellent yield and purity (Scheme 1). Low-intensity resonances at δ 5.25, 5.08, 4.33–4.19 and 3.72 in the ^{1}H NMR spectrum of the product indicated trace amounts of 1,2-dodecanoylglycerol and tridodecanoylglycerol side products (Haraldsson et al., 1995).

Carbodiimide-mediated coupling (Magnusson and Haraldsson, 2010; Andrews et al., 2008; Fraser et al., 2007; Halldorsson et al., 2003) of dihalogenated fatty acid (**2**) with 1,3-didodecanoylglycerol (**5**) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) in dichloromethane at room temperature gave (after chromatography) 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoylglycerol (**6**). The observation of two ^{13}C NMR resonances and an $(AB)_2X$ ^{1}H NMR splitting pattern for nuclei in the glycerol backbone of **6** demonstrated the symmetry of this triacylglycerol product, in accord with the reported absence of acyl-migration side products under these coupling conditions (Magnusson and Haraldsson, 2010; Andrews et al., 2008; Halldorsson et al., 2003). Also, no mono- or diacylglycerols were detected by ESI(+)MS.

3.3. Synthesis of two unsymmetrical chlorinated triacylglycerols (13 and 14)

The preparation of unsymmetrical triacylglycerols containing one dichlorinated chain in a terminal position or two dichlorinated chains at adjacent positions (Scheme 2) started with a protected glycerol (Andrews et al., 2008; Fraser et al., 2007). Separate carbodiimide-mediated couplings of



Scheme 2. Synthesis of the unsymmetrical triacylglycerols **13** and **14**. Reagents and conditions: (a) EDCI, DMAP, CH_2Cl_2, 24 h; (b) cation-exchange resin, MeOH/CH_2Cl_2, 4 h; (c) EDCI, DMAP, CH_2Cl_2, 33 h.

9,10-dichlorooctadecanoic acid (**2**) and dodecanoic acid (**7**) with *rac*-1,2-isopropylideneglycerol (**8**) added an acyl group specifically at a terminal position. Hydrolysis of the acylated isopropylidenes **9** and **10** was achieved in high yield under the acidic conditions provided by a sulfonated polystyrene ion-exchange resin. The racemic monoacylglycerol products **11** and **12** contained about 6–7% of their regioisomer by ^{1}H NMR analysis (Haraldsson et al., 1995), indicating only small amounts of acyl group migration. Carbodiimide coupling of dodecanoic acid (**7**) and 9,10-dichlorooctadecanoic acid (**2**) to the monoacylglycerols **11** and **12**, respectively, yielded chlorinated unsymmetrical triacylglycerols (**13** and **14**) with complementary substitution patterns.

3.4. Synthesis of hexahalogenated triacylglycerols

Tri-(9,10-dichlorooctadecanoyl)glycerol (**16**) was obtained in high yield (90%) by treating commercially available tri-(*cis*-9-octadecenoyl)glycerol (**15**) with excess sulfuryl chloride. Similarly, tri-(9,10-dibromooctadecanoyl)glycerol (**17**) was prepared by adding excess bromine to tri-(*cis*-9-octadecenoyl)glycerol (**15**). The glyceryl resonances in the ^{1}H NMR spectrum of each product appeared as the typical pattern of a triacylglycerol (Haraldsson et al., 1995), and no olefinic resonances were detected in the ^{1}H and ^{13}C NMR spectra, indicating complete conversion of unsaturated triacylglycerol to the hexahalogenated product.

3.5. Mass spectrometry

When subjected to electrospray ionization mass spectrometry (ESI(+)MS), 9,10-dichlorooctadecanoic acid (**2**) and all mono-, di-, and triacylglycerols (i.e., **5**, **6**, **9**–**14**, **16**, **17**) were detected as sodium adduct ions. The $[M+Na]^+$ ions of the chlorinated compounds showed distinctive isotope clusters (e.g., Fig. 1A inset and Table 1) in which the relative intensities of the individual peaks closely corresponded to the values calculated (Ramaley and Herrera, 2008) using the expected elemental compositions (including two, four or six chlorine atoms) and natural isotopic abundances.

The sodiated ions of the chlorinated compounds (i.e., **2**, **6**, **9**, **11**, **13**, **14**) showed no fragmentation upon electrospray ionization. By contrast, the ammonium adduct ions of methyl 9,10-dichlorooctadecanoate (Mu et al., 1996a; Wesén et al., 1995a) and methyl dichlorooctadecenoate (Mu et al., 1996b) fragmented under chemical ionization conditions, losing Cl_2, as well as one or two molecules of HCl from methyl dichlorooctadecenoate (Mu et al., 1996b). When subjected to plasma spray ionization mass spectrometry (Sundin et al., 1992), chlorinated triacylglycerols readily lost a fatty acid residue from the $[M+H]^+$ ions. Upon plasma spray ionization of tri-(9,10-dichlorooctadecanoyl)glycerol, loss of the fatty acid was accompanied by up to four molecules of HCl, but, unlike the CID fragmentations described below, there was no preference for losses of an even number of HCl molecules. For triacylglycerols containing one chlorine per acyl group, no loss of HCl was observed upon plasma spray ionization.

Loss of a fatty acid and its sodium salt are typically observed upon CID of the sodiated ions of di- and triacylglycerols (Herrera et al., 2010; Hsu and Turk, 2010; Segall et al., 2004); however, the ESI(+)MS/MS spectra showed predominantly two losses of HCl (e.g., Figs. 1A and 2) from the sodium adduct ions of 9,10-dichlorooctadecanoic acid (**2**) and all chlorinated mono-, di-, and triacylglycerols (i.e., **6**, **9**, **11**, **13** and **14**) containing 9,10-dichlorooctadecanoyl groups in both terminal and central positions. The lithium adduct ion of **13** showed a similar pattern of HCl losses. The isotopic composition of the product ions reflected that of the ion selected within the isotopic cluster. For example, losses of two $H^{35}Cl$ and $H^{35}Cl + H^{37}Cl$ from the $^{35}Cl_3{}^{37}Cl$ isotopologue (m/z 967.7) of sodiated **14** (Fig. 1A) yielded product ions at m/z 895.5 and 893.5 with relative abundances matching the calculated ratio (i.e., 76:100 vs. 79:100 (Ramaley and Herrera, 2008)). Double losses of HCl also were observed as in-source fragmentations. Subsequent CID of the $[M+Na - 2HCl]^+$ ion of compound **14** (Fig. 1B) and the $[M+Na - 2HCl]^+$ and $[M+Na - 4HCl]^+$ ions of compound **16** also led to minor single and major double losses of HCl.

Similar minor single and major double losses of HBr were observed upon CID of sodiated tri-(9,10-dibromooctadecanoyl)

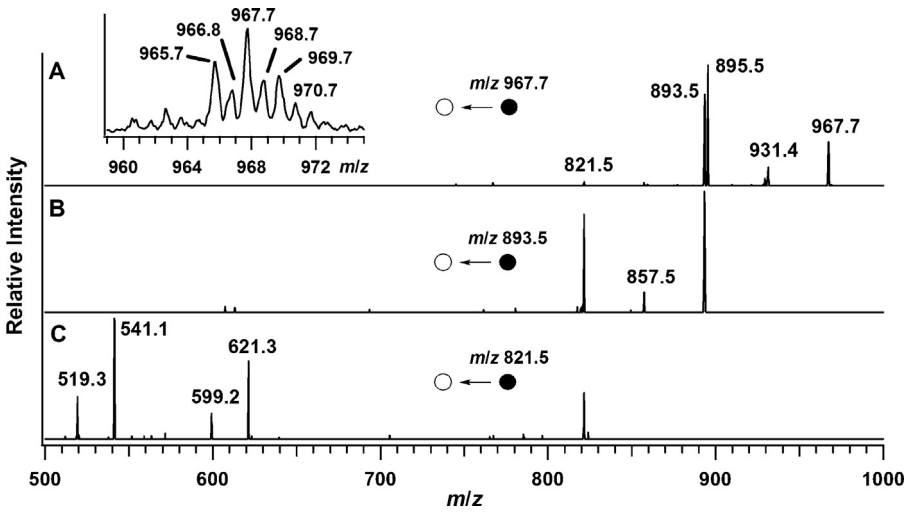


Fig. 1. Mass spectra of 1,2-di-(9,10-dichlorooctadecanoyl)-3-decanoylglycerol (**14**). A: CID (30%) spectrum of $[M+Na]^+$. B: CID (30%) spectrum of in-source generated $[M+Na - 2HCl]^+$. C: CID (36%) spectrum of in-source generated $[M+Na - 4HCl]^+$.

Table 1
Isotope clusters observed upon ESI(+)MS of the chlorinated compounds. The program Isotope Pattern Generator was used to provide the calculated relative intensities (Ramaley and Herrera, 2008).

Comp'd	$[M+Na]^+$ formula	*m/z* (Observed relative intensity, calculated relative intensity)						
		A	A+1	A+2	A+3	A+4	A+5	A+6
2	$C_{18}H_{34}Cl_2O_2Na$	375.3 (100,100)	376.3 (24,20)	377.3 (68,66)	378.3 (15,13)	379.2 (11,12)	–	–
6	$C_{45}H_{84}Cl_2O_6Na$	813.6 (100,100)	814.6 (49,50)	815.5 (73,77)	816.5 (31,34)	817.5 (20,19)	818.4 (9,7)	–
9	$C_{24}H_{44}Cl_2O_4Na$	489.3 (100,100)	490.1 (36,27)	491.3 (75,68)	492.1 (22,17)	493.1 (15,13)	–	–
11	$C_{21}H_{40}Cl_2O_4Na$	449.2 (100,100)	450.1 (28,23)	451.1 (68,67)	452.1 (16,15)	453.1 (22,12)	–	–
13	$C_{45}H_{84}Cl_2O_6Na$	813.5 (100,100)	814.5 (50,50)	815.5 (72,77)	816.5 (32,34)	817.4 (19,19)	818.5 (6,7)	–
14	$C_{51}H_{94}Cl_4O_6Na$	965.7 (71,69)	966.8 (44,39)	967.7 (100,100)	968.7 (52,52)	969.7 (58,57)	970.7 (30,27)	971.7 (20,17)
16	$C_{57}H_{104}Cl_6O_6Na$	1117.5 (60,47)	1118.6 (35,30)	1119.5 (100,100)	1120.5 (56,59)	1121.5 (95,91)	1122.5 (50,50)	1123.6 (48,47)

glycerol (**17**). Losses of HBr occurred at lower CID energies and isotopic clusters corresponding to $[M+Na]^+$, $[M+Na - 2HBr]^+$ and $[M+Na - 4HBr]^+$ were prominent in the electrospray mass spectrum. The relative peak intensities within each isotope cluster were consistent with calculated values (Ramaley and Herrera, 2008), and product ions with the expected isotopic compositions were obtained upon CID of the individual isotopologues of $[M+Na]^+$.

When all halogen atoms were lost by in-source fragmentation, then the expected neutral losses of acyl groups as fatty acids and their sodium salts (Herrera et al., 2010; Hsu and Turk, 2010; Segall et al., 2004) were observed (e.g., Figs. 1C and 2 (inset spectra)). The losses corresponded to dodecanoic acid and a doubly unsaturated C_{18} acid, the expected product formed upon elimination of two HCl. Preferred cleavage of acyl groups from the terminal position of sodiated triacylglycerol ions (Herrera et al., 2010; Hsu and Turk, 2010; Segall et al., 2004) allowed the isomeric triacylglycerols **6** and **13** to be distinguished by mass spectrometry (Fig. 2 inset spectra). For the symmetrical triacylglycerol **6**, the loss of a dodecanoyl group from either position 1 or 3 of was about four times more likely than the loss of the 9,10-dichlorooctadecanoyl group from the central position (Fig. 2B inset). On the other hand, the loss of a dodecanoyl group from position 1 or 2 of the unsymmetrical triacylglycerol **13** was favored by about 2:1 over the loss of the 9,10-dichlorooctadecanoyl group from position 3 (Fig. 2A inset). For comparison, the loss of a doubly unsaturated group from position 1 or 2 of the unsymmetrical triacylglycerol **14** with the complementary substitution pattern was also about twice as frequent as the loss of the dodecanoyl group from position 3 (Fig. 1C).

By contrast, HCl losses occurred readily from 9,10-dichlorooctadecanoyl substituents at both the central and terminal positions of sodiated triacylglycerols (Fig. 2) and from ions having only one fatty acid chain (i.e., the sodium adduct ions of **2**, **9** and **11**). The latter results identify the dichlorinated alkyl chain, one oxygen-containing functional group and a cation as the minimum common structural requirements and suggest that each double loss of HCl observed for the sodiated tetra- and hexachlorinated triacylglycerols **14** (Fig. 1) and **16** occur from a single 9,10-dichlorooctadecanoyl group. Also, the conjugated diene formed by two HCl losses from the same dichloroalkyl group provides another favorable binding site for the sodium ion and more favorable energetics than the formation of two separate double bonds.

A mechanistic hypothesis incorporating the minimum required structural features is presented in Scheme 3. Sodium ions bind to a wide range of functional groups (Hoyau et al., 1999; Rodgers and Armentrout, 2000), including chlorine in HCl, and some of the ions formed by ESI will be stabilized by interaction of the sodium ion with both a carbonyl oxygen and a chlorine at C9, or C10, in the same acyl group. The intervening alkyl chain in **18** is able to adopt a favorable chair-chair conformation, and the relative stereochemistry of the two chlorine atoms is fixed by the initial *anti* addition to a *cis* double bond (Section 3.1). Upon CID of **18**, C—Cl bond cleavage is assisted by sodium ion coordination and possible anchimeric assistance. With participation by the vicinal chlorine (Denmark et al., 2010), an intermediate chloronium ion (**19**) is formed, and abstraction of an acidic hydrogen from C8 and/or C11 by the proximate chloride ion facilitates double bond formation. Only the *cis* and *trans* product ions (**20** and **22**, respectively) resulting from loss of hydrogen at C11 are shown in Scheme 3. In the *cis* isomer **20**, interaction with the sodium ion weakens the C—Cl bond and increases the acidity of the allylic

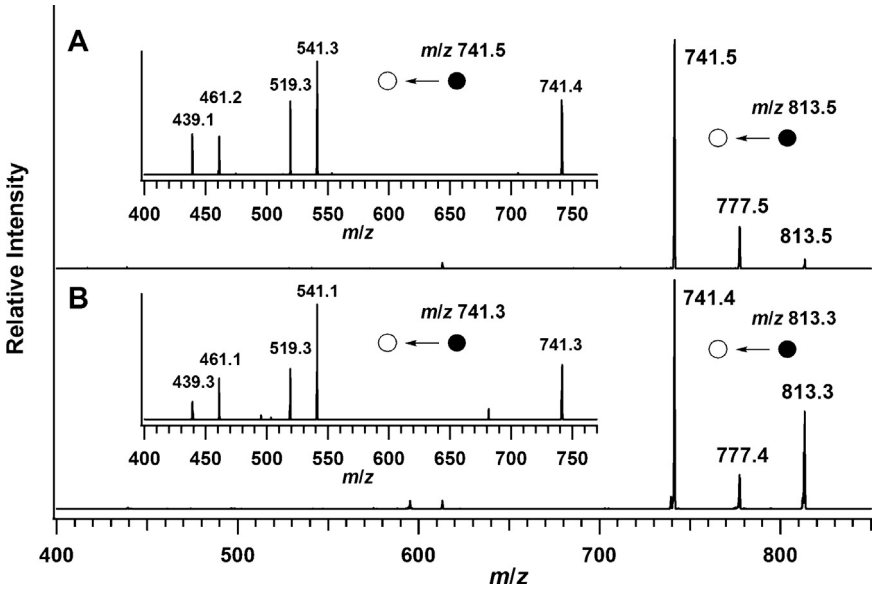
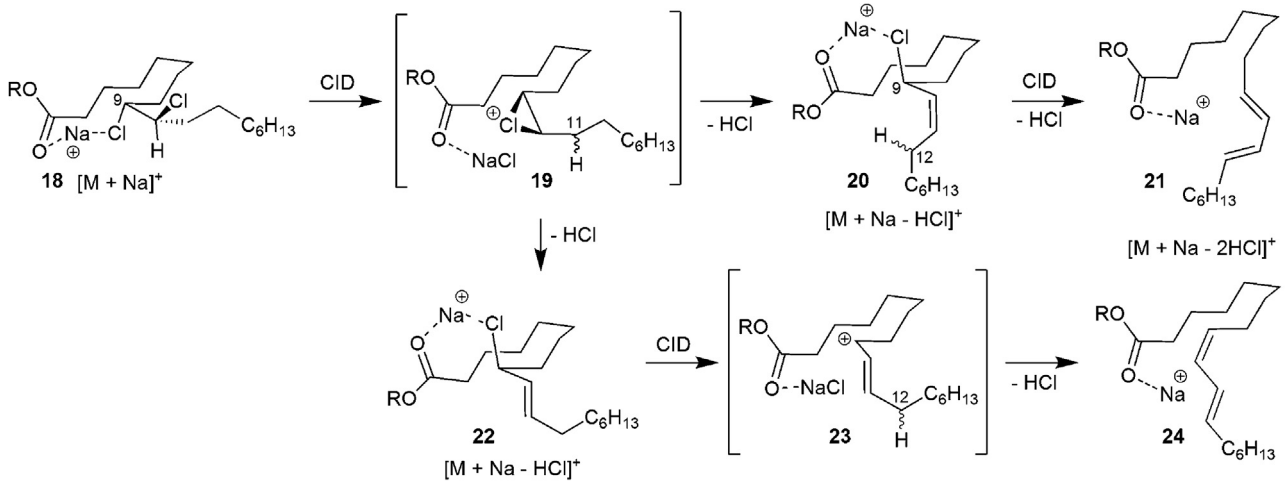


Fig. 2. CID spectra of A: 1-(9,10-dichlorooctadecanoyl)-2,3-didodecanoylglycerol (**13**, 33% CID) and B: 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoylglycerol (**6**, 36% CID). The CID (35%) spectra of the corresponding in-source generated $[M+Na-2HCl]^+$ ions are shown as insets.



Scheme 3. Mechanistic hypothesis for CID-initiated, sequential HCl losses from a 9,10-dichlorooctadecanoyl group, the common structural subunit in the sodiated ions formed from **2**, **6**, **9**, **11**, **13**, **14** and **16**. The two possible proton abstractions from C11 in **19** are shown. Analogous routes follow from C8 proton abstractions in **19** and from initial binding of the sodium ion to the chlorine at C10, rather than C9 as shown in **18**.

hydrogen at C12, which, in turn, promote HCl loss and conjugated diene (**21**) formation via an electrocyclic rearrangement. Alternatively, a conjugated diene product ion (e.g., **24**) could arise by allylic cation formation (e.g., **23**) and hydrogen abstraction from C12. The reactions presented in Scheme 3 are a subset of the possible routes arising from two initial binding sites for sodium ion with chlorine and the alternative sites for proton abstraction. A mixture of product ions differing in the position and stereochemistry of conjugated double bonds likely results from the observed HCl losses from 9,10-dichlorooctadecanoyl substituents (e.g., Figs. 1A, B and 2).

3.6. Conclusions

A series of halogenated triacylglycerols was synthesized using enzyme-catalyzed and carbodiimide-mediated coupling reactions. Little or no acyl migration was detected in the acylglycerol reaction products. Upon CID, the sodiated ions formed by ESI showed predominantly double losses of HCl (or HBr) in contrast to the typical losses of neutral fatty acids and their sodium salts from sodiated triacylglycerols. A mechanism was proposed for the double loss of HCl from a single acyl chain to yield a conjugated diene. The availablity of chlorinated triacylglycerols and their now documented characteristic mass spectral behavior will facilitate the development of LC-MS/MS methods for the quantitative determination of similar compounds in complex mixtures extracted from biological sources.

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