Isolation and Characterization of Vasoactive Lipids from the Needles of *Pinus ponderosa*

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Ingestion of ponderosa pine needles by late-pregnant cattle causes premature parturition induced by a profound and progressive decrease in uterine blood flow. *In vitro* perfused placentomes obtained from late-pregnant cows were used in the bioassay-guided fractionation of *Pinus ponderosa* pine needles to isolate a new class of vasoactive lipid substances. Chromatographically inseparable mixtures of active compounds were isolated by Soxhlet extraction and flash column chromatography. The structures of intact myristate and/or laurate diesters of 1,14-tetradecanediol or 1,12-dodecanediol in these extracts were determined by ^1H and ^{13}C NMR, IR, and high- and low-resolution FAB mass spectrometric analyses. Saponification of small samples of isolated diesters, derivatization, and GC/MS verified major components as lauric and myristic acids and 1,14-tetradecanediol. Structures of individual components of mixtures of lipids were confirmed by MS–MS analysis.

Keywords: *Vasoactive lipids; Pinus ponderosa*

INTRODUCTION

Pinus ponderosa (Pinaceae) is abundant in the western and midwestern United States and in western Canada (U.S. Department of Agriculture, 1964). Needles from *P. ponderosa* consumed during late gestation cause cattle to abort (McDonald, 1969; Stevenson et al., 1972; James et al., 1977; Short et al., 1992). *P. ponderosa* ingestion induces premature parturition in cattle by causing prolonged vasoconstriction (i.e., increased vascular tone) of the caruncular arterial bed partially through increases in potential sensitive calcium channel (PSC) activity (Ford et al., 1992; Christenson et al., 1993) resulting in a decrease in uterine blood flow. In pregnancy, blood flow to the gravid bovine uterus increases about 40-fold from conception to term (Ferrell and Ford, 1980). After day 200, 80–85% of the uterine arterial blood flows through the caruncular arterial bed (Macowski et al., 1968) as a consequence of marked decreases in caruncular arterial tone (Ford, 1994). It is believed that components in pine needles increase the tone of the caruncular artery, resulting in a reduction of blood flow to the fetal–maternal interface. Decreases in uterine arterial blood flow appear to result from activation of α2-adrenergic receptors on the vascular smooth muscle membrane of the artery (Ford, 1989; Ford et al., 1992). Specifically, α2 activation facilitates extracellular uptake of calcium via PSC, resulting in decreased vessel diameter (i.e., increased vessel tone) and uterine blood flow.

P. ponderosa is the only known species of *Pinus* to cause abortion in cattle (Pammel, 1911; James et al., 1989; Allison and Kitts, 1964), resulting in large economic losses each year to the beef industry (Lacey et al., 1988). Green and dry needles appear to cause abortion (Jensen et al., 1989), and bark and branch tips appear to contain abortifacient principles (Panter et al., 1990). Antiestrogenic activities of pine needle water extracts have been shown in mice (Cook and Kitts, 1964; Cook, 1960) but not in sheep (Call and James, 1978). Water-insoluble toxins have been implicated (Anderson and Lozano, 1979; James et al., 1994). Chemical constituents of *P. ponderosa* include volatile terpenes, terpene acids and resins, and others (James et al., 1989, 1994). Terpene resins have been implicated in embryotoxic effects in mice (Kubic and Jackson, 1981). Isocupressic acid identified in *P. ponderosa* (Zinkel and Magee, 1991) induced early parturition in pregnant cattle (Gardner et al., 1994), thus identifying one abortifacient principle in ponderosa pine needles. Even luteolytic agents such as prostaglandins or mycotoxins and the presence of infectious microorganisms have been suggested as causative agents in pine needle abortion in cattle (Adams et al., 1979).

Many plant leaves have surfaces of epicuticular wax with inner cuticular membranes composed of cutin (James et al., 1977; Kalviainen et al., 1985; Franich and Volkman, 1982; Dayal et al., 1989; Hu et al., 1988). Cutin waxes are complex polymer mixtures of very similar compounds that are difficult to isolate and characterize chemically and spectrally. Crude samples of cutin waxes are usually obtained by solvent extraction, and component acids and alcohols are identified only after alkaline hydrolysis (saponification), methylation of the resulting fatty acids, and GC/MS analyses (Kalviainen et al., 1985; Franich and Volkman, 1982). Mixtures of 12-hydroxydodecanoic acid, 14-hydroxytetradecanoic acid, 16-hydroxyhexadecanoic acid, and dihydroxyhexadecanoic acids were obtained from *Pinus* species in this manner (Franich and Volkman, 1982; Dayal et al., 1989; Hu et al., 1988).

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This paper provides the first evidence that lipid diesters of 1,14-tetradecanediol and 1,12-dodecanediol and lipid triesters comprised of alkanediols, ω-hydroxy fatty acids, and fatty acids may play a role in causing early parturition in pregnant beef cattle ingesting *P. ponderosa* needles.

MATERIALS AND METHODS

Chemicals. Bis(trimethylsilyl)trifluoroacetamide (BSTFA), boron trifluoride (BF_3), lauric acid, myristic acid, palmitic acid, 10-hydroxydecanoic acid, methyl laurate, methyl myristate, methyl palmitate, dodecan-1-ol, 1,12-dodecanediol, and 1,14-tetradecanediol were all from Aldrich Chemical Co., Milwaukee, WI. The purities of fatty acids and alcohols and their identities were confirmed by thin layer chromatography (TLC), gas chromatography (GC), and mass spectrometry (MS) before use. Common solvents used include methylene chloride (CH_2Cl_2), methanol (CH_3OH), acetonitrile (CH_3CN), ethanol (EtOH), hexane (C_6H_{14}), benzene (C_6H_6), and diethyl ether (Et_2O).

Plant Material. *P. ponderosa* needles were collected in Custer County, Montana, in the winter of 1989.

General Experimental and Equipment. Infrared (IR) spectra were obtained using a Nicolet 205 FT-IR spectrometer connected to a Hewlett-Packard ColorPro plotter.

Chemical ionization mass spectral (CIMS) analyses were obtained using a Nermag R 1010c instrument. Fast atom bombardment (FAB) experiments were performed on a ZAB-HF reversed geometry (BE configuration, where B is a magnetic sector and E is an electrostatic analyzer) mass spectrometer (MS) (VG Analytical, Inc.). It is equipped with an Ion Tech saddle-field FAB gun and commercial FAB ion source. Samples were bombarded with 8 keV of Xe atoms at an atom gun current of 1.5 mA. 3-Nitrobenzyl alcohol (Aldrich) (3-NBA) and Magic Bullet or 5:1 dithiothreitol/dithioerythritol (Sigma) were the FAB matrices used. Samples were dissolved in methylene chloride, and then 1 μL was added to the matrix on the FAB probe tip (Adams, 1990; Jensen and Gross, 1987). Tandem mass spectrometry experiments (MS-MS) were also performed on the ZAB-HF MS. The technique of mass-analyzed ion kinetic energy spectrometry (MIKES) was used to detect the unimolecular ion decompositions in the region between B and E. A particular precursor ion was selected by the magnet and, by scanning E, product ions that were formed by unimolecular ion decompositions in this region can be observed. The MS-MS spectra are the result of averaging 8-10 scans using VG software.

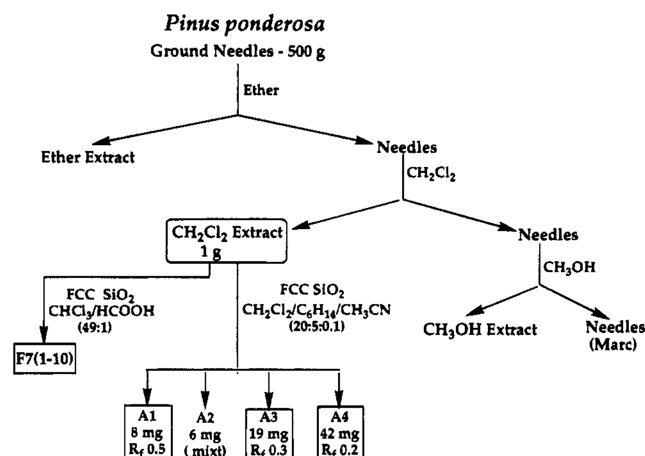
Nuclear magnetic resonance (NMR) spectra were obtained on Bruker NM 360 MHz and Varian NMR 500 MHz high-field spectrometers equipped with an IBM Aspect 2000 processor and with VNMR software version 4.1b, respectively. ^1H-(360.134 and 499.843 MHz) and ^{13}C-NMR (90.15 and 125.697 MHz) spectra were recorded using tetramethylsilane ($\delta = 0$) or solvent peaks as internal standards.

Chromatography. TLC was performed on 0.25 mm layers of silica gel GF_{254} (Merck) prepared on 5 × 20 cm or 20 × 20 cm glass plates with a Quikfit Industries spreader (London, U.K.). Plates were air-dried and activated at 120 °C for 1 h prior to use. Plates were developed in a solvent mixture of $CH_2Cl_2/C_6H_{14}/CH_3CN$ (20:5:0.1 v/v/v), and developed chromatograms were visualized by spraying with a solution of H_2SO_4/EtOH (1:5 v/v) before warming with a heat gun to develop black spots. Flash column chromatography (FCC) was performed using J. T. Baker glassware with 40 μm silica gel (Baker) as the stationary adsorbent phase. Solvent compositions similar to those described for TLC were used in the elution of samples from flash columns.

Pine Needle Fractionation. *P. ponderosa* pine needles were milled using a Fitzpatrick Model D hammer mill (The Fitzpatrick Co., Elmhurst, IL) at medium speed with knives forward and fitted with a No. 8 mesh stainless steel screen.

For Soxhlet extractions, 500 g of milled pine needles was exhaustively extracted in a Soxhlet apparatus with 2.5 L each of Et_2O, CH_2Cl_2, and CH_3OH. Extracts were filtered and concentrated under reduced pressure to give the following amounts of extracts: Et_2O, 40 g; CH_2Cl_2, 12 g; and CH_3OH, 80 g (Scheme 1).

Scheme 1. Extraction and Chromatographic Resolution of Extracts of *P. ponderosa*



Chromatographic Fractionation of CH_2Cl_2 Extracts. Chromatographic purification was achieved by adsorbing 1 g of CH_2Cl_2 extract onto 80 g of silica gel (40 μm) held in a 2.5 cm × 60 cm column and eluting stepwise with 500 mL of hexane, mixtures of $CH_2Cl_2/C_6H_{14}/CH_3CN$ beginning with 20:20:0.1 (v/v/v) to 20:1:0.1 (v/v/v), CH_2Cl_2/CH_3CN (20:0.1 v/v), and 20 and 50% CH_3OH in CH_2Cl_2. TLC ($CH_2Cl_2/C_6H_{14}/CH_3CN$, 20:5:0.1 v/v/v) analysis showed the following fraction compositions: fractions 27-33 (A1), R_f 0.5, 8 mg; fractions 34-37 (A2), 6 mg (mixture); fractions 38-49 (A3), R_f 0.3, 19 mg; fractions 64-135, (A4), R_f 0.2, 42 mg; fractions 140-475 (A5), a mixture of spots at R_f 0.5, 0.15, and 0.01 with $CH_2Cl_2/C_6H_{14}/CH_3CN$ (20:2:0.5 v/v/v), 117 mg; and fractions 500 or higher (A6), 487 mg (mixture). All fractions except (A6) and later fractions which eluted with 20 and 50% CH_3OH in CH_2Cl_2 appeared as white crystalline solids.

Sample Hydrolysis, Methylation, and O-Trimethylsilylation for GC/MS Analysis. For hydrolysis and derivatization, samples of approximately 1 mg were dissolved in 2 mL of 5% methanolic KOH and refluxed for 10 h. Mixtures were evaporated to dryness under N_2, 2 mL of H_2O was added to each residue, and the resulting aqueous mixtures were each extracted with CH_2Cl_2 (2 × 2 mL). Typically, this CH_2Cl_2 extract was dried over anhydrous Na_2SO_4 and evaporated to give 0.5 mg of neutral substances. The remaining aqueous phases were acidified with 2 N HCl, extracted with CH_2Cl_2 (2 × 2 mL), dried over anhydrous Na_2SO_4, and evaporated to typically give 0.4 mg of acidic substances. These neutral and acidic substances were subjected to derivatization for GC/MS analysis.

a. Methylation. The acid fraction after saponification was dissolved in 0.4 mL of HPLC grade CH_3OH, mixed with 1 mL of BF_3/CH_3OH, and refluxed for 2-3 h. The solvent was removed under N_2, 1 mL of distilled H_2O was added, and the aqueous mixture was extracted with CH_2Cl_2 (2 × 1 mL). After drying over anhydrous Na_2SO_4, rotary evaporation gave methylated products for GC analysis or further derivatization with BSTFA.

b. O-Trimethylsilylation. This was accomplished by adding 35 μL of BSTFA to a solution of 0.7 mg of neutral substances in 1 mL of CH_2Cl_2 or 20 μL of BSTFA to a solution of 0.4 mg of methylated acidic compounds in 1 mL of CH_2Cl_2. The reactants were shaken for 20-30 min before samples were directly subjected to GC/MS analysis without further workup.

c. GC/MS Analysis. GC was routinely performed with a Hewlett-Packard 5890A gas chromatograph equipped with a fused silica capillary (SPB-5) column with a bonded poly (5% diphenyl/95% dimethylsiloxane) phase, 30 m × 0.32 mm i.d., 0.20 μm film thickness (Supelco Inc., Bellefonte, PA), linked to a Hewlett-Packard 3390A integrator. Nitrogen was used as carrier and makeup gas at flows of 30 and 10 mL/min, respectively, and eluting compounds were detected by flame

ionization detection (FID). Column, injector, and detector temperatures were maintained at 180–210 (5 °C/min), 220, and 300 °C, respectively. Both column head carrier gas and hydrogen pressures were held at 35 psi. We used this method to evaluate the structures of hydroxy and oxo fatty acids derived from the hydration and subsequent oxidation of oleic acid (El-Sharkawy et al., 1992). Low-resolution MS were obtained either by direct inlet probe sample admission or by GC using a methyl silicone (DB-1) column (50–250 °C, 20 °C/min) in a Trio 1 mass spectrometer linked to a Hewlett-Packard 5890A GC.

Bioassays. Samples (5–10 mg) of extracts or chromatographic fractions were triturated into 50 mL of bovine plasma in a glass mortar. Samples were diluted with bovine plasma before being used in the placentome bioassay.

Placentomes were collected at a commercial slaughter facility from the gravid uterine horn of late-pregnant cows between 240 and 270 days of gestation as determined by measurement of fetal crown rump length (Evans and Sack, 1973). At the laboratory, placentomes were placed in an open perfusion chamber which was submerged in oxygenating Krebs–Ringer solution, the caruncular artery was connected to polyethylene tubing, and the artery was gently perfused to remove blood from the vascular tree. The chamber was then sealed, and a Harvard variable-speed peristaltic pump was used to deliver continuously oxygenated Krebs–Ringer solution at 37 °C. The extralumenal flow was maintained at 10 mL/min, and the intraluminal flow to each artery was maintained at 5 mL/min to achieve intraarterial pressures of approximately 80 mmHg as measured by Statham pressure transducers and recorded on a Hewlett-Packard 7700 chart recorder. A pulse similar to that seen in the live animal was imposed on the intraarterial flow with the use of a physiological perfusion pump (Medical Engineering Consultants, Los Angeles, CA). A 0.5 h equilibration period was allowed before the start of sample perfusions which allowed the placentome to establish a constant baseline perfusion pressure. Ponderosa pine needle extracts or chromatographic fractions dissolved in plasma were infused into the caruncular artery preparation at a rate of 0.5 mL/min with a Harvard dual syringe constant infusion pump (Conley, 1986). In general, two placentomes were perfused first for 20 min with vehicle, then samples with increasing concentrations of compounds or extracts for 20 min each, and then vehicle for 20 min. Vascular tone was estimated as the perfusion pressure established by the placentome preparation by the end of each 20 min perfusion period. At the end of each 20 min perfusion period (vehicle and samples), perfusion pressure was recorded. Then, a depolarizing bolus dose of 0.2 M KCl (200 μL) was injected into the intraluminal flow (5 mL/min), and the resulting increased perfusion pressure was used to estimate the maximal PSC activity as previously described (Christenson et al., 1993). Each sample's vasoactivity was determined by comparing the measurements of vascular tone and PSC activity at the end of its 20 min perfusion. Baseline values were determined by averaging values at the end of both 20 min vehicle perfusion periods. Each sample was tested simultaneously in two placentomes on each experimental day, and each sample was evaluated on at least 2 days.

RESULTS AND DISCUSSION

Early calving following pine needle consumption is accompanied by a profound constriction of caruncular arteries and ischemic necrosis at the placental attachment site (Stuart et al., 1989). The placentome preparation is the only *in vitro* system that allows the study of the intact caruncular arterial bed, allowing investigations of vasoactive compounds in ponderosa pine needle extracts (Ford et al., 1992). Although our initial placentome assays were plagued by irreproducibility caused largely by the use of solvent vehicles needed to solubilize lipophilic pine needle extracts, we discovered that when samples were dissolved in bovine plasma, placentome bioassay results were reproducible and useful in detecting vasoactive substances in complex extracts and chromatographic fractions.

Table 1. Vasoactivity Evaluation of Pine Needle Fractions in the Placentome Bioassay

fraction	μg/mL perfused	% increase in BPP[a] at end of 20 min	% response to KCl above BPP
F7(1–10)	10	417	362
	5	28	233
	2.5	28	40
A1	10	93	150
	5	43	50
	2.5	20	20
A3[b]	10	50	253
	5	22	138
	2.5	19	35
A4	10	23	5
A6	10	20	18

[a] BPP, baseline perfusion pressure. [b] A2 and A5 were not tested because they were mixtures.

Extraction and Isolation of Vasoactive Lipids. For Soxhlet extraction, the best system involved sequential contacts with Et_2O, CH_2Cl_2, and CH_3OH (Scheme 1) to obtain relatively large yields of pine needle extractables. Et_2O removes relatively nonpolar substances in 8% (w/w) yield, CH_2Cl_2 gave 2.4% yield of a more polar fraction, while CH_3OH gave 16% yield (w/w) of the most polar mixture of components. In all preliminary placentome assays, CH_2Cl_2 extracts and chromatographic subfractions of the CH_2Cl_2 extracts were active in increasing PSC activity and vascular tone of the caruncular artery. *P. ponderosa* CH_2Cl_2 extracts were colorless, semicrystalline mixtures (TLC) of components. Fractions A1–A6 and a mixture known as F7(1–10) were obtained by FCC (Scheme 1) and subjected to placentome bioassays.

Bioassay Results. The results of placentome bioassays are presented in Table 1. F7(1–10) gave significant increases in both perfusion pressure (tone) and PSC activity when perfused through the placentome at 10 μg/mL. When diluted to 5 μg/mL, this sample exhibited little effect on tone, but the increase in PSC activity remained remarkably high (233%). At the lowest dose of 2.5 μg/mL, little response to either measure of vasoactivity was observed. Fraction A1 caused dose-related increases in both tone and PSC activities of the vessels, while A3 primarily increased the KCl response, indicating a more specific effect on PSC activity. Neither fraction caused increases in vascular tone or PSC activity as high as those observed with F7(1–10). The results suggest that mixtures of compounds affect **both** the tone (i.e., vessel diameter) and PSC activity of the uterine vasculature and that the effects of these compounds are synergistic in nature. By spectral and chromatographic analysis, these samples contained no diterpene acids such as isocupressic acid. Fractions A4 and A6 showed little activity, and samples A2 and A5 were not assayed because they were mixtures. The bioassay active fractions (A1 and A3) were subjected to spectral and chemical analysis.

LRFAB MS Analysis of Fraction F7(1–10). Because fraction F7(1–10) was a complex mixture of substances, it was not subjected to complete spectral analysis. However, the LRFAB MS of F7(1–10) in 3-NBA saturated with LiI as shown in Figure 1 shows prominent ions at m/z 573 (m/z 566 + Li), 601 (m/z 594 + Li), 629 (m/z 622 + Li), and 657 (m/z 650 + Li). These ions are also the primary components found in fraction A1. Lower intensity ions at m/z 771 (m/z 764

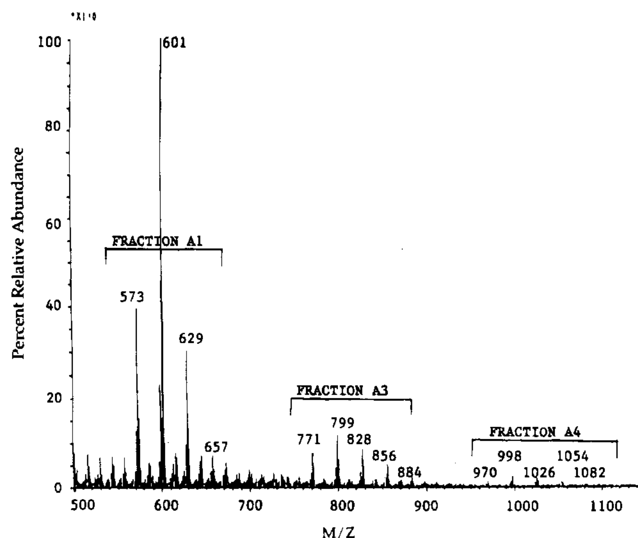


Figure 1. Ion clusters in the low-resolution FAB-MS (matrix 3-NBA-Li) of *P. ponderosa* fraction F7(1−10) indicating mixtures of similar compounds.

+ Li), 799 (m/z 792 + Li), 828 (m/z 821 + Li), and 856 (m/z 849 + Li) were observed. These are the primary ions found in fraction A3. Those found at m/z 970, 998, 1026, 1054, and 1082 were the primary components in the bioassay inactive fraction A4. From this spectrum it is apparent that there are at least three groups of similar components contained in F7(1−10). The relative amounts of these cannot be directly deduced from the relative intensities of molecular ions in the spectrum, because each group of compounds displays molecular ions of different intensities.

Spectral Analyses of Fraction A1. Fraction A1 was subjected to IR, ^{1}H- and ^{13}C-NMR, and MS analyses. The IR spectrum showed strong ester absorption at 1735 cm^{-1}, but neither sharp nor broad bands between 3000 and 3600 cm^{-1}, indicating the absence of free COOH or OH groups. ^{1}H NMR (Figure 2A) revealed a 4-proton triplet signal at 4.05 ppm (−$\mathbf{CH_2}$-OCO−), a 4-proton triplet at 2.28 ppm (−$\mathbf{CH_2}$COO−), an 8-proton multiplet at 1.6 ppm (−$\mathbf{CH_2}CH_2$COO− and −$\mathbf{CH_2}CH_2$OCO), a large 56-proton singlet at 1.26 ppm for numerous overlapping −CH_2− functional groups typical of those found in fatty acids, and a 6-proton triplet at 0.88 ppm representing terminal methyl groups. The ^{13}C-NMR spectrum (Figure 2B) exhibited signals for ester carbonyl carbons (174.02 ppm), ether carbons (64.38 ppm), and methyl carbons (14.12 ppm) together with numerous methylene group carbon signals.

LRFAB MS with Magic Bullet as matrix (Figure 3) indicated the presence of three major molecular ions of m/z 595.5 $(M_1 + H)^+$, 623.5 $(M_2 + H)^+$, and 651.6 $(M_3 + H)^+$. Confirming results were obtained by cationizing with lithium, which resulted in $(M + Li)^+$ ions of m/z 601.5, 629.6, and 657.5, respectively. These MS results indicated that the chromatographically pure material was a mixture of at least three major compounds. Assuming that relative intensities of the molecular ions reflect the actual concentrations of the three components in fraction A1, the major compound was that of m/z 623 (48%). Ions of m/z 595 and 651 represented 34 and 18% of fraction A1, respectively. The HRFAB analyses for $(M_1 + 1)^+$ and $(M_2 + 1)^+$ ions resulted in masses of m/z 595.5674 for $C_{38}H_{75}O_4$ (theoretical 595.5665) and m/z 623.5926 for $C_{40}H_{79}O_4$ (theoretical 623.5977), respectively. The $(M + H)^+$ ion of m/z 651.6 can be regarded as possessing two additional methylene groups in its structure for $C_{42}H_{83}O_4$.

Additional ions in the MS indicated that each of the major components fragment and lose units of m/z 182, which result in the formation of ions of m/z 413, 441, and 469, respectively. Another major fragmentation pathway produces low mass ions of m/z 183, 201, 211, and 229. HRFAB experiments have confirmed that ions of m/z 183 and 211 can be attributed to the acylium ions of lauric and myristic acids, respectively. The identities of ions of m/z 201 and 229 were confirmed by HRFAB analyses to be protonated lauric and myristic acids, respectively. These MS data suggested that the structures of compounds contained in A1 consisted of fatty acid diesters of alkanediols.

Tandem mass spectrometry experiments (MS−MS) were used to further analyze these compounds. MIKES scans were used to observe and characterize the unimolecular ion decompositions of the above $(M + H)^+$ ions (Adams, 1990; Jensen and Gross, 1987). The precursor ion of m/z 595.5 (Figure 4) decomposed to produce ions of m/z 578 via loss of H_2O, m/z 413 via loss of 183 or $C_{12}H_{23}O$, and m/z 395 via loss of 201 or loss of H_2O from the ion of m/z 413 ($C_{12}H_{25}O_2$). The low mass product ions include m/z 183, 195, and 201. The fragment ion of m/z 195 is thought to arise by loss of two dodecanoic (lauric) acid fragments from a tetradecanediol diester.

The parent ion at m/z 623.6 gave m/z 605 by loss of H_2O, m/z 441 by loss of m/z 183 or $C_{12}H_{23}O$, m/z 423 by loss of m/z 201 fragment or $C_{12}H_{23}O_2$, m/z 413 by loss of m/z 211 or $C_{14}H_{27}O$, m/z 395 by loss of m/z 229 or $C_{14}H_{27}O_2$, and m/z 195 by loss of both dodecanoic (lauric) and tetradecanoic (myristic) acids from a tetradecanediol diester.

The precursor ion at m/z 651.6 gave a much more complex and noisy MS−MS spectrum, indicating that this ion actually consisted of a mixture of different compounds possessing the same molecular mass. Useful information from this spectrum showed loss of H_2O to give m/z 635 and numerous other ions as well. Losses of fragments of masses of 183 for $CH_3(CH_2)_{10}$-CO from lauric acid gave m/z 469, of 211 for $CH_3(CH_2)_{12}$-CO from myristic acid gave m/z 441, and of 239 for $CH_3(CH_2)_{14}$CO from palmitic acid gave m/z 413. Ions at m/z 396, 422, and 450 can be explained by losses of palmitate [$CH_3(CH_2)_{14}$COOH, m/z 256], myristate [$CH_3(CH_2)_{12}$COOH, m/z 229], and laurate [$CH_3(CH_2)_{10}$-COOH, m/z 200] from the molecular ion. At the lower end of the MS−MS spectrum, fragment ions for protonated palmitic acid (m/z 257), myristic acid (m/z 229), and lauric acid (m/z 201) were observed along with their accompanying m/z 239, 211, and 183 fragments, respectively. These results confirmed the presence of these three fatty acids as parts of the structures in the mixture giving rise to m/z 652. Noteworthy in this spectrum was the presence of two fragment ions at m/z 195 and 223, typical for ions arising from 1,14-tetradecanediol and 1,16-hexadecanediol, respectively.

Fraction A1 was subjected to alkaline saponification to confirm the composition of presumed diesters contained in this mixture of lipids. Neutral and acidic fractions were trimethylsilylated and methylated, and the derivatized components of A1 were subjected to GC/MS comparisons with standard compounds (Eglinton et al., 1968). Table 2 lists GC retention times and key fragments obtained for fatty acid methyl esters and trimethylsilylated alkanols and α,ω-alkanediol standards. From the total ion current chromatogram, and

Figure 2. NMR spectra of fraction A1: (A) ^1H NMR ($CDCl_3$) at 360 MHz; (B) ^{13}C NMR ($CDCl_3$) at 90.5 MHz.

fragmentation patterns, the major neutral components were identified as 1,14-tetradecanediol and 1,16-hexadecanediol (Figure 5A). The acidic fraction (Figure 5B) contained methyl esters of lauric acid, myristic acid, and palmitic acid in relative proportions of 1:4:1 and 1,14-tetradecanediol and 1,16-hexadecanediol in relative proportions of 4.6:1. Peaks with R_T at 10.8 min are unknown artifacts that appeared in the GC chromatograms of both unknown and authentic standards derivatized in this work.

The results from MS and from hydrolysis and derivatization confirmed that the structures of compounds found in fraction A1 are esters of lauric, myristic, and/or palmitic acids esterified with 1,14-tetradecanediol and/or 1,16-hexadecanediol. The MS–MS results confirmed the presence of 1,14-tetradecanedioyl dilaurate (12:14:12) (**2**), and 1,14-tetradecanedioyl 1-laurate 14-myristate (12:14:14) (**3**) for molecular ions at m/z 595 and 623. (A convenient shorthand method for indicating the structures of these compounds has been devised to

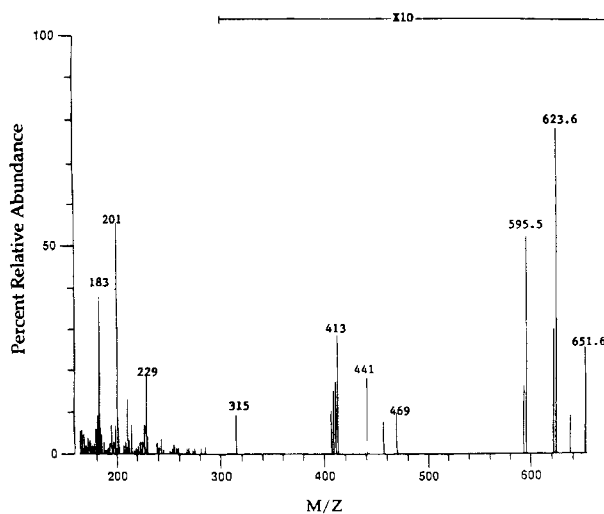


Figure 3. FAB MS spectrum (3-NBA) of fraction A1.

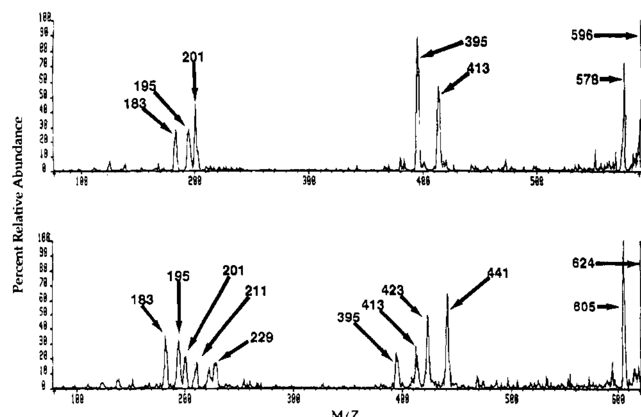


Figure 4. Metastable ion spectra of fraction A1 peaks m/z 596 and 624.

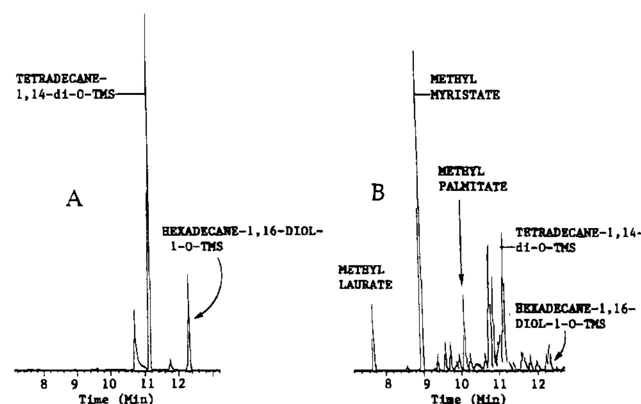


Figure 5. GC total ion current chromatograms of neutral (A) and acidic (B) fractions obtained by saponification of fraction A1 and derivatization with BSTFA and CH_3OH/BF_3.

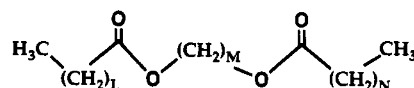
Table 2. GC Retention Times and Key MS Fragments of Fatty Acid Methyl Esters, Trimethylsilylated Alkanols, α,ω-Alkanediols, and TMS-ω-Hydroxyhexadecanoic Acid Methyl Ester

compound	m/z (M^+)	GC retention time (min)	key MS fragments m/z
methyl esters			
lauric (C_{12})	214	7.75	183, 74
myristic (C_{14})	242	8.98	211, 74
palmitic (C_{16})	270	10.11	239, 74
TMS derivatives			
dodecan-1-ol	258	8.12	243, 227, 185, 168, 75, 73
tetradecan-1-ol	286	9.28	271, 255, 213, 196, 75, 73
hexadecan-1-ol	314	10.35	299, 283, 224, 75, 73
octadecan-1-ol	342	11.34	327, 311, 75, 73
1,12-dodecanediol	346	10.05	315, 241, 75, 73
1,14-tetradecanediol	374	11.14	343, 284, 269, 75, 73
TMS, methyl ester			
16-hydroxyhexadecanoic acid	358	11.2	343, 284, 269, 75

indicate the numbers of carbon atoms on the acid components flanking α,ω-alkanediols. Thus, 12:12:12 refers to 1,12-dodecanedioyl dilaurate, for example.) The ion at m/z 651 is a mixture of compounds of isomers. On the basis of saponification and MS−MS analyses, there are only three possible combinations of fatty acid esters with alkanediols that match m/z 651. These are 1,14-tetradecanedioyl dimyristate (14:14:14) (**4**), 1,14-tetradecanedioyl laurate palmitate (12:14:16) (**5**), and 1,16-hexadecanedioyl laurate myristate (12:16:14) (**6**). The structures of these compounds are shown in Figure 6.

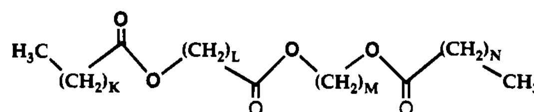
A separate isolation and analysis of A1 yielded a MS slightly different from that originally seen for A1. The major ions at m/z 595, 623, and 651 were evident, albeit

A-1 Components



	L	M	N	Shorthand Notation
1-	10	12	10	12:12:12
2-	10	14	10	12:14:12
3-	10	14	12	12:14:14
4-	12	14	12	14:14:14
5-	10	14	14	12:14:16
6-	10	16	12	12:16:14

A-3 Components



	K	L	M	N	Shorthand Notation
7-	10	15	14	12	12:16:14:14
8-	10	15	16	10	12:16:16:12
9-	10	15	14	10	12:16:14:12
10-	10	15	12	10	12:16:12:12
11-	10	13	12	10	12:14:12:12

Figure 6. Structures of compounds identified in fractions A1 and A3.

in different proportions from the previous A1 fraction. A new ion at m/z 568 consistent for the structure 1,12-dodecanedioyl dilaurate (12:12:12) (**1**) ($C_{36}H_{70}O_4$) was observed. MS−MS of this ion gave m/z 550 ($C_{36}H_{68}O_3$) by loss of H_2O, m/z 385 by loss of m/z 183 or $C_{12}H_{23}O$, m/z 367 by loss of m/z 201 or $C_{12}H_{23}O_2$, and m/z 168 for $C_{12}H_{24}$ derived from 1,12-dodecanedioyl dilaurate which eliminates the two ester fragments. This result confirmed the presence of the three compounds identified earlier in fraction A1 and an additional derivative dodecanedioyl dilaurate (**1**) in a second A1 fraction. The results also underscore the complexities in characterizing mixtures of highly similar lipid substances from pine needle extracts.

Spectral Analyses of Fraction A3. The IR and ^1H- and ^{13}C-NMR spectra of fraction A3 were essentially the same as for A1. By ^1H NMR, differences were found in the relative proton integrations for signals grouped at 4.05, 2.28, 1.60, 1.26, and 0.88 ppm. Interestingly, the triplet signal at 0.88 ppm, which represents end-chain methyl groups, was smaller than it was in the ^1H NMR spectrum of A1. The ^1H-NMR spectrum also indicated that relatively simple and terminally oxygenated alkanes and fatty acids were likely components of fraction A3. The ^1H-NMR results would rule out the presence of secondary alcohols or ether functional groups in the components of this fraction. The ^{13}C-NMR spectrum clearly indicated the presence of ester, numerous methylene, $-OCH_2CH_2-$, and terminal methyl group signals like A1.

For A3, LRFAB (Magic Bullet) (Figure 7) gave ions of m/z 793.7 $(M_1 + H)^+$, 821.7 $(M_2 + H)^+$, 849.7 $(M_3 + H)^+$, and 877.8 $(M_4 + H)^+$. MS results indicated that A3 was a mixture of at least four major compounds, each apparently differing in mass by two CH_2- units from one another. HRFAB in 3-NBA (Magic Bullet) matrix gave m/z 821.7584 for $C_{52}H_{101}O_6$ $(M + 1)^+$ (theoretical 821.7597). This compound differs in mass by $C_{12}H_{22}O_2$ versus the major compound in fraction A1 at m/z 623 for $C_{40}H_{79}O_4$ and identified as 1,14-tetradecanedioyl laurate myristate (12:14:14) (**3**). The difference in

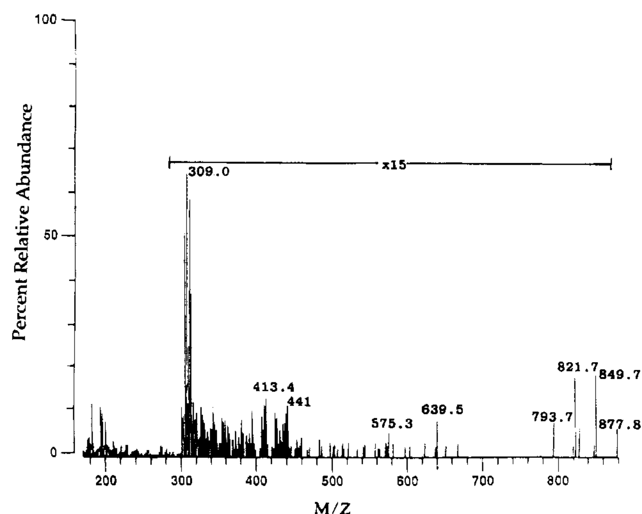


Figure 7. FAB MS spectrum (3-NBA) of fraction A3.

structure can be explained by the presence of an ω-hydroxy fatty acid.

Saponification of A3 and GC/MS of the resulting O-trimethylsilylated neutral CH_2Cl_2 extract indicated that 1,14-tetradecanediol-di-O-TMS was the major (90%) neutral component with about 10% of 1,16-hexadecanediol-1-O-TMS. These results were identical to those obtained with authentic compounds and seen earlier with fraction A1 (Figure 5). The acidic extract of A3 gave total ion chromatogram peaks and fragmentation patterns consistent with the presence of methyl myristate, methyl palmitate, *O*-trimethylsilyl-ω-hydroxyhexadecanoic acid methyl ester, 1,14-tetradecanediol-di-O-TMS, and 1,16-hexadecanediol-1-O-TMS (Table 2). The major difference in the acidic fraction of A3 was the presence of a major peak (about 90% of acids) (R_T = 11.2 min) matching with the O-TMS derivative of 16-hydroxyhexadecanoic acid methyl ester with ions of m/z 343 = (M − 15), 284 = (M − 74), 269 = (M − 15 − 74), 75, 74, and 73.

MS data and derivatization of saponified samples suggest the presence of much more complex components in fraction A3. The major diol is 1,14-tetradecanediol, and palmitic and myristic acids appear to be common fatty acid components of compounds in A3. The presence of ω-hydroxyhexadecanoic acid (16-hydroxypalmitic acid) suggests that the structures of esters found in A3 are comprised of two fatty acids esterified together with one alkanediol and one ω-hydroxy fatty acid. We suggest the structures of several compounds, **7–11** (Figure 6), fit the MS and chemical derivatization GC/MS data. The compound with m/z 821 is represented as structure **10** for $C_{52}H_{101}O_6$. In all but the compound with lowest mass at m/z 792, ω-hydroxypalmitic acid (16-hydroxyhexadecanoic acid) exists as the hydroxy fatty acid component of the esters. To obtain m/z 792, 14-hydroxytetradecanoic acid replaces 16-hydroxyhexadecanoic acid shown in all other structures, even though direct evidence for its presence in saponified samples was not seen. Compounds with m/z 876 can be represented by more than one isomeric form such as **7** or **8**. Attempts to derive confirming information by MS−MS analysis of peaks for fraction A3 were unsuccessful.

Structural classes of vasoactive lipids dominated by the presence of alkanediols esterified with myristic and/or lauric acids have been identified for the first time. The fact that components of A1 and A3 appear to have differential effects on vascular tone and PSC activities suggests that reductions in blood flow *in vitro* and perhaps *in vivo* may be due to the presence of structurally similar compounds with two types of vasoactivity. The physiological basis for these differences is unknown. It is noteworthy that a new guinea pig model system developed by Ford et al. (1994) has been used to demonstrate that abortion was induced within 3 days of feeding when the related derivative 14:12:14 was administered orally in doses of 500 μg/kg to pregnant guinea pigs. This finding confirms the abortifacient activities of this class of compounds in guinea pigs, and it remains to be demonstrated in beef cattle. This work provides a basis for the synthesis and biological evaluation of a series of related compounds that is the subject of another paper.

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