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Ozone Exposure *In Vivo* and Formation of Biologically Active Oxysterols in the Lung

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d) Abbreviations:

6-oxo-3,5-diol	cholestan-6-oxo-3,5-diol
β-epoxide	5β,6β-epoxycholesterol
BAL	bronchoalveolar lavage
BSTFA	bis(trimethylsilyl)fluoroacetamide
HBSS	Hank's buffered salt solution
MRM	multiple reaction monitoring
NAAQS	national ambient air quality standards
norcholestane-6-carboxy	3β-hydroxy-5β-hydroxy-B-norcholestan-6β-carboxaldehyde
RP-HPLC	reversed phase high pressure liquid chromatography
*Seco*sterol	3β-hydroxy-5-oxo-5,6-secocholestan-6-al
S.E.M.	standard error of the mean
TMS	trimethylsilyl

e) Gastrointestinal, Hepatic, Pulmonary, Renal

ABSTRACT

Ozone toxicity in the lung is thought to be mediated by products derived from the reaction of ozone with components of the lung epithelial lining fluid. Cholesterol is an abundant component of this epithelial lining fluid, and it is susceptible to ozonolysis, yielding several stable products including 3β-hydroxy-5-oxo-5,6-secocholestan-6-al and 5β,6β-epoxycholesterol. Both 5β,6β-epoxycholesterol and its metabolite, cholestan-6-oxo-3,5-diol, have been shown to cause cytotoxicity *in vitro*, suggesting that they may be potential mediators of ozone toxicity *in vivo*. An ozone sensitive mouse strain, C57BL/6j was exposed to varying concentrations of ozone (0.5 to 3.0 ppm), and subsequently the levels of these cholesterol ozonolysis products were quantitated by electrospray ionization mass spectrometry in bronchoalveolar lavage fluid, lavaged cells, and lung homogenate. An ozone dose dependent formation of these biologically active oxysterols was observed *in vivo*, supporting a role for these compounds in ozone toxicity. Since the 5β,6β-epoxycholesterol metabolite, cholestan-6-oxo-3,5-diol, was isobaric with other cholesterol ozonolysis products, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al and its aldol condensation product, 3β-hydroxy-5β-hydroxy-B-norcholestan-6β-carboxaldehyde, detailed mass spectral analysis using electron impact ionization was utilized to differentiate these isobaric cholesterol ozonolysis products. The specific detection of cholestan-6-oxo-3,5-diol in lung homogenate after ozone exposure established formation of 5β,6β-epoxycholesterol within the lung after exposure to 0.5 ppm ozone.

INTRODUCTION

Ozone is a major air pollutant that has been known to cause toxic pulmonary effects in animals and man for decades (Stokinger, 1965). Various adverse sequelae of ozone exposure have been documented including increased airway hyperresponsiveness, epithelial sloughing, and neutrophil influx in the airways (Schelegle, 1991; Hyde, 1992; Park, 2004a). The primary mechanism for this observed ozone toxicity has not been defined. Calculations suggest that the high reactivity of ozone and its low solubility in water would prevent it from passing through the lung epithelial lining fluid to act directly with the underlying epithelial cells (Pryor, 1992a). Lung epithelial lining fluid, which contains pulmonary surfactant, is composed of almost 95% lipids (Sadana, 1988; Hall, 1985); thus, it has been proposed that ozone exerts its toxic effects via a lipid mediator, which is formed during the interaction of ozone with lipids in the pulmonary surfactant (Postlethwait, 1998; Pryor, 1995). Various studies have shown that oxidized lipids can act as signaling molecules (Kafoury, 1998; Uhlson, 2002); for example, lysophospholipids, which could potentially be formed during ozonolysis, have been shown to initiate PAF-like activity (Marathe, 1999).

Cholesterol, which is the most abundant neutral lipid in human pulmonary surfactant (Sadana, 1988), has a double bond that is susceptible to attack by ozone (Bailey, 1957). Multiple products have been described to form during the reaction of ozone with cholesterol and the product yields have been shown to depend on ozonolysis conditions (Gumulka, 1983, Jaworski, 1988). Recently, our laboratory found that 5β,6β-epoxycholesterol (β-epoxide) (Scheme 1) was a major product of cholesterol ozonolysis in a lipid environment (Pulfer, 2004). This oxysterol has been studied as a product of cholesterol autooxidation and lipid peroxidation (Sevanian, 1987) and has been shown to cause cytotoxicity in *in vitro* systems (O'Callagan, 2001; Sevanian,

1991). Recent studies have shown that a major metabolite of β-epoxide in cultured human bronchial epithelial cells, cholestan-6-oxo-3,5-diol (6-oxo-3,5-diol), is also cytotoxic (Pulfer, 2004). Therefore, formation of β-epoxide and 6-oxo-3,5-diol in lung surfactant during ozone exposure could play a role in the lung epithelial cell necrosis and sloughing observed soon after ozone exposure (Hyde, 1992).

The formation of cholesterol derived products has been studied after ozone exposure in animals (Pryor, 1992b). The products monitored were 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (*seco*sterol) and its aldol condensation product, 3β-hydroxy-5β-hydroxy-B-norcholestan-6β-carboxaldehyde (norcholestane-6-carboxy) (Scheme 1), and the method of detection for these products involved only molecular weight analysis of the DNPH derivatives. Formation of cholesterol epoxides has not been studied in relationship to ozone exposure; however, cholesterol epoxide formation in the lung was previously studied after exposure of rats to high levels of NO_2, which induces lipid peroxidation (Sevanian, 1979). A modest increase in the levels of epoxide was seen after exposure to this potent oxidant, suggesting that cholesterol in pulmonary surfactant is susceptible to oxidation. To further assess the effect of ozone on cholesterol oxidation, we used an *in vivo* model of acute ozone exposure (Park et al, 2004a; Park et al, 2004b) to study the formation of β-epoxide and its cellular metabolite, 6-oxo-3,5-diol. In the present study, we describe the formation of these cholesterol oxidation products in the lungs of mice exposed to ozone concentrations as low as 0.5 ppm.

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METHODS

Materials. Cholesterol and bis(trimethylsilyl)fluoroacetamide (BSTFA) were purchased from Sigma (St. Louis, MO). Stable isotope labeled 2,2',3,4,4',6-d_6-cholesterol (98%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Solvents were purchased from Fisher Chemical (Pittsburgh, PA).

Quantitation of Epoxycholesterol and 6-oxo-3,5-diol. The alpha and beta isomers of 5,6-epoxycholesterol were synthesized as previously described (Pulfer et al., 2004). For reversed phase high pressure liquid chromatography (RP-HPLC) solvents used were solvent A, methanol:water:acetonitrile (v:v:v; 60:20:20) with 1 mM ammonium acetate and solvent B, methanol with 1 mM ammonium acetate. Quantitation was carried out using stable isotope dilution mass spectrometry. Samples were introduced onto the mass spectrometer using a 150 x 1.0 mm Columbus C18 column (Phenomenex; Rancho Palos Verdes, CA). The flow rate was 50 µl/min and the gradient increased from 75% to 100%B over 10 min followed by 100%B for 20 min. Multiple reaction monitoring (MRM) analysis was carried out on a Sciex API-2000 mass spectrometer (Perkin-Elmer Life Sciences, Thornhill, Ontario, Canada). The oxidized cholesterol products formed $[M+NH_4]^+$ ions in the positive ion mode with an ionspray voltage of 4500V, declustering potential of 40V, focusing potential of 350V and collision energy of 12V. Nitrogen was used in the collision cell with a collision gas thickness of 2.17×10^{15} mol/cm^2. The transitions monitored were m/z 420 → 385 for the 5,6-epoxycholesterol isomers, m/z 426 → 391 for d_6-5β,6β-epoxycholesterol, m/z 404 → 369 for cholesterol, and m/z 436 → 383 for 6-oxo-3,5-diol, *seco*sterol, and norcholestane-6-carboxy. The dwell time for each transition was 800 ms. The standard curve was linear for a range of 0.31 ng to 320 ng for β-epoxide and 6-oxo-3,5-diol. The limit of detection was defined as 0.16 ng, which yielded a peak that was 4-fold greater

than background noise. Therefore, for points that were lower than the limits of detection, a value of <0.16 ng was assigned to the sample.

Animals. Eight to 12-week old female C57BL/6j mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were bred and housed under pathogen-free conditions and maintained in the Biological Research Center at National Jewish Medical and Research Center (NJMRC). All protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee of NJMRC.

Ozone Exposure. Mice were exposed to different concentrations (0.5-3.0 ppm) O_3 for 3 hr (n=4). They were placed in stainless steel wire cages set inside 240-liter laminar flow inhalation chambers. HEPA-filtered room air was passed through these chambers at 25 changes/hr. Room temperature was maintained at 20-25°C. Ozone was generated by directing compressed medical-grade oxygen through an electrical discharge O_3 generator (Sander Ozonizer, Model 25, Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) located upstream of the exposure chamber. The O_3-air mixture was metered into the inlet air stream with mass flow controllers (MKS Instruments Inc., Model #1359C, Andover, MA). Simultaneous exposure to HEPA-filtered air was carried out in a separate chamber with age- and treatment-matched control animals. Ozone concentrations were continuously monitored at mouse nose levels within the chamber with a photometric O_3 analyzer (Advanced Pollution Instrumentation, Inc., Model 400A, San Diego, CA) and recorded on a strip-chart recorder. Calibration of the O_3 analyzer was performed by the Colorado Department of Public Health and Environment.

Bronchoalveolar Lavage and Lung Preparation. Mice were euthanized 6 hr after the ozone exposure,and the,lungs were lavaged via the tracheal tube with 1 ml of Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY). The bronchoalveolar lavage fluid (BAL) was

centrifuged at 2,000 RPM for 10 min and the supernatant was transferred to glass tubes. The cell pellet was resuspended in 1ml HBSS and subjected to a second round of centrifugation. The supernatant was combined with that from the first centrifugation, and the cell pellet was resuspended in 200 µl of HBSS. Deuterated β-epoxide (40 ng in 25 µl) was added to the samples of cell-free lavage fluid and cells. A 10 µl aliquot of the cell suspension was counted using a hemocytometer for total cell count, excluding red blood cells. Whole lungs were dissected from the mice, and 400 ng of internal standard in 25 µl ethanol was added prior to homogenation in HBSS.

Lung homogenate, lavaged cells and cell free lavage supernatants were diluted to a final volume of 1ml with water. Neutral lipid extraction was achieved by the addition of 2 ml methanol and 3 ml iso-octane, and the organic phase was dried under nitrogen. The lipid extract from lung homogenate was resuspended in 2ml ethanol, and a 40µl aliquot was diluted with 40 µl solvent A for mass spectral analysis. Total extracts from the lavage fluid and cells were resuspended in 80µl solvent A for analysis. Samples were quantitated by MRM as described above.

Synthesis of Cholestan-6-oxo-3,5-diol and 3β-Hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde and Purification of *in vivo* Unknown. Cholestan-6-oxo-3,5-diol was synthesized as previously described (Pulfer, 2004). Briefly, cholestanetriol was synthesized by opening the epoxide moiety of β-epoxide (10 mg) to a vicinal diol by treatment with 0.5 ml perchloric acid in 4 ml of THF:H_2O:acetone (v:v:v; 4:1:0.5). The resulting cholestanetriol was extracted in dichloromethane and purified by RP-HPLC using the conditions described above. Cholestanetriol (10 mg) was dissolved in 4.5 ml ether, 750 µl methanol, and 750 µl water and stirred with N-bromosuccinimide (108 mg) for 3 hr at room temperature to yield the product, 6-

oxo-3,5-diol. The reaction was diluted with water and extracted with dichloromethane. Purification was achieved by RP-HPLC.

The cholesterol ozonolysis product, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (norcholestane-6-carboxy), was synthesized by modification of published methods (Gumulka, 1983; Wentworth, 2003; Miyamoto, 2001). Cholesterol (5 mg) was suspended in 2ml of THF:H_2O (1:1;v:v) and exposed for 3 minutes to ozone generated by passing oxygen at 1ml/min through a tesla coil (Supelco, Belefonte, PA) fitted with a glass sleeve covering the high voltage electrode. Lipids were then extracted from the solution with methylene chloride and dried under nitrogen. The primary ozonide was resuspended in 2.5 ml acetic acid:H_2O (9:1;v:v) and reduced by stirring with 32.5 mg zinc dust for 3 hr at room temperature. The reaction mixture was washed with water (5 x 4 ml) and the resulting *seco*sterol was purified by RP-HPLC as described above. Purified *seco*sterol (5 mg) in benzene (2.5 ml) was stirred overnight with excess activated alumina (2 hr at 110˚C). Benzene was evaporated under nitrogen and lipids extracted using the method of Bligh and Dyer (1959). The lipid extract was dried under nitrogen and norcholestane-6-carboxy was purified by RP-HPLC.

The total neutral lipid extract from the lung homogenate of 3 mice lungs exposed to 3 ppm ozone for 3 hr was pooled and dried under vacuum. The sample was resuspended in 100 µl of solvent A and chromatographed by RP-HPLC on an Ultramex 5 C18 (250 x 4.6 mm) reverse phase column (Phenomenex, Torrance, CA) with a flow rate of 1ml/min using the gradient listed above. A small fraction of the effluent (20µl/min) was split to the mass spectrometer for on-line MRM analysis of the mass transition 436→383. The remaining effluent was collected in one-minute fractions, and the fractions corresponding to the aforementioned mass transition were pooled and dried under vacuum.

NMR analysis of the synthesized norcholestane-6-carboxy was kindly provided by Dr. David Jones, Ph.D. (University of Colorado Health Sciences Center). Samples were dissolved in deuterated chloroform for proton NMR analysis (100 atom % D; Aldrich chemical; Milwaukee, WI). Spectra were recorded on a Varian Inova Spectrometer with a proton frequency of 500 MHz. COSY and TOCSY proton assignments for the A and B rings of the synthesized compound were consistent with the norcholestane-6-carboxy structure: ^{1}H NMR ($CDCl_3$) δ 9.721 (d, CHO), 4.141 (m, H-3), 2.256 (m, H-4a), 2.349 (m, H-6), 2.214 (m, H-7), 1.837 (m, H-4b), 1.763 (m, H-2a), 1.724 (m, H-2b), 1.483 (m, H-1).

GC/MS. For analysis of standards, 5µg of sample was dried under vacuum. Samples were derivatized by the addition of 50µl BSTFA and 50µl acetonitrile followed by heating to 65˚C, yielding the trimethylsilyl derivatives. For the unknown *in vivo* sample (molecular weight, 418 amu) a small aliquot (5%) was dried under vacuum and similarly derivatized with BSTFA. An aliquot (2 µL) of each derivatization solution was analyzed by a gas chromatograph mass spectrometer using electron ionization at +70 eV (Trace 2000, Thermo-Finnigan, San Jose, CA). The temperature gradient ran from 150ºC to 260ºC at 20˚C/min and 260ºC to 310ºC at 4ºC/min on a 30 meter ZB-1 column (Phenomenex, Torrance, CA) with a 0.25 mM ID and a 0.25 µ stationary film thickness.

Data Analysis. Statistical calculations were performed using Excel (Microsoft, Redmond, WA). Data are presented as mean ± SEM. For analysis of significance, the data were analyzed by Student's *t*-test. Values of $p<0.05$ were considered significant.

RESULTS

The mouse strain C57BL/6J was chosen for analysis of β-epoxide and 6-oxo-3,5-diol *in vivo* because of the unique susceptibility of this mouse strain to ozone toxicity (Kleeberger, 1997). Quantitation of cholesterol ozonides in mice after ozone exposure was performed by stable isotope dilution mass spectrometry. Representative MRM traces from the BAL fluid of a control mouse and an ozone treated mouse are shown in Figure 1. Relative to added internal standard, greater ion intensities were observed for the peaks corresponding to cholesterol ozonide products in the ozone treated mouse BAL than in the control mouse BAL.

Ozone-Dose Dependent Formation of Oxysterols *In Vivo.* Mice were treated with room air or 3 ppm ozone for 3 hr, and at various time points after exposure, the cholesterol ozonide products were quantitated in lung lavaged fluid (supernatant), lavaged cells, and lung homogenate. The concentration of both β-epoxide and its metabolite, 6-oxo-3,5-diol, were elevated above filtered air treated mice for 24 hr after exposure, with the highest levels found at 6 hr post exposure (data not shown). The 6 hr time point was therefore chosen for the analysis of BAL fluid and whole lung homogenate from mice exposed to increasing concentrations of ozone (0.5, 1.0, 2.0 or 3.0 PPM). Quantitative results for cellular lipids were normalized to the number of cells in the lavage sample and reported as ng per 10^5 cells. A trend for increased β-epoxide was seen in the lipid extract from lavaged cells over the ozone dose range studied (Figure 2a). The lipid extract from the lavage supernatant also showed a trend for increased β-epoxide, and levels were significantly different from control mice at all ozone exposure doses (Figure 2b; $p<0.05$, $n=4$). A dose response was not observed for increased β-epoxide in lung homogenates from ozonized mice (Figure 2c).

The levels of the β-epoxide metabolite, 6-oxo-3,5-diol, in bronchoalveolar lavaged cells of control C57BL/6j mice were below the limits of detection (<0.16 ng). However, in mice exposed to 3.0 ppm ozone, 16.8 ng of 6-oxo-3,5-diol per 10^5 cells were detected (Figure 3a). Quantitation of 6-oxo-3,5-diol in the lavage supernatants revealed significant increases over control mice for all doses of ozone studied (Figure 3b; $p<0.05$; $n=4$). In remarkable contrast to β-epoxide, there was a dose dependent increase in the formation of 6-oxo-3,5-diol in lung homogenates (Figure 3c).

Trypan blue exclusion analysis of cells collected from bronchoalveolar lavage (with the exclusion of RBCs) from mice exposed to filtered air or ozone (0.5, 1.0, 2.0, or 3.0 ppm) was performed to determine if there was a relationship between ozone dose and percent cell death (Figure 4). Values were normalized to control mice, and a significant increase in the percent of cells that were permeable to trypan blue dye was observed in mice exposed to 3.0 ppm ozone.

Mass Spectral Analysis of Unknown *In Vivo* Oxysterol. Previous investigators have used *seco*sterol and norcholestane-6-carboxy as biomarkers of ozone exposure, since the formation of these products has been reported to be unique to the properties of ozone chemistry (Pryor, 1992b; Wentworth, 2003). These two compounds have very different structures, as one has an open B ring and the other has a closed ring system (Scheme 1). They are isobaric with a molecular weight of 418 amu; however, the difference in lipophillicity due to ring structure leads to good separation on reverse phase HPLC. The *seco*sterol eluted from a C18 column at approximately 8 minutes with the gradient used in this study, while norcholestane-6-carboxy eluted at approximately 10.5 minutes. The identification of 6-oxo-3,5-diol as a major cellular metabolite of the cholesterol ozonide, β-epoxide, further complicated this situation as it was also isobaric with *seco*sterol and norcholestane-6-carboxy (molecular weight, 418 amu). The

structure of 6-oxo-3,5-diol is very similar to norcholestane-6-carboxy, containing a fused ring system with hydroxyls at the carbon-3 and carbon-5 positions of cholesterol and a carbonyl at the carbon-6 position. As a result, norcholestane-6-carboxy and 6-oxo-3,5-diol eluted closely together on RP-HPLC, both with retention times near 10.5 minutes.

Gas chromatography (GC) and electron ionization mass spectrometry were utilized in order to unambiguously determine if the compound observed *in vivo* was norcholestane-6-carboxy or 6-oxo-3,5-diol. Standard preparations of norcholestane-6-carboxy and 6-oxo-3,5-diol were synthesized as described in the methods, and then derivatized with BSTFA and subjected to GC/MS analysis. Both compounds yielded $M^{+\bullet}$ ions observed at m/z 562 which had distinct gas chromatographic elution times (Figure 5). The retention time of 6-oxo-3,5-diol was 13.23 minutes. Extraction of m/z 562 from the norcholestane-6-carboxy spectrum yielded two abundant peaks at 11.96 minutes and 12.84 minutes, which were also the most abundant peaks in the total ion chromatogram. The formation of two peaks was likely due to the formation of isomeric norcholestane-6-carboxy products, with stereoisomerism at carbon 6. A full electron ionization (70eV) mass spectrum, from m/z 50 to 700, was collected for both compounds (Figure 6).

While the spectra of the two major peaks from norcholestane-6-carboxy were found to be similar (Figure 6B and 6C), there were large differences between the mass spectral data for norcholestane-6-carboxy and 6-oxo-3,5-diol. Several ions were consistent for all the spectra, including m/z 472, an $[M-90]^+$ ion that was characteristic for the loss of OTMS from the molecular ion (m/z 562). Ions at m/z 547, $[M-15]^+$, and m/z 457, $[M-105]^+$, presumably arose from the loss of a methyl group from an OTMS moiety of the molecular ion and the m/z 472 ion, respectively. A striking difference between the two spectra was the ion at m/z 382, $[M-180]^+$,

which was consistent with the loss of both OTMS groups from the molecular ion. This was a prominent ion in the spectra of norcholestane-6-carboxy, but not in the spectrum of 6-oxo-3,5-diol, suggesting that loss of the second OTMS group from this compound was hindered. This was consistent with the proposed structures of the ether TMS derivatives of 6-oxo-3,5-diol and norcholestane-6-carboxy (Figure 5), since there are fewer protons adjacent to the OTMS group at the carbon-6 position of 6-oxo-3,5-diol. The most remarkable contrast between the two spectra was the presence of an ion at m/z 321, which was the most abundant ion in the mass spectrum of norcholestane-6-carboxy. This ion was not observed in the mass spectrum of 6-oxo-3,5-diol.

The lung homogenates from mice exposed to 3.0 ppm ozone were pooled and injected onto RP-HPLC. The fractions containing the compound with a molecular weight of 418 amu ($[M+NH_4]^+$ ion, m/z 436), which eluted between 10 and 12 minutes, were collected for derivatization with BSTFA for GC/MS analysis. The electron ionization (70eV) GC/MS spectrum (Figure 6d) was consistent with the spectrum of 6-oxo-3,5-diol. Comparative chromatograms for the molecular ion, m/z 562, further supported the identification of the *in vivo* compound as 6-oxo-3,5-diol (Figure 5c). The *in vivo* unknown yielded a single peak, observed at m/z 562, with a retention time of 13.22 min (compared to 13.23 min for synthetic 6-oxo-3,5-diol).

DISCUSSION

Previous reports have suggested that cholesterol epoxide levels are higher in the lung than in any other tissue, but its formation was assumed to be a result of free radical based mechanisms of peroxidation. The pollutant gas NO_2 has been found to induce peroxidation; therefore, not surprisingly, epoxide formation was observed after administration of NO_2 to rats (Sevanian et al, 1979). In those studies, rats were exposed to 6 ppm NO_2 for 48 hr, which far exceeds the National Ambient Air Quality Standards (NAAQS) of 0.053 ppm (US EPA, 2001). Even at this high NO_2 concentration, the levels of epoxide in BAL were only 2-fold higher than in filtered air treated controls. In mice treated with 0.5 ppm ozone for 3 hr (NAAQS of 0.12 ppm for 1 hr) (US EPA, 2001) there was nearly a 20-fold increase in the level of epoxide found in both the fluid and cells of BAL. The unique chemical reactivity of ozone may be the reason for this difference. Ozone can initiate free radical production (Pryor, 1994) and the β-epoxide observed *in vivo* could be derived from lipid peroxidation mediated by this process. Additionally, ozone can act directly to form epoxides (Bailey, 1985), and as discussed previously (Pulfer, 2004), this mechanism of ozone chemistry becomes more prominent when ozone attacks compounds with a sterically hindered double bond, such as cholesterol.

The rationale for choice of the levels of ozone exposed to mice in these studies was based on previous work (Park et al, 2004a; Park et al, 2004b) which studied alterations in airway responsiveness, neutrophilic inflammation, changes in BAL protein control, and changes in epithelial cell integrity. These changes were shown to be very ozone dose-dependent as changes in airway function and epithelial cell damage were seen at 2.0 ppm ozone exposure for 3 hr; at 1.0 ppm, these changes were much less obvious, although lung neutrophilic inflammation

persisted. At 0.5 ppm ozone, there were little discernable effects in the lung as assessed by any of these parameters.

It is important to note that activated neutrophils recruited to the lung after ozone exposure could be responsible for secondary formation of β-epoxide and 6-oxo-3,5-diol. The elevated levels of these products for 24 hr after ozone exposure suggests that continued formation due to neutrophil action may occur. Additional studies where neutrophils are depleted may shed some light on this question, since depletion does not affect changes in airway function or epithelial damage induced by ozone (Park et al, 2004a). Activated neutrophils can initiate free radical chemistry, which would potentially yield these products; however, recent studies have suggested that neutrophils may also generate ozone *in vivo* (Babior, 2003). In order to distinguish between ozone generated products and free radical mediated production of oxidized cholesterol products by neutrophils, unequivocal detection of 5-hydroperoxy-B-homo-6-oxa-cholestan-3β,7a-diol or *seco*sterol would be required, as these are unique products from the reaction of ozone with cholesterol.

Auto-oxidation of cholesterol during work-up of samples has been shown to interfere with quantitative analysis of cholesterol epoxides (Wasilchuk, 1992). This may explain why a dose response was not seen in the lung homogenate samples. One suggested solution to this problem with sample preparation is to add deuterated cholesterol to samples prior to work-up to determine if deuterated auto-oxidation products are formed. However, this was not compatible with the quantitative method in this study, which employed deuteratium labeled internal standards. Therefore, the best way to quantitate epoxide formation may be to quantitatively assess the formation of 6-oxo-3,5-diol. Formation of this β-epoxide metabolite likely requires living cells, since it is thought to form via enzymatic mechanisms, and is not formed in media.

The mouse lung homogenates and BAL fluid were placed in organic solvents immediately, which inactivated enzymatic activity, and prevented conversion of any β-epoxide formed during sample work-up to 6-oxo-3,5-diol. Consistent with this, the 6-oxo-3,5-diol seemed to better correlate with ozone exposure than the epoxide itself, as seen by the dose response curve in lung homogenate (Figure 3).

The levels of 6-oxo-3,5-diol in the lung homogenate from control mice were very low, ranging from 1.5 to 5 ng in whole lung and there was a significant increase after ozone exposure, reaching levels of greater than 180 ng in some samples. An observed parallel change in β-epoxide levels would be expected since it is the precursor for 6-oxo-3,5-diol formation. Surprisingly, the levels of β-epoxide in the control mouse lungs were very high, ranging from 69 to 120 ng in whole lung, and there was not a significant increase in β-epoxide levels after ozone exposure. Therefore, there was an abundant amount of β-epoxide in control lungs that did not undergo conversion to 6-oxo-3,5-diol. The unambiguous structural determination of 6-oxo-3,5-diol in the *in vivo* samples excluded the possibility that another structurally similar cholesterol ozonolysis product was increasing in the ozone treated mice lungs.

The absence of measurable metabolites in the lungs and BAL fluid of filtered air-treated mice suggested that either the β-epoxide measured in these samples was formed due to auto-oxidation during sample preparation, or that there was a compartmentalization of the epoxycholesterol that prevented its metabolism via a cholesterol epoxide hydrolase. A distinctly active cholesterol epoxide hydrolase activity has been reported to exist (Watabe, 1986), which acts preferentially on 5,6-cholesterol epoxides; however, the protein(s) responsible for this activity have not been identified (Fretland, 2000). As a result it is not possible to determine which of the 40 different cell types in the lung could potentially metabolize β-epoxide. The

activity of cholesterol epoxide hydrolase in lung microsomal fractions is lower than that of liver microsomal fractions (Sevanian *et al.*, 1980), suggesting that only a fraction of the cells in the lung possess this activity. Another possibility was that induction of enzymes to metabolize the epoxide might occur during ozonolysis. Further study of the formation of β-epoxide metabolites and the enzymes involved in their formation is thus warranted.

The levels of 6-oxo-3,5-diol and β-epoxide in control cell extracts were below the limits of detection in this assay. These samples were therefore assigned quantitative values of <0.16ng oxysterol. Since these samples contained fewer numbers of cells in BAL than the ozone exposed mice, this might overestimate the levels of β-epoxide and 6-oxo-3,5-diol in the control samples. As a result, the levels of these oxysterols in mice exposed to low doses of ozone were not significantly increased compared to control mice.

It should be noted that the levels of β-epoxide in individual cells was likely quite variable as cells in different regions of the lung were presumably exposed to drastically different concentrations of ozone due to non-homogenous distribution of this gas in the lung (Hu, 1994; Schelegle, 2001). On average, cells recovered from BAL of mice exposed to 0.5 ppm ozone, contained 1.8 ng β-epoxide/10^5 cells. Considering that many of these individual cells may not have incorporated β-epoxide, there are likely some cells in this recovered lavage that have accumulated much higher concentrations of β-epoxide. When cultured bronchial epithelial cells were treated with 1 μM β-epoxide for 24 hr, the amount incorporated into the membranes was only 12 ng/10^5 cells (data not shown). This concentration, which leads to potent suppression of cholesterol synthesis, is within the same range as the oxysterol levels in the membranes of bronchoalveolar lavaged cells from ozone treated mice. Both β-epoxide and 6-oxo-3,5-diol have been shown to cause cell death in cultured lung epithelial cells (16-HBE) as assayed by trypan

blue exclusion analysis (Pulfer, 2004). The mechanism of action for β-epoxide and 6-oxo-3,5-diol is likely independent of cell type, and high concentrations of ozonized lipids could potentially lead to cytotoxicity in numerous cell types, including epithelial cells and macrophages. The overall increase in percentage of non-viable lavaged cells after ozone exposure paralleled the increased levels in the oxidized lipid products, consistent with a potential role of oxidized lipid products in ozone induced cytotoxicity.

These oxysterols have not been studied extensively, and therefore they may bind to unknown receptors which initiate inflammatory signaling directly. For instance, 6-oxo-3,5-diol was reported to be a PMA analog that binds to a cytosolic-nuclear receptor (Endo, 1993). Alternatively, β-epoxide and 6-oxo-3,5-diol could build up to concentrations lower than those that initiate cell death, but still cause disruptions in the cellular membrane and alter signaling abilities. There are several studies which have provided evidence that cholesterol depleted cells have altered signaling properties due to disruption of lipid rafts (Westover, 2003; Pike, 1998). Decreased function in cells depleted of cholesterol is restored by cholesterol addition, but cholesterol analogs such as 4-cholesten-3-one do not restore activity (Nguyen, 2002). Increased proportional amounts of β-epoxide and 6-oxo-3,5-diol relative to cholesterol could lead to similar effects.

In summary, ozone exposure led to a dose dependent formation of β-epoxide and 6-oxo-3,5-diol in the lungs of mice exposed to ozone concentrations from 0.5 to 3.0 ppm. These oxysterols have been observed to have cytotoxic effects in cultured lung cells, and they were formed *in vivo* at similar concentrations that could potentially lead to epithelial cell damage, which is an effect frequently associated with ozone exposure. Furthermore, this work revealed that molecular weight analysis alone is not sufficient for identification of cholesterol ozonide

products formed *in vivo*, since three products, 6-oxo-3,5-diol, *seco*sterol, and norcholestane-6-carboxy are isobaric with a molecular weight of 418 daltons. This may be of particular relevance in studies suggesting that neutrophils generate ozone as revealed by formation of cholesterol ozonide products.

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FOOTNOTE

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FIGURE LEGENDS

Figure 1 Representative data for the increase of cholesterol ozonides in the BAL fluid from a mouse exposed to ozone (1.0 ppm) as compared to a control mouse. A-C) Representative MRM chromatograms of cholesterol derived ozonides from the BAL fluid of the control mouse. A) The MRM transition for the 5,6-cholesterol epoxides (420→385) yielded a single peak, with a retention time consistent with α-epoxide. B) The MRM transistion for *seco*sterol and 6-oxo-3,5-diol yielded no peaks in the BAL from the control mouse. D-F) Representative MRM chromatograms of cholesterol derived ozonides from the BAL fluid of an ozone treated mouse. D) The MRM transition for the 5,6-cholesterol epoxides revealed two peaks, with the earlier peak corresponding to β-epoxide, as compared to the retention time of the deuterated standard (C and F). E) The MRM transition for *seco*sterol and 6-oxo-3,5-diol (436→383) revealed that both compounds were formed in the BAL of the ozone exposed mouse, and that these products eluted with distinct chromatographic retention times.

Figure 2 Formation of β-epoxide *in vivo* following ozone exposure. Ozone sensitive C57BL/6j mice were exposed to filtered air or to increasing concentrations of ozone (0.5, 1.0, 2.0 or 3.0 ppm). A) Nanograms of β-epoxide found in bronchoalveolar lavaged cells, normalized for the number of cells recovered in the lavage. B) Nanograms of β-epoxide detected in the lavage supernatant (ng/ml). C) Nanograms of β-epoxide detected in whole lung homogenate. For samples that were below the limits of detection in this quantitative assay, values of 0.16 ng per sample were assigned. * $p < 0.05$ compared to control mice (n=4).

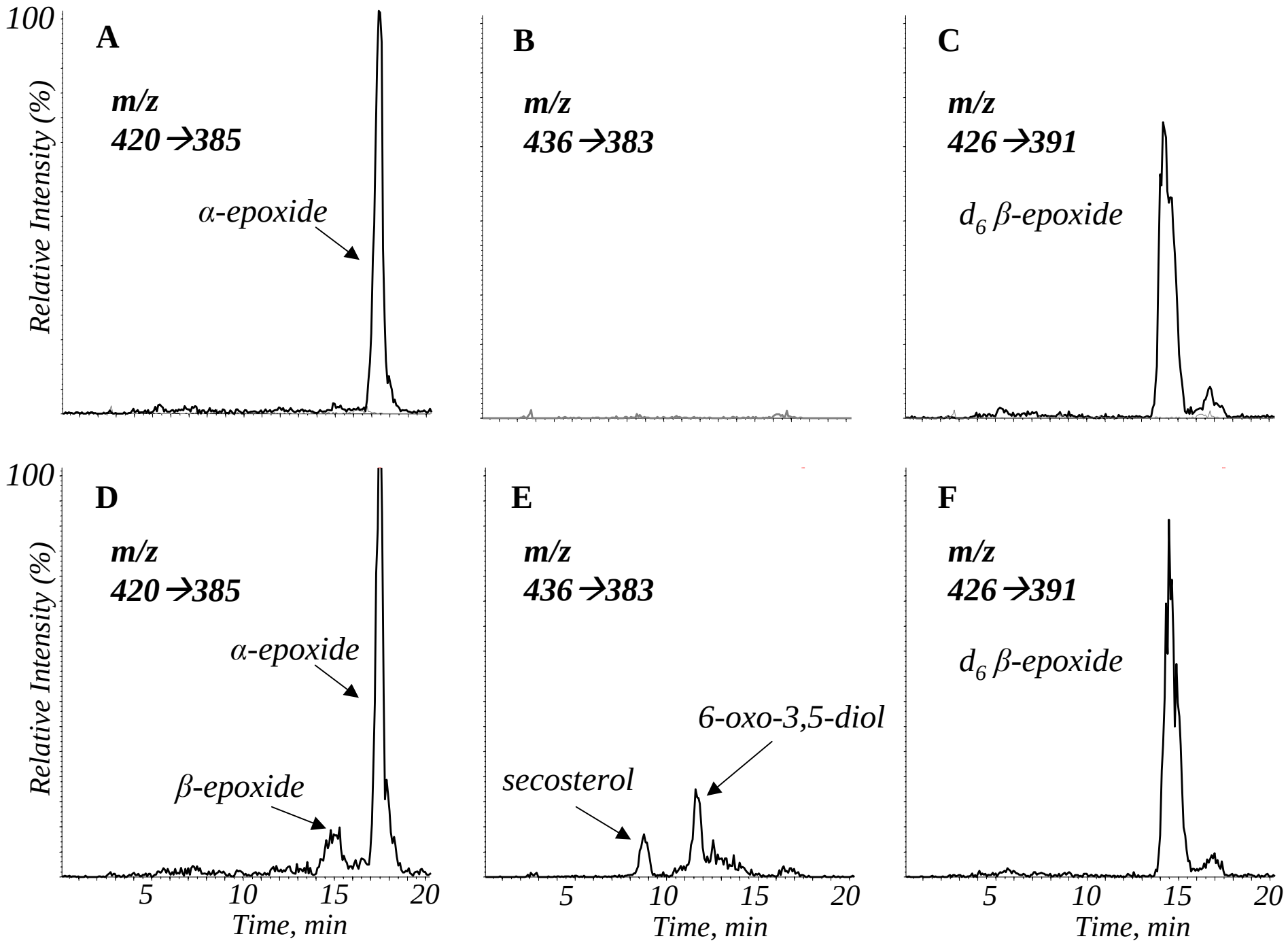
Figure 3 Formation of 6-oxo-3,5-diol *in vivo* following ozone exposure. The ozone sensitive mouse strain, C57BL/6j was exposed filtered air or increasing concentrations of ozone (0.5, 1.0, 2.0 or 3.0 ppm). A) Nanograms of 6-oxo-3,5-diol found in bronchoalveolar lavaged cells, normalized for the number of cells recovered in the lavage. B) Nanograms of 6-oxo-3,5-diol detected in lavage supernatant (ng/ml). C) Nanograms of 6-oxo-3,5-diol detected in whole lung homogenate *$p < 0.05$ compared to control mice (n=4).

Figure 4 Trypan blue exclusion analysis of cells recovered from bronchoalveolar lavage. Percent cell death for lavaged cells from mice exposed to 0.5, 1.0, 2.0, or 3.0 ppm ozone were normalized to those from control mice, and expressed as percent cell death relative to control. *$p < 0.05$ compared to control mice (n=4).

Figure 5 Chromatograms from GC/MS analysis of A). 6-oxo-3,5-diol, B) norcholestane-6-carboxy, and C) the *in vivo* unknown product with a molecular weight of 418 daltons. The chromatograms represent extraction of m/z 562. The peaks observed corresponding to this ion were the most abundant peaks on the total ion chromatogram (data not shown).

Figure 6 Electron ionization (70 eV) mass spectrum of the TMS derivatives of A) 6-oxo-3,5-diol, B) norcholestane-6-carboxy (retention time 11.96 min), C) norcholestane-6-carboxy (retention time 12.84 min) and D) *in vivo* unknown.

Figure 1.



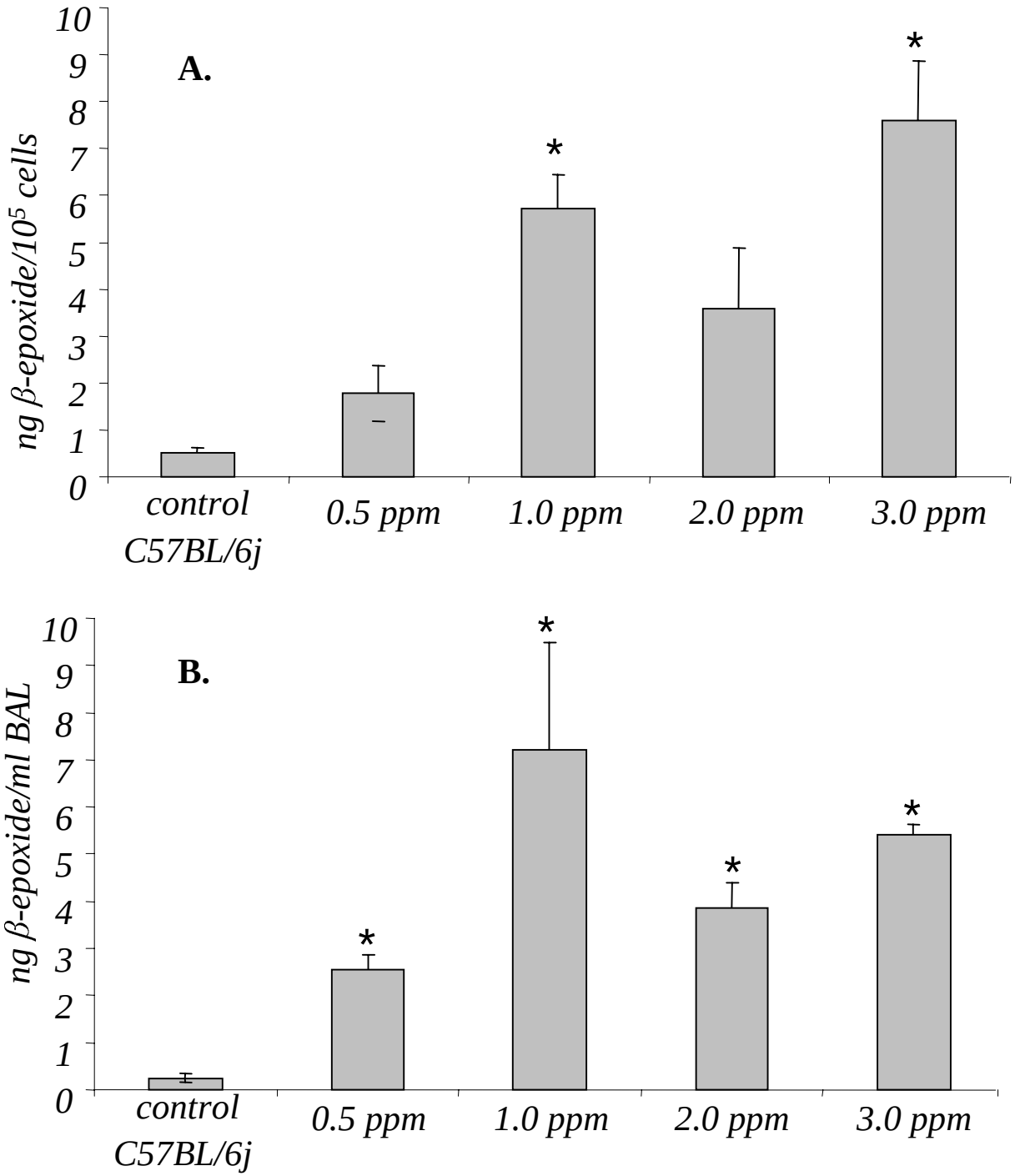


Figure 2.

Figure 2C

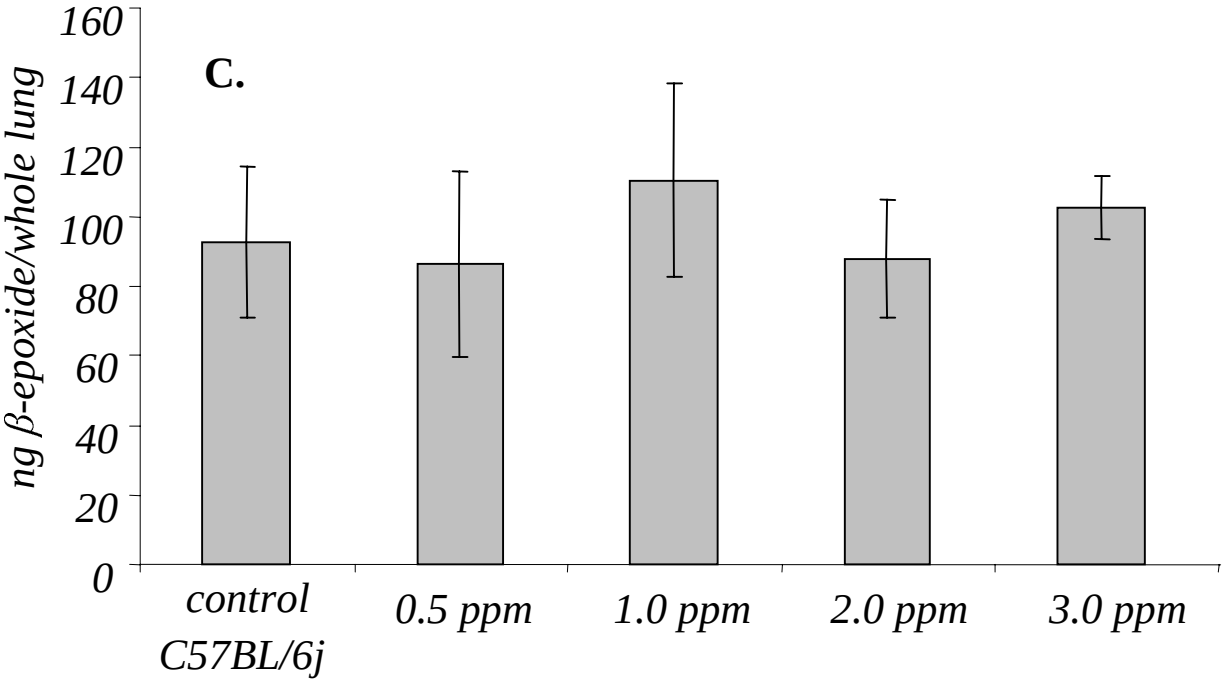


Figure 3

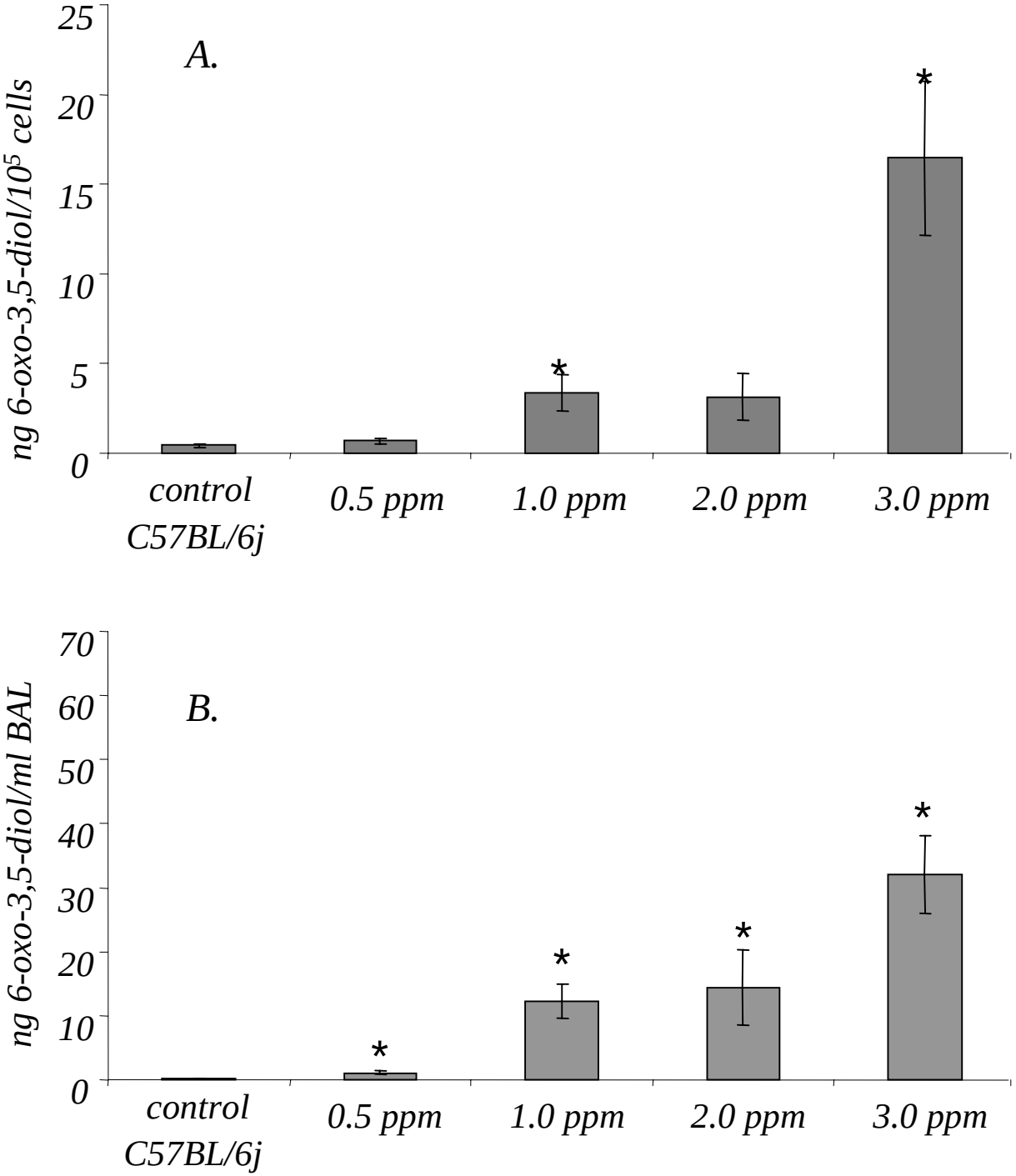


Figure 3C

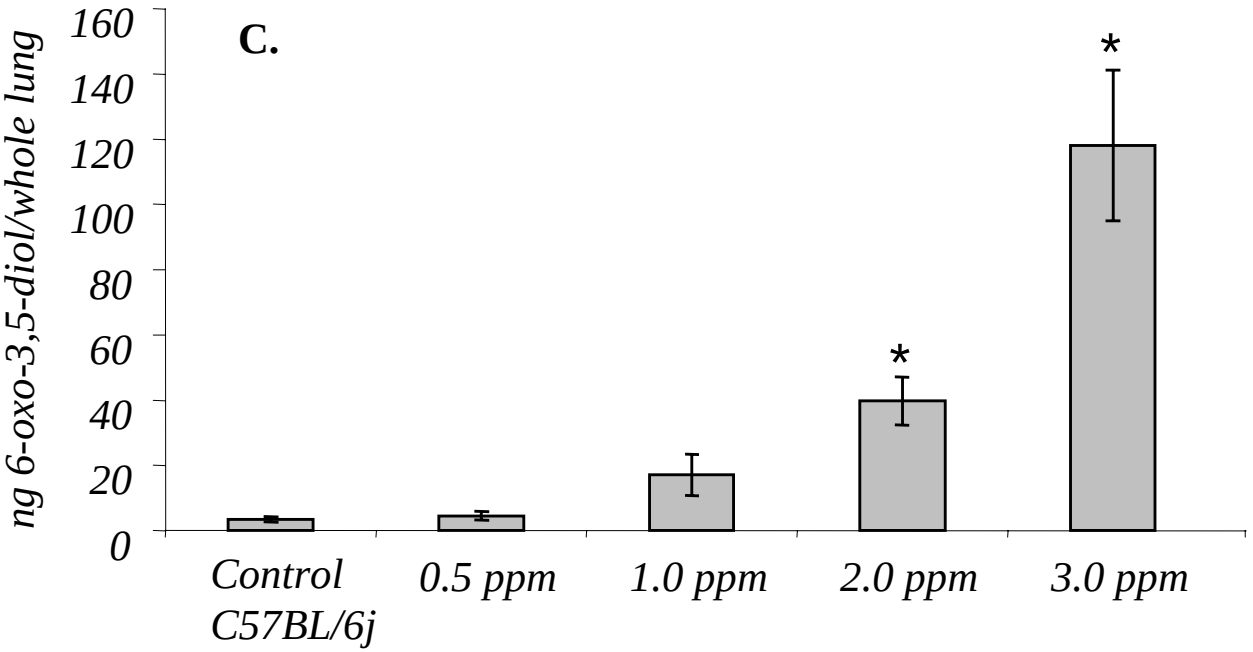


Figure 4

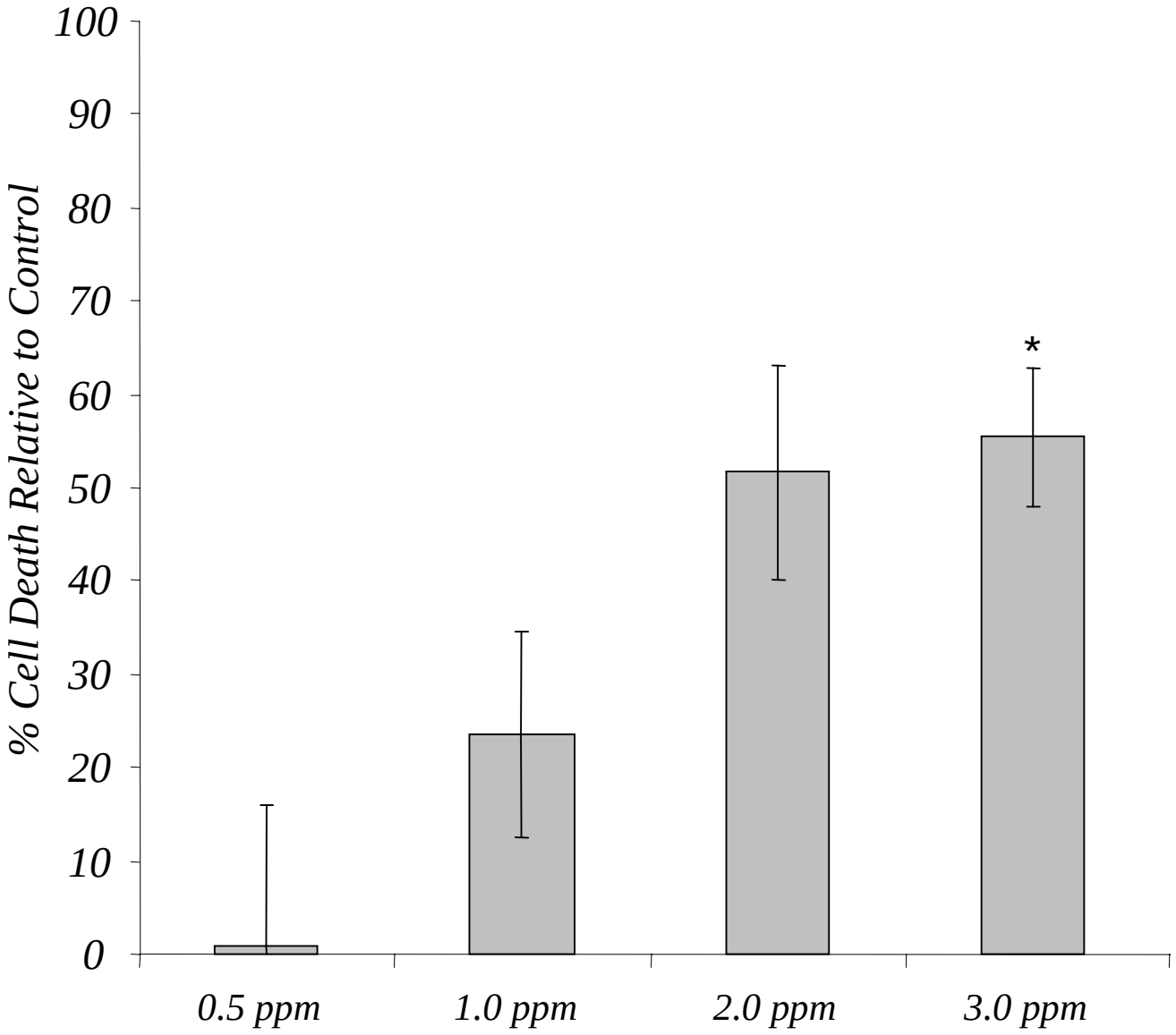


Figure 5

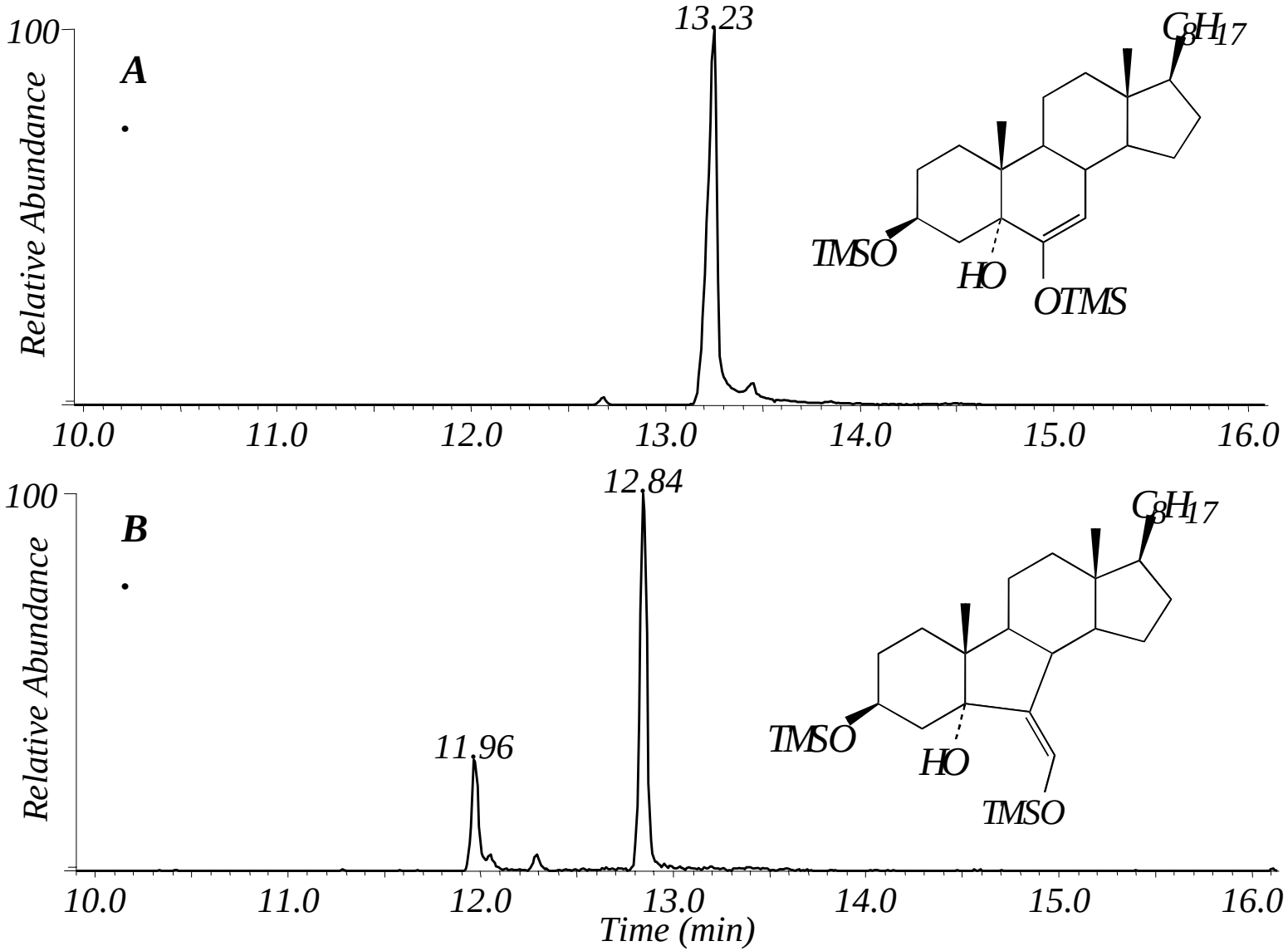


Figure 5C

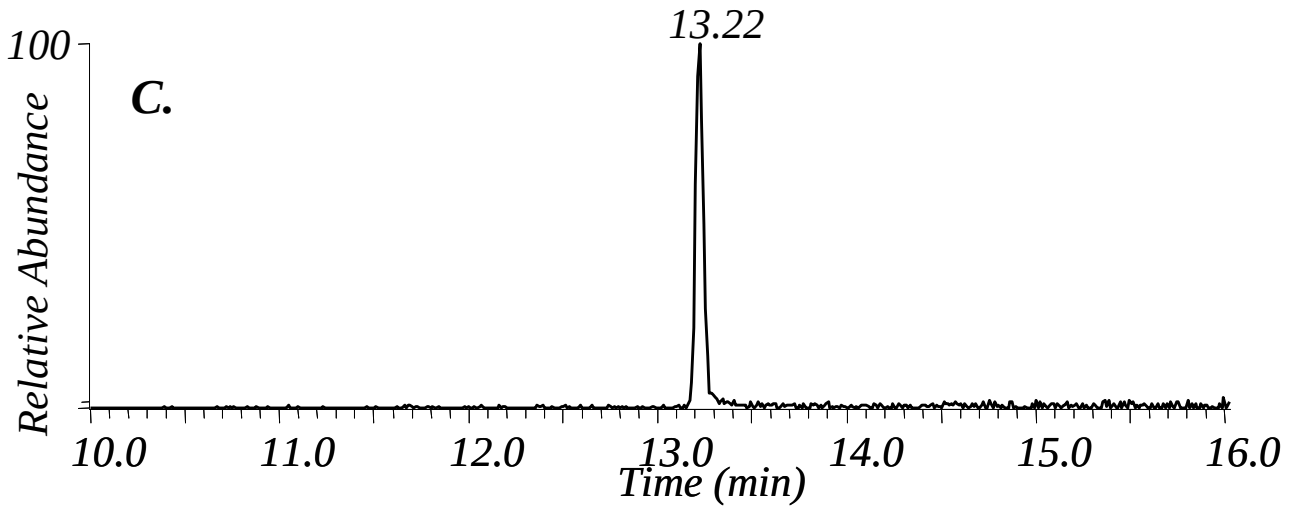


Figure 6

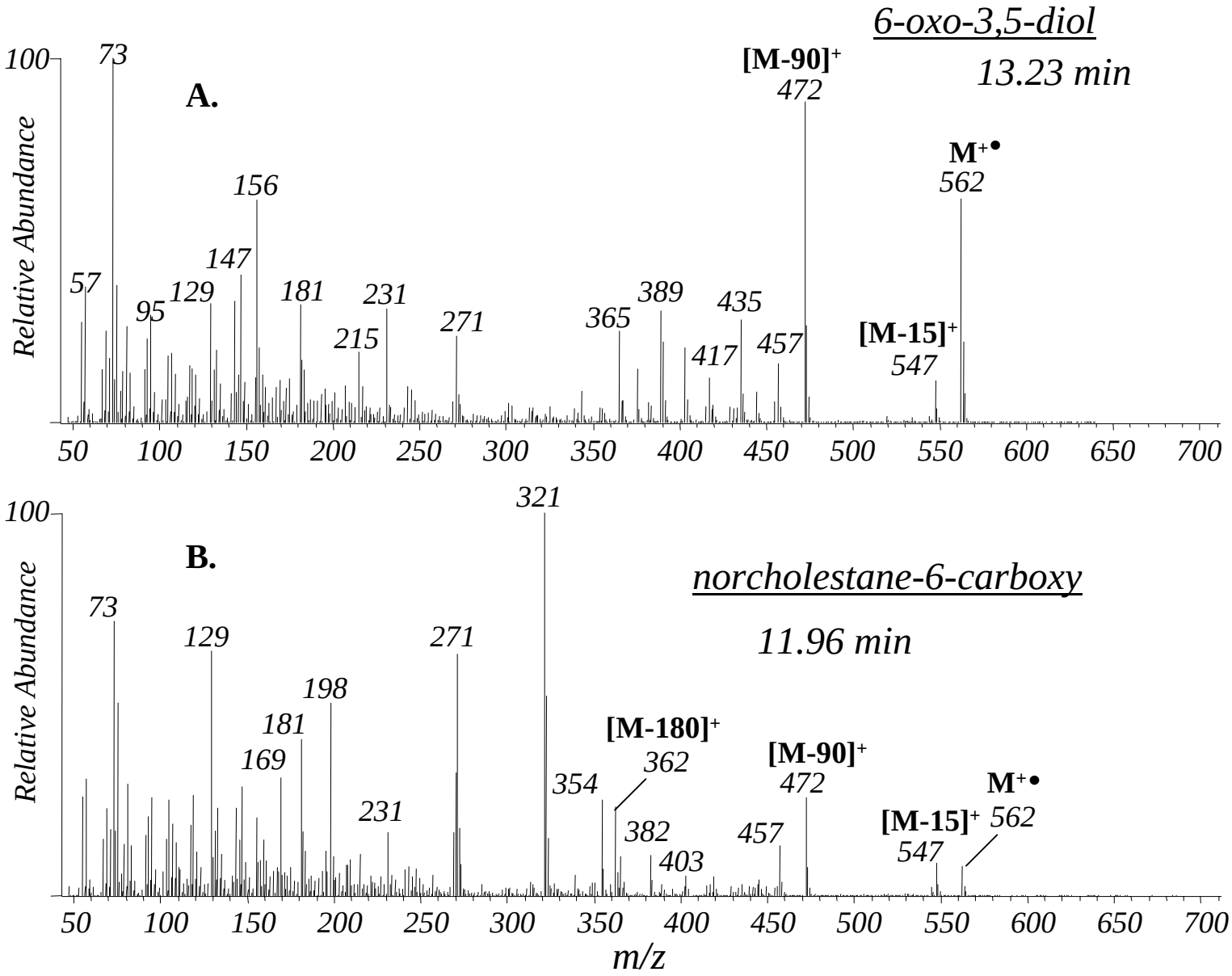
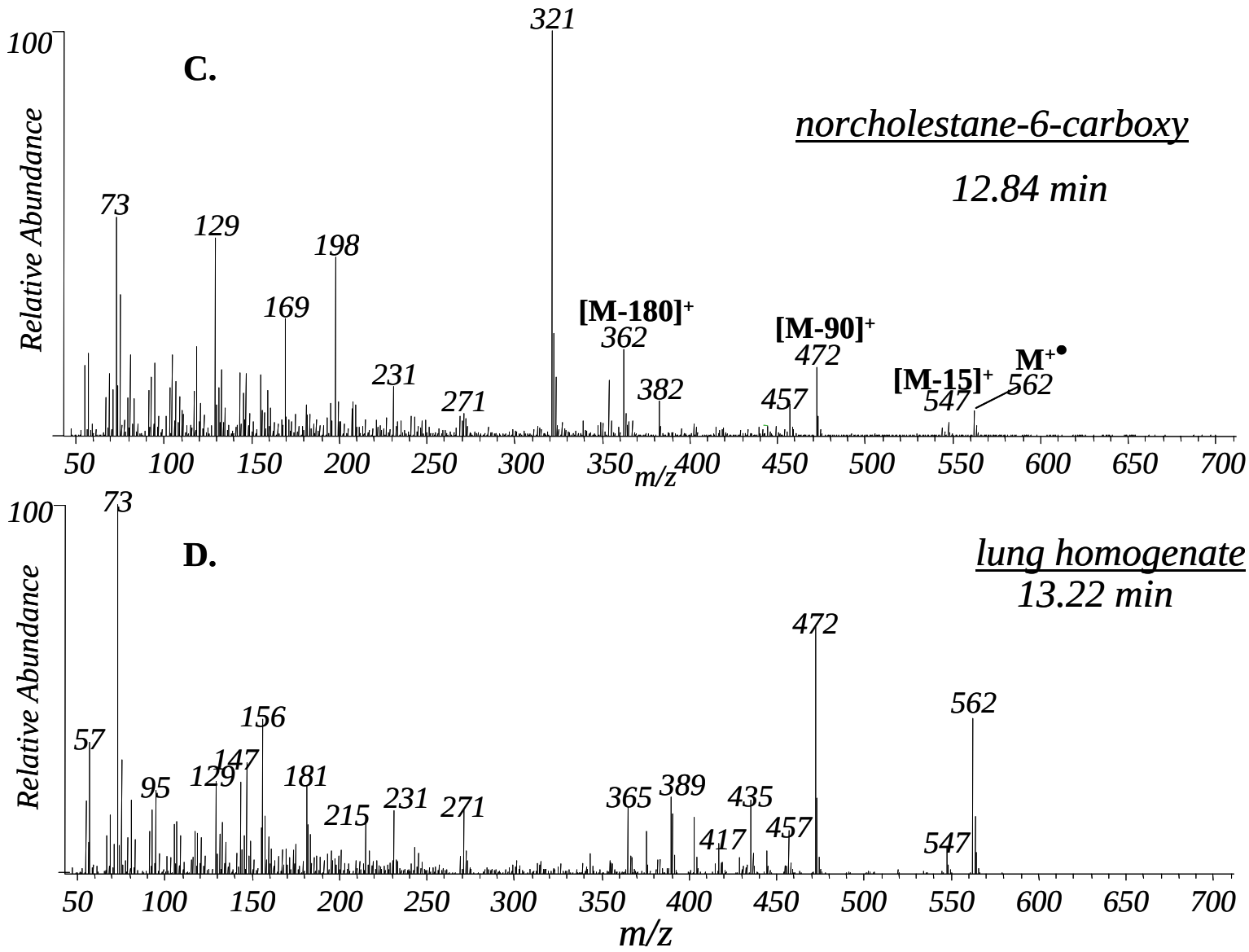
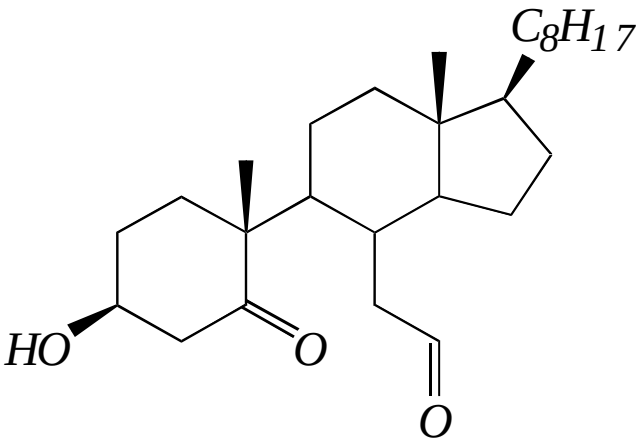


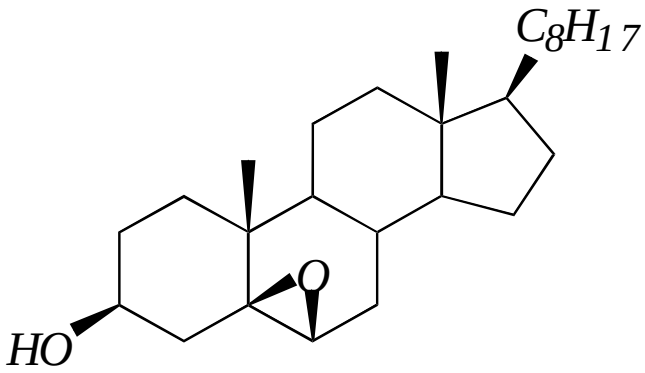
Figure 6C,D



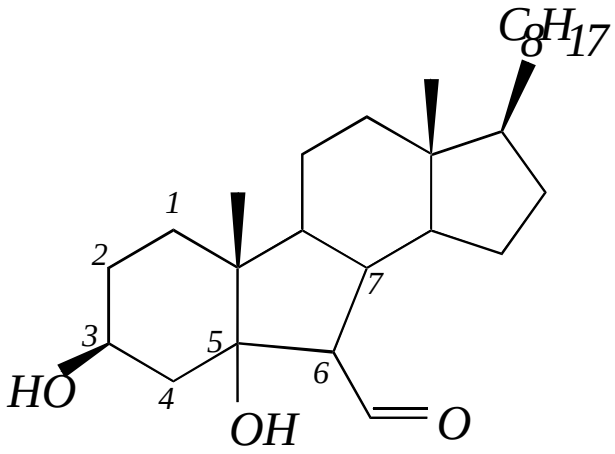
Scheme 1



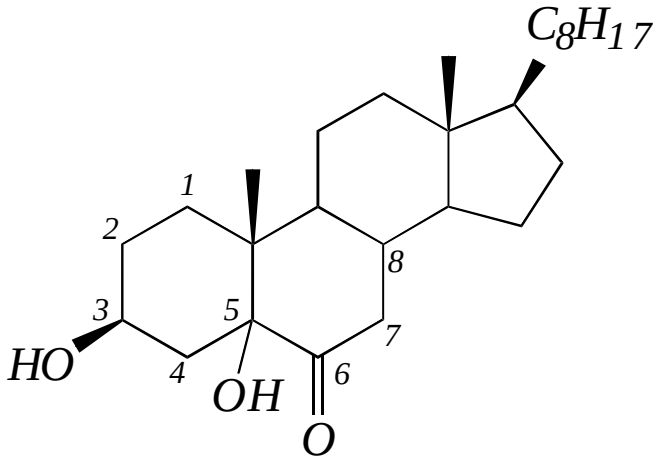
***seco*sterol**



β-epoxide



norcholestane-6-carboxy



6-oxo-3,5-diol