Synthesis of a Fatty Tetrahydroxyamide Using Peroxygenase from Oat Seeds

George J. Piazza* and Thomas A. Foglia

USDA, ARS, Wyndmoor, Pennsylvania 19038

ABSTRACT: Prior work has shown that oat (*Avena sativa*) seeds are a rich source of lipase and peroxygenase. Partial epoxidation of the isobutyl amide derivative of α-linolenic acid with peroxygenase gave *N-i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide, a diepoxide product in which the epoxides reside only at the formerly external double bond positions. No amide hydrolysis occurred during the epoxidation procedure. Hydrolysis of the diepoxide gave *N-i*-butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide, a polyol derivative with relatively high polarity, potentially useful in developing new materials from fats and oils.

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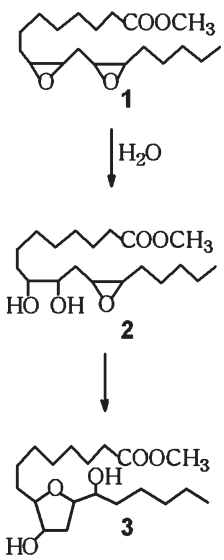
KEY WORDS: *t*-Butylhydroperoxide, *N-i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide, *N-i*-butyl-9(*Z*),12(*Z*),15(*Z*)-octadecatrienamide, *N-i*-butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide, hydroperoxide, linolenate amide, peroxygenase.

Ricinoleic acid from castor oil is the only commercially available hydroxylated FA. It is used in a number of industrial formulations that require a fatty material with increased viscosity and polarity. It would be highly desirable to find an easy synthetic route to fatty substances with multiple hydroxy groups, as these could be components of emulsifiers, high-temperature greases, and metal-working fluids. Epoxy fatty esters are commercially available, and it would seem that their hydrolysis would be a simple route to polyhydroxy fatty esters. The monoepoxide derived from methyl oleate is cleanly converted to its diol derivative by hydrolysis (1). In contrast, it has been shown that hydrolysis of methyl 9,10-12,13-diepoxyoctadecanoate **1** (Scheme 1) with epoxide hydrolase, sulfuric acid-activated bleaching earth, alumina, or perchloric acid gives cyclic products (1–3). Diepoxide **1** is partially hydrolyzed to give the diol **2**, which rapidly rearranges to give various stereo- and regioisomers of dihydroxy THF, such as **3** (methyl 9,12-epoxy-10,13-dihydroxystearate). The tetrahydroxy fatty ester is difficult to form; there is a single report of its synthesis by diepoxide hydrolysis using extremely high levels of epoxide hydrolase (4). Recently we devised a new method for immobilization of the enzyme peroxygenase from oat seeds on hydrophobic membranes (5). We found that the diepoxide produced from



SCHEME 1

α-linolenic acid (ALA) by this peroxygenase is epoxidized almost exclusively at the 9 and 15 positions of the skipped triene double bonds of ALA (6). In the research reported here, we accomplished three goals. First, we showed that ground oat seeds can serve as a source of crude peroxygenase and present optimized reaction conditions to give high yields of fatty epoxides. Second, since the ground oat seeds contain an active lipase activity (7), we improved on the stability of the epoxy product by using an amide derivative rather than an ester. Third, we determined that the fatty amide diepoxide prepared from the amide of ALA could be hydrolyzed to a fatty tetraol, rather than to a THF derivative.

MATERIALS AND METHODS

Materials. Oat seeds (*Avena sativa* L.) were obtained from Davis Feed Mills (Perkasie, PA). Nu-Chek-Prep, Inc. (Elysian, MN) supplied trilinolenin. Isobutylamine was from Aldrich (Milwaukee, WI). Water was purified to a resistance of 18 megohm-cm using a Barnstead E-pure system. All other reagents were of the highest purity available.

Chromatographic and instrumental methods. Solutions of the amides in dichloromethane were filtered through 13 mm, 0.45 μm syringe filters (PVDF; Scientific Resources, Eatontown, NJ). Dichloromethane was removed under a stream of nitrogen, and the amide residues were dissolved in 1 mL

*To whom correspondence should be addressed at USDA, ARS, ERRC, 600 East Mermaid Lane, Wyndmoor, PA 19038.
E-mail: gpiazza@errc.ars.usda.gov.

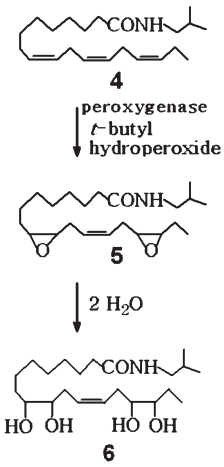
Abbreviations: ALA, α-linolenic acid; APCI, atmospheric pressure chemical ionization; RT, retention time; TMS, trimethylsilyl.

isopropanol. Reaction mixtures were separated on two Symmetry 3.5 μm C_{18} reversed-phase columns (150 × 2.1 mm and 50 × 2.1 mm) (Waters, Milford, MA) connected in series. Quantification of products was made using a Varex MK III ELSD (Alltech, Deerfield, IL) operated at 55°C, with N_2 as the nebulizing gas at a flow rate of 1.5 L/min. Mobile phase composition and gradient were 0–5 min H_2O/CH_3CN (40:60 vol/vol); 5–30 min H_2O/CH_3CN (40:60 vol/vol) to CH_3CN (100); 30–54 min CH_3CN (100) at a flow rate of 0.25 mL/min. Products were characterized by HPLC with mass detection using EI-MS (Thermabeam Mass Detector; Waters) and atmospheric pressure chemical ionization (APCI) HPLC-MS (Micromass ZMD; Waters). The EI-MS detector was set to scan the mass range of *m/z* 55–600 at 1000 amu/s and had an ionization energy of 70 eV. Ionization source, nebulizer, and expansion region temperatures were 200, 64, and 75°C, respectively. When using the APCI-MS, the HPLC gradient contained 0.1% formic acid, and the APCI-MS detector was set to scan in the mass range of *m/z* 150–550 at 400 amu/s. The corona, cone, and extractor voltages were 3700, 20, and 5 eV, respectively. The source and APCI heater temperatures were 150 and 400°C, respectively. ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectra were obtained as described previously (8).

N-i-*Butyl-9(*Z*),12(*Z*),15(*Z*)-octadecatrienamide* **4** (Scheme 2). Isobutyl amine (1.54 g, 21.0 mmol, Aldrich) was mixed with 2 g (2.29 mmol) trilinolenin (Nu-Chek-Prep) in a stoppered glass vial. The mixture was allowed to stand at 22°C for 10 d. Excess amine was removed by dissolving the mixture in 50 mL diethyl ether, washing with 2 × 30 mL 0.1 M HCl or until the pH was acidic, followed by 3 × 30 mL water. The amide **4** was obtained in 96% yield as a yellow oil.

HPLC-MS analysis of amide **4**: Retention time (RT): 26.5 min. APCI-MS: *m/z* (fragment, intensity), 334 ($[M + H^+(=1)]^+$, 100%). EI-MS: *m/z* (fragment, intensity), 333 ($[M]^+$, 4.2%), 276 ($[M - CH_2CH(CH_3)_2$ (=57)$]^+$, 5.4%), 198 ($[(CH_3)_2CHCH_2NHCO(CH_2)_7]^+$, 5.8%), 142 ($[(CH_3)_2$ CH–$CH_2NHCO(CH_2)_3]^+$, 14.2%), 128 ($[(CH_3)_2CHCH_2NH$–$CO(CH_2)_2]^+$, 86.6%), 115 ($[(CH_3)_2CHCH_2NHCOCH_3]^+$, 92.5%), 74 ($[(CH_3)_2CHCH_2NH]^+$, 100%). ^{1}H NMR (400 MHz, $CDCl_3$, δ_H): 0.88 (6H, *d*, *J* = 6.4, $(CH_3)_2$CHR), 0.95 (3H, *t*, *J* = 7.4, CH_3CH_2), 1.23–1.48 (10H, *m*, CH_2), 1.73 (1H, *m*, $(CH_3)_2CHCH_2NH$), 2.03 (4H, *m*, CH_2CH=CH), 2.14 (2H, *t*, *J* = 7.4, $CH_2C(O)NH$), 2.78 (4H, *m*, CH=CHCH_2CH=CH), 3.05 (2H, *t*, *J* = 6.8, CH_2NH), 5.32 (6H, *m*, CH=CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ_C): 14.3 (CH_3CH_2), 20.1, 20.2 ($(CH_3)_2$CHR), 25.6, 25.7, 27.3, 28.6, 29.2, 29.3, 29.6, 37.0 (CH_2CONH), 46.8 (CONHCH_2), 127.1, 127.6, 128.1, 130.1, 131.9 (*C*=*C*), 172.9 (*C*ONH).

N-i-*Butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide* **5** (Scheme 2). Crude preparations of peroxygenase were obtained from oat seeds as follows: Oat seeds (5 g) were ground in the mini cup of a Waring commercial blender for 1 min and then were washed with 2 × 40 mL diethyl ether. The washed ground oats were placed in a vacuum desiccator for 2 h and then added to 24 mL of pH 7.5 buffer (50 mM Hepes [*N*(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)] and 0.1% (wt/vol) Tween 20) and 50 mg (150 μmol) fatty amide **4**. The suspension was agitated at 25°C. The oxidant *t*-butyl hydroperoxide (Sigma) was added in a stepwise manner, since previous work showed that higher product yields are obtained as a result of less peroxygenase deactivation (5). At 0, 1, 2, and 4 h, 37.2 μmol of *t*-butyl hydroperoxide was added. At 6 h 226 μmol *t*-butyl hydroperoxide was added. The reaction was allowed to proceed for 24 h, and then the product, diepoxide **5**, was extracted with 2 × 50 mL diethyl ether. The combined ether extracts were washed with 2 × 40 mL water. The amide diepoxide **5** was obtained in 80–90% yield as a yellow oil.



SCHEME 2

HPLC-MS analysis of diepoxide amide **5**: RT: 7.2 min. APCI-MS: *m/z* (fragment, intensity), 366 ($[M + 1]^+$, 100%). EI-MS: *m/z* (fragment, intensity), 184 ($[(CH_3)_2CHCH_2NH$–$CO(CH_2)_6]^+$, 10.7%), 156 ($[(CH_3)_2CHCH_2NHCO(CH_2)_4]^+$, 21.4%), 128 ($[(CH_3)_2CHCH_2NHCO(CH_2)_2]^+$, 53.6%), 115 ($[(CH_3)_2CHCH_2NHCOCH_3]^+$, 78.6%), 74 ($[(CH_3)_2CH$–$CH_2NH_3]^+$, 94.6%). ^{1}H NMR (400 MHz, $CDCl_3$, δ_H): 0.91 (6H, *d*, *J* = 6.8, $(CH_3)_2$CHR, 1.06 (3H, *t*, *J* = 7.6, CH_3CH_2), 1.22–1.42 (14H, *m*, CH_2), 1.76 (1H, *m*, $(CH_3)_2CHCH_2NH$), 2.18 (2H, *t*, *J* = 7.6, $CH_2C(O)NH$), 2.30 (4H, *m*, CH(O)CH–CH_2CH=CH), 2.95 (4H, *m*, CH(O)CH), 3.08 (2H, *t*, *J* = 6.4, CH_2NH), 5.61 (2H, *bs*, CH=CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ_C): 10.6 (CH_3CH_2), 20.0, 21.0 ($(CH_3)_2$CHR), 25.2, 25.7, 26.3, 26.4, 26.8, 27.1, 27.7, 28.4, 29.1, 29.2, 36.8 (CH_2CONH), 46.7 (CONHCH_2), 54.1, 56.2, 57.1, 58.3 (*C*H(O)), 126.8 (*C*=*C*), 173.2 (*C*ONH).

N-i-*Butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide* **6** (Scheme 2). Diepoxide **5** (100 mg) was placed into a screw-capped glass vial together with 6 mL of THF/water (3:2 vol/vol) containing 0.5% (by vol) $HClO_4$. The vial was attached to a laboratory rotator (radius of 17 cm) and rotated at 3.5 revolutions per minute for 24 h. Water (10 mL) was added to the hydrolysis mixture, and the product was extracted with 2 × 30 mL diethyl ether. The combined ether fractions were washed with 2 × 20 mL 2% $NaHCO_3$. The tetrahydroxy amide **6** was obtained in 90% yield as a yellow oil.

HPLC-MS analysis of tetrahydroxy amide **6** (Scheme 2): RT: 2.8 min. APCI-MS: *m/z* (fragment, intensity), 402 ($[M + 1]^+$, 100%). EI-MS: *m/z* (fragment, intensity), 258 ($[(CH_3)_2CH–CH_2NHCO(CH_2)_7CH(OH)CH(OH)]^+$, 26.3%), 228 ($[258 – CH(OH)\ (=30)]^+$, 54.4%), 184 ($[(CH_3)_2CHCH_2NH–CO(CH_2)_6]^+$, 11.4%), 156 ($[(CH_3)_2CHCH_2NHCO(CH_2)_4]^+$, 6.1%), 128 ($[(CH_3)_2CHCH_2NHCO(CH_2)_2]^+$, 36.8%), 115 ($[(CH_3)_2CHCH_2NHCOCH_3]^+$, 67.5%), 74 ($[(CH_3)_2CH–CH_2NH_3]^+$, 100%). NMR: See Table 1.

Trimethylsilyl (TMS) derivative of **6**: APCI-MS: *m/z* (fragment, intensity), 690 ($[M + 1]^+$, 100%). EI-MS: *m/z* (fragment, intensity), 558 ($[M – ([CH_3CH_2CH(OTMS)\ (=131)]^+$, 0.8%), 402 ($[(CH_3)_2CHCH_2NHCO(CH_2)_7CH(OTMS)CH(OTMS)]^+$, 19.6%), 312 ($[402 – TMSOH\ (=90)]^+$, 19.5%), 300 ($[402 – CH(OTMS)\ (=102)]^+$, 46.4%), 233 ($[CH_3CH_2CH(OTMS)–CH(OTMS)]^+$, 12.5%), 131 ($[CH_3CH_2CH(OTMS)]^+$, 25.0%).

RESULTS AND DISCUSSION

The synthetic pathway for production of the tetrahydroxy amide **6** is shown in Scheme 2. The synthesis of isobutyl amide **4** was based on our earlier work with fatty amides in which an excess of amine is added to a TG (9). The alkyl amine functions as both a reactant and a catalyst. The amide product **4** was produced in 96% yield and had an RT on HPLC of 26.5 min. The APCI-MS spectrum of amide **4** gave a base peak at *m/z* 334 ($[M +1]^+$, 100%) (Fig. 1). The EI-MS spectrum of **4** gave prominent ions at *m/z* 333 ($[M]^+$, 4.2%), 115 ($[(CH_3)_2CHCH_2NHC(O)CH_3]^+$, 92.5%), and 74 ($[(CH_3)_2CH–CH_2NH]^+$, 100%), confirming amide formation. The ^{1}H NMR spectrum of **4** had triplet resonances at δ_H 2.14 (2H, *t*, *J* = 7.4, C*H*$_2$C(O)NH) and 3.05 (2H, *t*, *J* = 6.8, C*H*$_2$NH), and the ^{13}C NMR spectrum had resonances at δ_C 37.0 (*C*H$_2$CONH), 46.8 (CONH*C*H$_2$), and 172.9 (*C*ONH), thus confirming the amide formation. The presence of three double bonds was demonstrated by signals at δ_H 5.32 (6H, *m*, C*H*=C*H*), although only five resonances were visible in the ^{13}C NMR spectrum: δ_C 127.1, 127.6, 128.1, 130.1, 131.9 (*C*=*C*).

In our previous work with methyl linolenate, epoxidation ceased after diepoxide formation and little triepoxide was formed. However, with the amide substrate, some triepoxide (*N-i*-butyl-9,10-12,13-15,16-triepoxyoctadecamide) was obtained, and the amount of triepoxide increased with increasing amounts of *t*-butyl hydroperoxide up to a 45% yield of triepoxide in 24-h reactions (highest amount of *t*-butyl hydroperoxide shown in Fig. 2). Further increases in the amount of *t*-butyl hydroperoxide gave lower overall yields of di- and triepoxide, presumably as a result of peroxygenase inactivation, as we

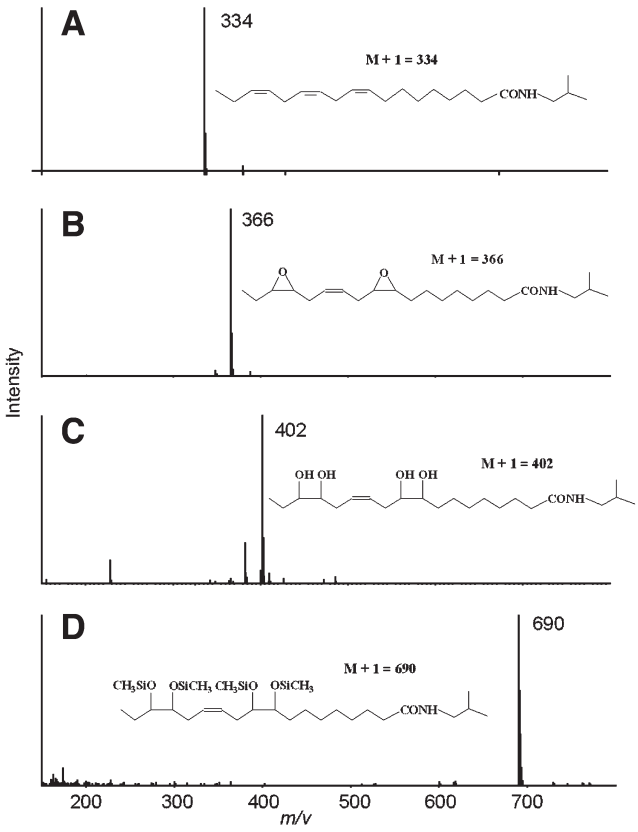


FIG. 1. Atmospheric pressure chemical ionization-MS of (A) *N-i*-butyl-9(*Z*),12(*Z*),15(*Z*)-octadecatrienamide, **4**, (B) *N-i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide, **5**, (C) *N-i*-butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide, **6**, and (D) its trimethylsilyl derivative.

TABLE 1
^{1}H NMR and ^{13}C NMR[a] of *N-i*-Butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide, 6 (retention time: 2.8 min)

Chemical shift (400 MHz, $CDCl_3$, δ_H)	Number of protons	Appearance	Assignment	Coupling constant (Hz)
0.83	6H	*d*	(C*H*$_3$)$_2$CHR	*J* = 6.4
0.94	3H	*t*	C*H*$_3$CH$_2$	*J* = 7.2
1.22–1.64	14H	*m*	C*H*$_2$	
1.74	1H	*m*	(CH$_3$)$_2$C*H*CH$_2$NH	
2.16	2H	*t*	C*H*$_2$C(O)NH	*J* = 7.6
2.33	4H	*m*	(HO)CHC*H*$_2$CH=CH)	
3.06	2H	*t*	C*H*$_2$NH	*J* = 6.4
3.90	4H	*m*	C*H*OH	
5.56	2H	*bs*	C*H*=C*H*	

[a]^{13}C NMR (100 MHz, $CDCl_3$, δ_C): 9.1 (*C*H$_3$CH$_2$), 20.1 ((*C*H$_3$)$_2$CHR), 24.7, 25.7, 28.5, 28.8, 28.9, 33.9, 35.5, 36.8 (*C*H$_2$CONH), 46.7 (CONH*C*H$_2$), 77.6, 80.3, 80.9, 82.0 (*C*HOH), 127.0 (*C*=*C*), 173.9 (*C*ONH).

observed in our previous work with immobilized peroxygenase (10). Preliminary analysis of products obtained after triepoxide hydrolysis indicated substantial conversion to cyclic products. As we wished to avoid the generation of these and to maximize synthesis of the diepoxide **5**, it was necessary to determine the optimal amount of *t*-butyl hydroperoxide to be used. For a 24-h reaction period, the best yields of diepoxide **5** were obtained when the molar ratio of *t*-butyl hydroperoxide to amide **4** was 2.5:1 (lowest amount of *t*-butyl hydroperoxide shown in Fig. 2). Under these conditions, the mean conversion to diepoxide (*N*-*i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide) **5** was 82%. It is noted, however, that high yields of diepoxide **5** can be obtained even with high levels of *t*-butyl hydroperoxide by shortening the reaction time. For example when an eightfold higher amount of *t*-butyl hydroperoxide was added at 0, 1, and 2 h, and the product was extracted at only 4 h, a yield of diepoxide **5** of over 80% was still obtained. It is also noted that optimal conditions (reaction time and oxidant levels) for diepoxide formation will vary depending on the activity of the oat seed peroxygenase. Therefore, it is always necessary to assay the level of diepoxide and to adjust conditions to maximize this level.

The APCI-MS spectrum of diepoxide amide **5** gave a base peak at *m/z* 366 ($[M + 1]^+$, 100%) (Fig. 1). The EI-MS of **5** gave prominent ions at *m/z* 184 ($[(CH_3)_2CHCH_2NHCO(CH_2)_6]^+$, 10.7%), 115 ($[(CH_3)_2CHCH_2NHCOCH_3]^+$, 78.6%), and 74 ($[(CH_3)_2CHCH_2NH_3]^+$, 94.6%), which confirmed the amide structure. The ^{1}H NMR spectrum had a multiplet resonance at δ_H 2.95 (4H, *m*, C*H*(O)C*H*), and the ^{13}C NMR spectrum showed resonances at δ_C 54.1, 56.2, 57.1, 58.3 (*C*H(O)), establishing the presence of two epoxide rings on the fatty amide chain. The presence of the remaining double bond is shown by the resonance at δ_H 5.61 (2H, *bs*, C*H*=C*H*) and the resonance at δ_C 127.0 (*C*=*C*). Only the central double bond at C-12–C-13 remains because the double bond proton resonances are a broad singlet, and this can occur only if the diepoxide structure is nearly symmetrical. In our prior research we found that immobilized peroxygenase also converted linolenic acid to a symmetrical diepoxide (6).

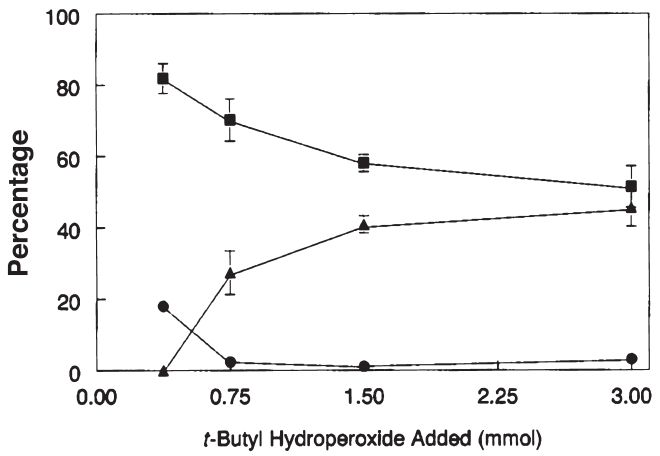


FIG. 2. Percentage of *N*-*i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide, **5** (■), *N*-*i*-butyl-9,10-12,13-15,16-triepoxyoctadecamide (▲), and *N*-*i*-butyl-9(*Z*),12(*Z*),15(*Z*)-octadecatrienamide, **4** (●), after 24 h reaction of starting material **4** with peroxygenase using different amounts of *t*-butylhydroperoxide as the oxidant. The amount of *t*-butylhydroperoxide added, from left to right, is as follows: The first entry is the amount in millimoles, and the entry in parentheses is the time of addition in hours: .0372 (0), .0372 (1), .0372 (2), .0372 (4), .226 (6); .0744 (0), .0744 (1), .0744 (2), .0744 (4), .452 (6); .149 (0), .149 (1), .149 (2), .149 (4), .905 (6); .298 (0), .298 (1), .298 (2), .298 (4); 1.810 (6). Other reaction conditions are described in the Materials and Methods section. Each data point is the mean ± SE (n = 4–6).

We investigated hydrolysis of diepoxide **5** with dilute perchloric acid and found that a 90% yield of tetraol **6** was obtained based on diepoxide **5**. The APCI-MS spectrum of tetrahydroxy amide **6** gave a base peak at *m/z* 402 ($[M + 1]^+$, 100%, and its TMS derivative gave a base peak at *m/z* 690 ($[M + 1]^+$, 100% (Fig. 1). The EI-MS spectrum of **6** had prominent ions at *m/z* 258 ($[(CH_3)_2CHCH_2NHCO(CH_2)_7CH(OH)CH(OH)]^+$, 26.3%, demonstrating the presence of a diol at C-9 and C-10. This is also shown by the presence of the EI-MS ion for the TMS derivative of **6** at *m/z* 402 ($[(CH_3)_2CHCH_2NHCO(CH_2)_7$–$CH(OTMS)CH(OTMS)]^+$, 19.6%). The presence of hydroxy groups on C-15 and C-16 is demonstrated by the EI-MS ion for the TMS derivative of **6** at *m/z* 233 ($[CH_3CH_2CH(OTMS)$–$CH(OTMS)]^+$, 12.5%). The ^{1}H NMR and ^{13}C NMR spectra of **6** are shown in Table 1. The ^{1}H NMR spectrum showed multiplet resonance at δ_H 3.90 (4H, *m*, C*H*COH), and the ^{13}C NMR spectrum showed resonances at δ_C 77.6, 80.3, 80.9, and 82.0 (*C*HOH), establishing the presence of four hydroxy groups in the amide. The presence of one remaining double bond is shown by the resonance at δ_H 5.56 (2H, *bs*, C*H*=C*H*) and the resonance at δ_C 127.0 (*C*=*C*). As in compound **5**, only the central double bond at C-12–C-13 remains because the proton resonances are found as a broad singlet, and this can occur only if the structure is relatively symmetrical.

Finally, we emphasize that the reason that diepoxide **5** forms tetraol rather than the THF formed by diepoxide **1** is the presence of the intervening C-12–C-13 double bond in diepoxide **5**. Diepoxide **5** is also an amide derivative, but this does not influence the nature of its hydrolysis product. We have demonstrated this by preparing the tetraol from the diepoxide of the methyl ester of ALA. The methyl ester of the tetraol will form higher-M.W. estolides under a variety of conditions, whereas the amide derivative does not; therefore, amide tetraol **6** is more useful for certain applications. We are investigating ways to increase the scale of production of tetraol **6** using this synthetic route to provide material for testing in lubricant and functional fluid applications.

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