Preparation of Fatty Amide Polyols *via* Epoxidation of Vegetable Oil Amides by Oat Seed Peroxygenase

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ABSTRACT: Prior work has shown that oat (*Avena sativa*) seeds are a rich source of peroxygenase, an enzyme that promotes the oxidation of carbon–carbon double bonds to form epoxides. Ground and defatted oat seeds were used as a low-cost source of peroxygenase. A systematic study of the epoxidation of *i*-butyl amides from linseed oil was conducted. Hexane was used as the primary component of the reaction media to eliminate the need for extraction. We found that the addition of a small amount of buffered water containing Tween 20 enhanced the epoxidation activity when using *t*-butyl hydroperoxide and cumene hydroperoxide as oxidants. This activity could be further enhanced by the addition of isopropyl ether. Conditions for larger-scale reactions were developed and applied to amides prepared from linseed, soybean, and canola oils. Because of enzymatic selectivity, the epoxidation of adjacent double bonds was low, and monoepoxides from the amides of oleate and linoleate predominated; the diepoxide, *N*-*i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide, was obtained from the amide of linolenate. The enzymatically epoxidized amides from the oils were hydrolyzed in dilute acid, and the distribution of the various classes of polyols was determined. Reflecting the high proportion of starting monoepoxides, saturated diols and diols with one double bond were the major polyols obtained from soybean and canola oils. Because linseed oil contains a high proportion of linolenate, polyols obtained from the epoxides of this oil had a major amount of the tetrol, *N*-*i*-butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide. In contrast, the components of polyols obtained from the hydrolysis of commercial epoxide preparations of soybean and linseed methyl esters followed by amide formation were primarily saturated diols and furan derivatives resulting from the presence of adjacent epoxide groups in these preparations.

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KEY WORDS: *N*-*i*-Butyl-9,10-dihydroxyoctadecanamide, *N*-*i*-butyl-9,10-dihydroxy-12(Z)-octadecenamide, *N*-*i*-butyl-9,10-dihydroxy-12(*Z*),15(*Z*)-octadecadienamide, *N*-*i*-butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide, *N*-*i*-butyl-9,12-epoxy-10,13-dihydroxyoctadecanamide, *N*-*i*-butyl-10,13-epoxy-9,12-dihydroxyoctadecanamide, *N*-*i*-butyl-12,13-dihydroxy-9(*Z*)-octadecenamide, *N*-*i*-butyl-15,16-dihydroxy-9(*Z*),12(*Z*)-octadecadienamide, hydroperoxide, peroxygenase.

Epoxides of fats and oils and their ester derivatives are used as plasticizers and stabilizers of plastics. Since fatty epoxides can be converted to a number of chemical derivatives, there has been increasing interest in using these substances as intermediates in the production of new materials from fats and oils. As such, we have investigated the use of a peroxygenase enzyme found in oat seeds as a safe alternative to the currently used peracid epoxidation system (1–4). We used oat seed microsomes as a source of peroxygenase (1). Preparation of the oat seed microsomes is time-consuming, and the stability of the resulting peroxygenase preparations is limited. We immobilized peroxygenase on hydrophobic membranes to reduce its preparation time and improved its stability, thereby reducing its cost (2–4). To achieve more cost-effective preparations, we have been investigating the use of defatted, ground oat seeds as a source of peroxygenase. The reduction in cost achieved by using the ground oat seeds is offset by complications caused by the presence of an active lipase in the oat seeds. We found that secondary fatty amides are resistant to the action of the lipase but are still good substrates for peroxygenase. An additional advantage of using the fatty amides for substrates is that polyol hydrolysis products will not polymerize, and the physical properties of the resulting products can be modified by changing the chemical nature of the amine used for amide formation. Fatty amides have low reactivity and are used as detergents, shampoos, lubricants, cosmetics, foam control agents, and water repellents (5). Reported here are the results of our investigations of the epoxidation of amide derivatives of three common vegetable oils. These fatty amide epoxides were converted to polyols upon hydrolysis. The chemical structures of the epoxides and polyols were determined.

MATERIALS AND METHODS

Materials. Oat (*Avena sativa* L.) seeds were obtained from Davis Feed Mills (Perkasie, PA). Nu-Chek-Prep, Inc. (Elysian, MN) supplied trilinolenin. Raw linseed oil was purchased from Sunnyside Corp. (Wheeling, IL). Isobutylamine was from Aldrich (Milwaukee, WI). Water was purified to a resistance of 18 megohm-cm using a Barnstead E-pure system. Cumene hydroperoxide (80%) and *t*-butyl hydroperoxide (70%) were from Sigma (St. Louis, MO). Epoxidized methyl soyate (Vikoflex 7010) and epoxidized methyl linseedate (Vikoflex 9010) were gifts from Atofina Chemicals, Inc. (Philadelphia, PA). All other reagents were of the highest purity available.

Hydroxylated fatty isobutyl amides (*Scheme 1*). A three-step procedure was used. In the first step, fatty amides were prepared by mixing isobutylamine (8.0 g, 0.109 mol) with linseed, soybean, or canola oil (12 g) in a stoppered glass vial. The mixture

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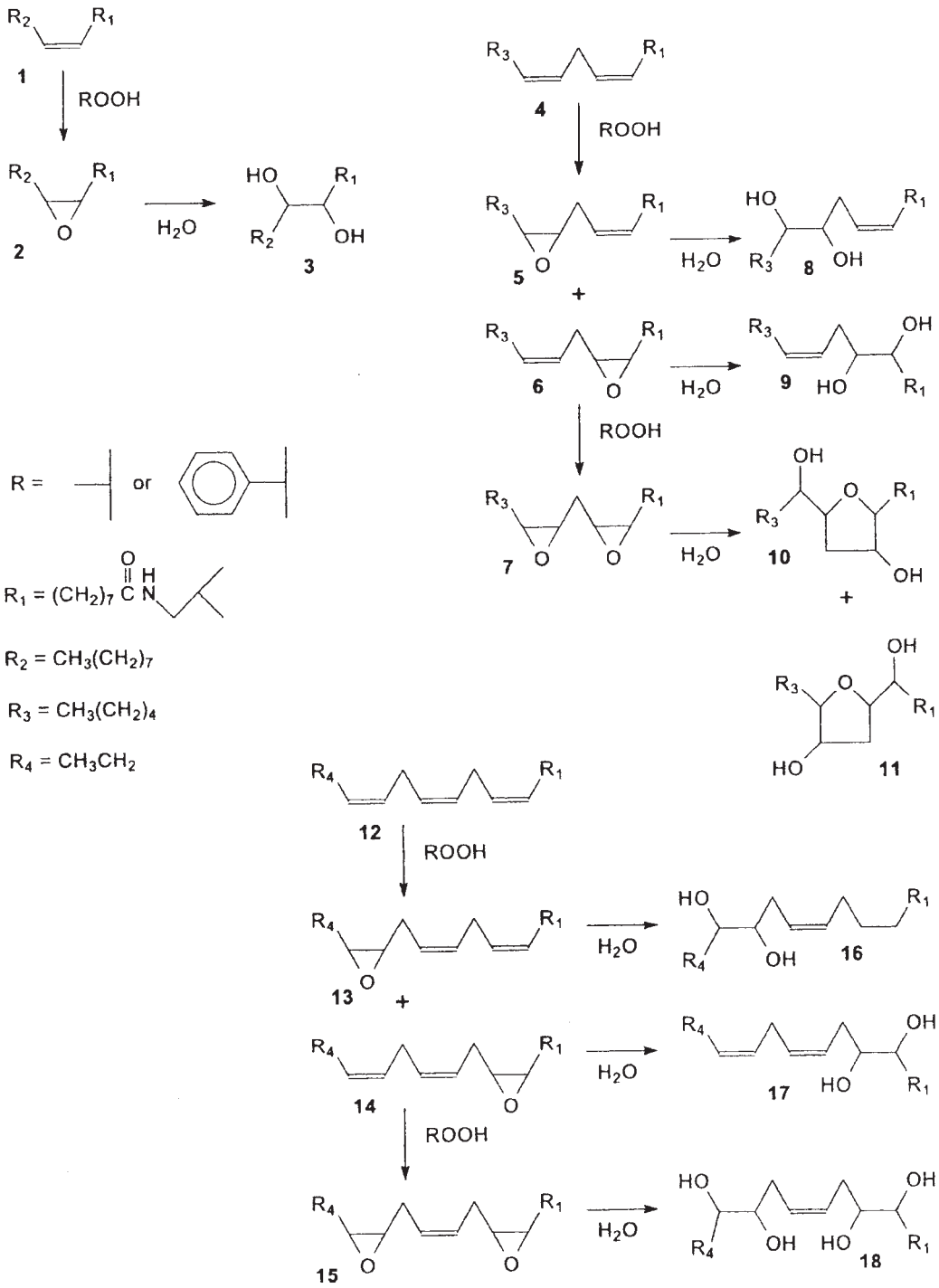

SCHEME 1

was allowed to stand at 22°C for 10 d. Excess amine was removed by dissolving the mixture in diethyl ether (500 mL) and washing with 0.1 M HCl until the pH of the aqueous layer was acidic, followed by three washes with water. The primary unsaturated fatty esters found in the oils are oleate, linoleate, and linolenate. Therefore, the unsaturated amides that were formed were *N-i*-butyl-9(*Z*)-octadecenamide **1**, *N-i*-butyl-9(*Z*),12(*Z*),15(*Z*)-octadecatrienamide **4**, and *N-i*-butyl-9(*Z*),12(*Z*)-octadecadienamide **12**. Analysis by TLC and HPLC showed that the amides contained no residual TG oil.

The unsaturated amides were exposed to the action of oat seed peroxygenase to promote epoxide formation. The oat seeds (60 g) were ground in a Magic Mill III Plus high-speed flour mill (Monsey, NY) and then defatted with diethyl ether (300 mL). In a typical reaction procedure, fatty isobutyl amides (0.9 g) were mixed with the ground, defatted oat seeds in 288

mL of hexane, 19.2 mL of isopropyl ether, and 4.8 mL of 0.15 M Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)] (pH 7.5) containing 0.1% Tween 20. The mixture was shaken at 15°C for 24 h. At 0, 1, and 6 h, 163.2 µL (0.9 mmol) of cumene hydroperoxide was added. At 2 and 4 h, 81.6 µL (0.45 mmol) of cumene hydroperoxide was added. At the end of the reaction, the mixture was sequentially vacuum filtered through Whatman 113 filter paper (150 mm), Whatman 1 filter paper (150 mm), and Millipore 0.45 µm Durapore Membrane (47 mm). The reaction vessel and all filters were rinsed with 200 L of diethyl ether. The filtrate and wash were combined, and the solvent was removed under flowing nitrogen to give the product (**2**, **5**, **6**, **7**, **13**, **14**, and **15**; Scheme 1). Smaller-scale reactions were run to determine the optimal reaction conditions for enzymatic epoxidation; these conditions are given in the figure legends. Approximately 40 µmol of fatty epoxide was generated per gram oat seeds in 24 h.

In the third step, the aforementioned fatty epoxidized isobutylamides were hydrolyzed by placing them in 115 mL of THF containing 76 mL of water and 950 µL of concentrated (70%) perchloric acid. The reaction mixture was shaken at 15°C for 24 h, diluted with 400 mL of diethyl ether, and washed with 2 × 100 mL of water. The water layers were reextracted with 200 mL of diethyl ether, and the combined ether fractions were washed with 100 mL of 2% $NaHCO_3$. The ether was removed under a stream of nitrogen to give polyol amides (**3**, **8**, **9**, **10**, **11**, **16**, **17**, and **18**; Scheme 1).

Chromatographic and instrumental methods. Fatty amides, epoxidized derivatives, and hydrolyzed epoxide products, dissolved in isopropanol, were separated on Symmetry 3.5 µm C_{18} reversed-phase columns (150 × 2.1 mm and 50 × 2.1 mm; Waters, Milford, MA). Quantification of products was done 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 compositions and gradients for the analysis of fatty amides and epoxidized derivatives 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); and 30–54 min CH_3CN (100) at a flow rate of 0.25 mL/min. Mobile phase compositions and gradients for the analysis of hydrolyzed epoxides were 0–5 min H_2O/CH_3CN (90:10 vol/vol); 5–30 min H_2O/CH_3CN (90:10 vol/vol) to CH_3CN (100); and 30–64 min CH_3CN (100) at a flow rate of 0.12 mL/min. Reported yields were determined by comparing the peak areas of the products to the total peak area.

Product characterization. Starting amides and their epoxy and polyol derivatives 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) using the aforementioned HPLC conditions, except that when using APCI-MS, the HPLC gradient contained 0.1% formic acid. The EI-MS detector was set to scan over the mass range of *m/z* 55–600 at 1000 amu per s and had an ionization energy of 70 eV. The ionization source, nebulizer, and expansion region temperatures were 200, 64, and 75°C, respectively. The APCI-MS detector was set to scan over the mass range of *m/z* 150–550 at 400 amu per 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.

RESULTS AND DISCUSSION

Optimizing reaction conditions for the epoxidation of linseed oil amides. Hexane was chosen as the main component of the reaction medium because workup is relatively easy compared with an aqueous medium. Epoxide products were separated from the oats by sequential filtration, and the solvent was removed by evaporation. Although the product epoxides are contaminated with residual buffer, this is removed after hydrolysis of the epoxides to polyols. After preparation of the isobutylamide derivatives of linseed oil, the ratio of ground oats, amide, and hydroperoxide oxidant was determined by trial and error to give the maximal yield of epoxides. After completing this screening, experiments were undertaken to determine the optimal amount of aqueous buffer (pH 7.5) to add to the hexane. The pH of the buffer was chosen based on prior work (3). The results of experiments using the oxidants cumene hydroperoxide and *t*-butyl hydroperoxide are shown in Figure 1, which illustrates that the addition of aqueous buffer resulted in a substantial increase in the yield of epoxides. The maximal yield of epoxides (approximately 70%) occurred with the addition of 0.8 mL of buffer. The results obtained with cumene hydroperoxide and *t*-butyl hydroperoxide were nearly identical. Increasing the amount of buffer beyond 0.8 mL caused the yield of epoxide to be reduced.

The starting fatty amides were not entirely soluble in either aqueous buffer or hexane. A more highly polar organic solvent might be beneficial. However, care must be taken as most polar

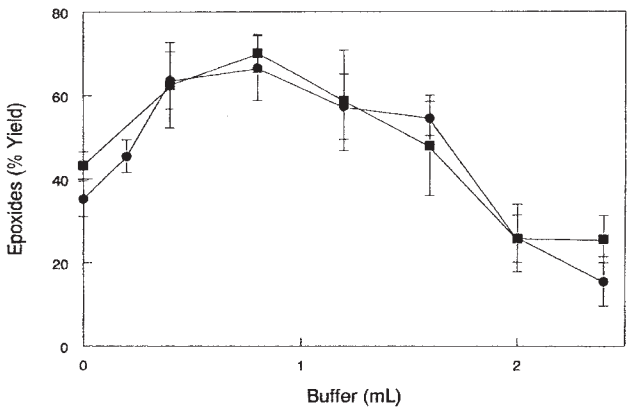

FIG. 1. The effect of aqueous buffer on the epoxidation of linseed isobutyl amides using cumene hydroperoxide (●) and *t*-butyl hydroperoxide (■) as the oxidants. Linseed isobutyl amides (75 mg) were added to ground, defatted oat seeds (5 g) in 24 mL of hexane, with different amounts 0.15 M Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)] buffer (pH 7.5) containing 0.1% Tween 20. The mixture was shaken at 15°C for 24 h. At 0, 1, and 6 h, 75 µmol of cumene hydroperoxide or *t*-butyl hydroperoxide was added. At 2 and 4 h, 37.5 µmol of cumene hydroperoxide or *t*-butyl hydroperoxide was added. Data are the mean ± SE for $n = 4$.

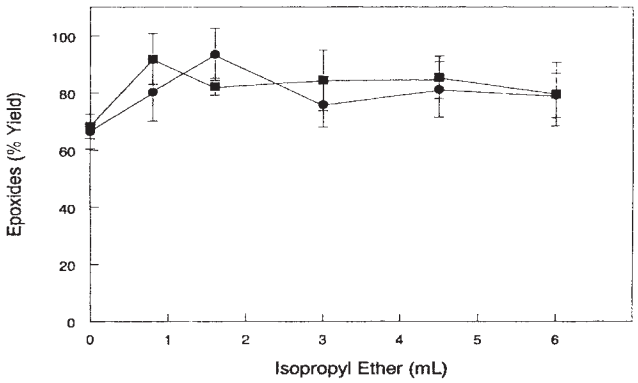

FIG. 2. The effect of isopropyl ether on the epoxidation of linseed isobutylamides using cumene hydroperoxide (●) and *t*-butyl hydroperoxide (■) as the oxidants. Linseed isobutylamides (75 mg) were added to ground, defatted oat seeds containing the indicated amounts of isopropyl ether, hexane, and 0.8 mL of Hepes/Tween buffer. The total volume of hexane and isopropyl ether was kept fixed at 24 mL. The reaction conditions and additions of oxidant were as described in Figure 1.

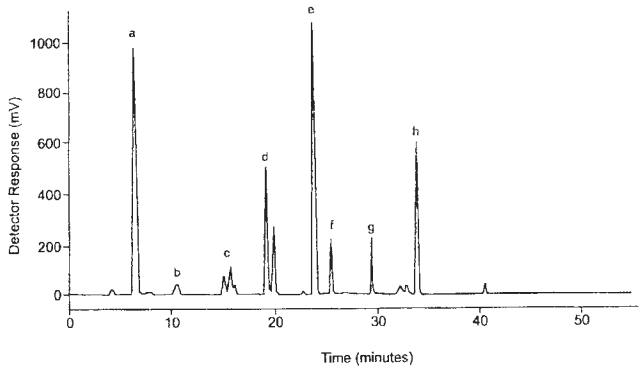

FIG. 3. Typical analysis of epoxidized linseed isobutylamides. Linseed isobutylamides were epoxidized as described in the Materials and Methods section, and the figure shows the signal trace of an ELSD from HPLC. The compositions of the gradient and the column are described in the Materials and Methods section. Composition of peaks: (a) diepoxide from linolenate amide (*N-i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide **15**); (b) diepoxide from linoleate amide (*N-i*-butyl-9,10-12,13-diepoxyoctadecanamide **7**); (c) monoepoxides from linolenate (*N-i*-butyl-9,10-epoxy-12(*Z*),15(*Z*)-octadecadienamide **14** and *N-i*-butyl-15,16-epoxy-9(*Z*),12(*Z*)-octadecadienamide **13**); (d) monoepoxides from linoleate amide (*N-i*-butyl-9,10-epoxy-12(*Z*)-octadecenamide **6** and *N-i*-butyl-12,13-epoxy-9(Z)-octadecenamide **5**); (e) epoxide from oleate amide (*N-i*-butyl-9,10-epoxyoctadecanamide **2**); (f) amide derivative of linolenate (*N-i*-butyl-9(*Z*),12(*Z*),15(*Z*)-octadecatrienamide **12**); (g) amide derivative of linoleate (*N-i*-butyl-9(*Z*),12(*Z*)-octadecadienamide **4**); (h) amide derivative of oleate (*N-i*-butyl-9(*Z*)-octadecenamide 1).

organic solvents rapidly reduce enzymatic activity, and indeed the addition of ethanol or diethyl ether reduced the yield of epoxide. However, the addition of isopropyl ether increased the yield of epoxide to approximately 90%, as shown in Figure 2.

Epoxides from vegetable oil amides. A typical HPLC analysis with detection by ELSD of a mixture of the isobutyl linseed amide precursors and their epoxidized products is shown in Figure 3. The epoxides were produced by reaction with peroxygenase using cumene hydroperoxide as the oxidant. The structures of the epoxide components and their precursors were determined using HPLC-MS with detection by APCI; the starting amides and their epoxidized products all gave strong M + 1 peaks. Structural confirmation was done using HPLC-MS with EI detection. Peaks f, g, and h are the remaining unreacted linseed oil amides derived from linolenate, linoleate, and oleate, respectively. The major oxidized products are the monoepoxides formed from oleate amide (*N-i*-butyl-9,10-epoxyoctadecanamide **2**; peak e), the isomeric monoepoxides formed from linoleate amide (*N-i*-butyl-9,10-epoxy-12(*Z*)-octadecenamide **6** and *N-i*-butyl-12,13-epoxy-9(*Z*)-octadecenamide **5**; peaks d), and the diepoxide formed from linolenate amide (*N-i*-butyl-9,10-15,16-diepoxy-12(*Z*)-octadecenamide **15**; peak a). Observed in lesser amounts were the diepoxide formed from linoleate amide (*N-i*-butyl-9,10-12,13-diepoxyoctadecanamide **7**; peak b) and the isomeric monoepoxides formed from linolenate (*N-i*-butyl-9,10-epoxy-12(*Z*),15(*Z*)-octadecadienamide **14** and *N-i*-butyl-15,16-epoxy-9(*Z*),12(*Z*)-octadecadienamide **13**; peaks c).

Products from the acid hydrolysis of epoxides. Table 1 lists the distribution of polyol products obtained after hydrolysis of the epoxide amides prepared from linseed, soybean, and canola oils. Five major polyol products were produced; all are either diols or a tetrol except for the second entry, which is labeled as a THF-diol. This cyclized product arises from the acid hydrolysis of adjacent epoxides separated by a methylene group as described previously (6–9). From linseed oil three products are dominant: the tetrol from the diepoxide prepared from linolenate amide (*N-i*-butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide **18**), the diols obtained from the monoepoxide of linoleate amide (*N-i*-butyl-9,10-dihydroxy-12(*Z*)-octadecenamide **9** and *N-i*-butyl-12,13-dihydroxy-9(*Z*)-octadecenamide **8**), and the diol obtained from the monoepoxide of oleate amide (*N-i*-butyl-9,10-dihydroxyoctadecanamide **3**). From soybean oil two products are dominant: the diols obtained from the monoepoxide of linoleate amide (*N-i*-butyl-9,10-dihydroxy-12(*Z*)-octadecenamide **9** and *N-i*-butyl-12,13-dihydroxy-9(*Z*)-octadecenamide **8**), and the diol obtained from the monoepoxide of oleate amide (*N-i*-butyl-9,10-dihydroxyoctadecanamide **3**). For canola oil there is one dominant product: the diol obtained from the monoepoxide of oleate amide (*N-i*-butyl-9,10-dihydroxyoctadecanamide **3**). For linseed, soybean, and canola oils, the levels of the hydrolysis products arising from the epoxidized amide of linolenate (products **16**, **17**, and **18**) were lower than expected from the level of linolenate in the oil. This decrease is explained by a loss of linolenate during amide synthesis.

For the purpose of creating new oleochemical products from epoxy fats and oils, it is very important to realize that the products obtained here are different from those that would be obtained from commercial epoxidized oils or esters because generally, the epoxidation level is very high in a commercial product. For example, it would be predicted that upon hydrolysis of a commercial epoxide preparation, only THF derivatives would be obtained from the di- and triepoxides of linoleate and linolenate. We were able to confirm this with commercially available epoxidized methyl linseedate and epoxidized methyl soyate.

TABLE 1
Distribution of Polyol Hydrolysis Products Obtained from Peroxygenase Epoxidation of Several Oil-Derived Isobutylamides

Oil	Tetrol, 1 db[a]	Diol, THF[b]	Diol, 2 db[c]	Diol, 1 db[d]	Diol[e]
			%		
Linseed	26.6	2.0	6.4	30.3	34.7
Soybean	2.0	1.9	4.1	50.1	41.9
Canola	ND[f]	2.7	ND	14.1	83.2

[a]Tetrol with one double bond (*N*-*i*-butyl-9,10,15,16-tetrahydroxy-12(*Z*)-octadecenamide **18**).
[b]Diol with a THF ring (*N*-*i*-butyl-9,12-epoxy-10,13-dihydroxyoctadecanamide **10** and *N*-*i*-butyl-10,13-epoxy-9,12-dihydroxyoctadecanamide **11**).
[c]Diol with two double bonds (*N*-*i*-butyl-9,10-dihydroxy-12(*Z*),15(*Z*)-octadecadienamide **17** and *N*-*i*-butyl-15,16-dihydroxy-9(*Z*),12(*Z*)-octadecadienamide **16**).
[d]Diol with one double bond (*N*-*i*-butyl-9,10-dihydroxy-12(*Z*)-octadecenamide **9** and *N*-*i*-butyl-12,13-dihydroxy-9(*Z*)-octadecenamide **8**).
[e]Diol with no unsaturation (*N*-*i*-butyl-9,10-dihydroxyoctadecanamide **3**).
[f]ND, not detected.

Direct analysis of these esters showed nearly complete double bond epoxidation. These epoxides were hydrolyzed and converted to their respective amides. Analysis of the amides using the same methods applied to the hydrolyzed enzymatically epoxidized samples showed almost exclusively the presence of THF derivatives and the 1,2-diol derived from the monoepoxide of oleate. The THF derivatives are very polar, but not as polar as the polyols prepared in this study; therefore, the hydrolyzed materials prepared from epoxides made with peroxygenase may in some instances be more suited for selected applications than those prepared from commercial epoxy oils or esters.

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