Soybean Lipoxygenase-Mediated Oxygenation of Monounsaturated Fatty Acids to Enones

Charles H. Clapp,*,+ Susan E. Senchak,+,† Theodore J. Stover,+,‡ Thomas C. Potter,+,§ Peter M. Findeis,+ and Mark J. Novak*,#

Department of Chemistry, Bucknell University Lewisburg, Pennsylvania 17837
Department of Medicinal Chemistry Walter Reed Army Institute of Research 503 Robert Grant Avenue, Silver Spring, Maryland 20910

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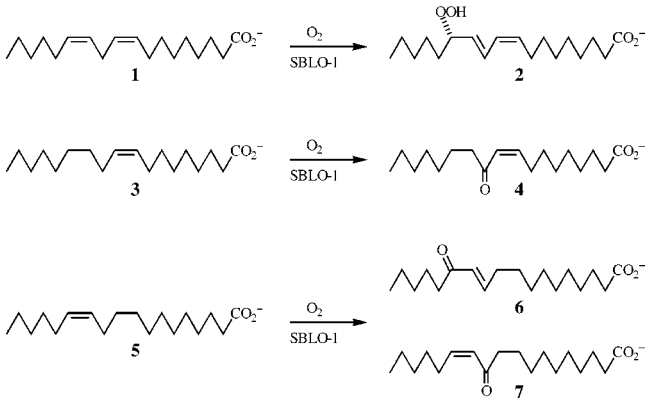
Soybean lipoxygenase-1 (SBLO-1) catalyzes the peroxidation of polyunsaturated fatty acids[1] and other 1,4-dienes[2] to produce chiral hydroperoxides. As illustrated in Scheme 1, SBLO-1 affords the conversion of linoleic acid (**1**) to 13(*S*)-hydroperoxy-9(*Z*),11-(*E*)-octadecadienoic acid (13-HPOD, **2**). We now report that SBLO-1 will catalyze the oxygenation of monounsaturated acids. Interestingly, the products are enones rather than the expected allylic hydroperoxides. Specifically, oleic acid (**3**) is converted primarily to 11-oxo-9(*Z*)-octadecenoic acid (**4**), and 12(*Z*)-octadecenoic acid (**5**) is converted to 13-oxo-11(*E*)-octadecenoic acid (**6**) plus a minor amount of 11-oxo-12(*Z*)-octadecenoic acid (**7**).

Initial experiments involved the incubation of 75 μM [1-^{14}C]-oleic acid[3] with 5 μM SBLO-1[4] and 5 μM 13-HPOD[5] at 25 °C in 50 mM borate buffer, pH 9.0. The reaction was monitored by reverse-phase HPLC with radioisotope and UV (234 nm) detection. After 2 h, the peak of radioactivity due to oleic acid had disappeared and was replaced by a lower mobility peak with two shoulders. Control experiments demonstrated that no reaction occurred in the absence of enzyme and that the reaction was much slower in the absence of 13-HPOD.[6] The products were also detectable by UV absorbance, and the major peak and the two shoulders could be resolved as distinct peaks with a ratio of 71:20:9. Utilizing a diode array detector it was found that the major product had an absorption maximum at 232 nm while the minor products had maxima at 225 nm. These data suggested that the products might be enones rather than the initially expected allylic hydroperoxides. Furthermore, no hydroperoxides could be detected during the course of the reaction by colorimetric assay with FOX reagent[7] or by enzymatic assay with glutathione peroxidase.[8]

Scheme 1



To obtain larger amounts of these products for structural characterization, we took advantage of the observation by Corey[9] that increased oxygen pressure increases the turnover rate of SBLO-1. Incubation of oleic acid (50 mg) with 250 mg of SBLO-1[10] with vigorous stirring under hyperbaric conditions (18 atm O_2) at 4 °C for 24 h in 50 mL of 0.1 M borate buffer, pH 9.0, led to the production of two new compounds in low yield. Control incubations in the absence of enzyme gave no measurable oxidation. After extractive workup and silica gel chromatography, HPLC and LC/MS analyses showed that the major product (12% isolated yield) was identical with the major product obtained from the preliminary experiments described above. ^{1}H and ^{13}C NMR analyses[11] confirmed that both products were enones and indicated that the major product was a cis enone and the minor product was a trans enone.[12] The EI mass spectrum of the major product gave a peak at *m*/*z* 296 (M^+) and a base peak at 127 (C_7H_{15}-CO^+), which are consistent with structure **4**. To establish conclusively the location of the keto group, both the major and minor products were treated with diazomethane and then hydrogenated.[14] These transformations yielded two identical compounds whose mass spectroscopic properties were consistent with methyl 11-oxooctadecanoate. The CI MS (isobutane) gave the expected M + 1 peak at *m*/*z* 313, and the EI MS gave diagnostic fragments at *m*/*z* 127 and 142 (cleavage on either side of the keto group), 228 and 213 (McLafferty rearrangements), and 171 (loss of ketene from the 213 fragment). This, along with the NMR analyses,

* To whom correspondence should be addressed.
+ Bucknell University.
# Walter Reed Army Institute of Research.
† Current address: 513 Wartik Laboratory, The Pennsylvania State University, University Park, PA 16802.
‡ Current address: Lexigen Pharmaceuticals, 125 Hartwell Ave, Lexington, MA 02421.
§ Current Address: Ceimic Corporation, 10 Dean Knauss Dr., Narragansett, RI 02882.

(1) Veldink, G. A.; Hilbers, M. P.; Nieuwenhuizen, W. F.; Vliegenthart, J. F. G. In *Eicosanoids and Related Compounds in Plants and Animals*; Rowley, A. F., Kuhn, H., Schewe, T., Eds.; Princeton University Press: Princeton, 1998; pp 69−96.

(2) (a) Brash, A. R.; Ingram, C. D.; Harris, T. M. *Biochemistry* **1987**, *26*, 5465−5471. (b) Eskola, J.; Laasko, S. *Biochim. Biophys. Acta* **1983**, *751*, 305−311. (c) Novak, M. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 31−34.

(3) [1-^{14}C]Oleic acid (56 mCi/mmol) was obtained from DuPont NEN and diluted with unlabeled oleic acid (Sigma) to a final specific activity of 1.0 mCi/mmol.

(4) Axelrod, B.; Cheesbrough, T. M.; Laasko, S. *Methods Enzymol.* **1981**, *71*, 441−451.

(5) Prepared enzymatically by the action of SBLO-1 on linoleic acid.

(6) This finding suggests that the Fe(III) form of SBLO-1 metabolizes oleic acid: Schilstra, M. J.; Veldink, G. A.; Vliegenthart, J. F. G. *Biochemistry* **1994**, *33*, 3974−3979.

(7) Jiang, Z.-Y.; Woollard, A. C. S.; Wolff, S. P. *Lipids* **1981**, *26*, 853−856.

(8) Tappel, A. L. *Methods Enzymol.* **1978**, *52*, 506−513.

(9) Corey, E. J.; Nagata, R. *J. Am. Chem. Soc.* **1987**, *109*, 8107−8108.

(10) Lipoxygenase (Type I, from soybeans) was obtained from Sigma and was used without further purification. The specific activity was determined by the standard assay procedure[4] with linoleic acid as substrate to be 16 μmol/min·mg.

(11) Major product, 11-oxo-9(*Z*)-octadecenoic acid (**4**): ^{1}H NMR ($CDCl_3$) δ 6.11 (2 H, m), 2.60 (2 H, q, J = 7.0 Hz), 2.43 (2 H, t, J = 7.4 Hz), 2.34 (2 H, t, J = 7.5 Hz), 1.60 (6 H, m), 1.40 (14 H, m), 0.87 (3 H, t, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 202.2, 178.8, 148.3, 126.6, 44.2, 33.8, 31.6, 29.3, 29.2, 29.1, 29.0, 28.9, 28.9, 24.6, 24.0, 22.5, 14.0. Minor product, 11-oxo-9(*E*)-octadecenoic acid (**8**): ^{1}H NMR ($CDCl_3$) δ 6.82 (1 H, dt, J = 15.8 Hz, J = 7.0 Hz), 6.09 (1 H, d, J = 15.8 Hz), 2.53 (2 H, t, J = 7.5 Hz), 2.36 (2 H, t, J = 7.4 Hz), 2.21 (2 H, q, J = 6.9 Hz), 1.60 (6 H, m), 1.55−1.25 (14 H, m), 0.88 (3 H, t, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 201.2, 178.0, 147.2, 130.4, 40.1, 33.7, 32.4, 31.7, 29.3, 29.1, 29.0, 28.9, 28.9, 28.0, 24.6, 24.3, 22.6, 14.1; FT-IR (neat, ATR) 2921, 1696, 1628, 1463 cm^{-1}.

(12) In the ^{1}H NMR spectrum of the major product, the two vinyl protons give overlapping multiplets near 6.1 ppm. Similar data have been reported for the vinyl protons of (*Z*)-5-octene-4-one[13a] and (*Z*)-3-undecen-2-one.[13b] Homonuclear decoupling of the allylic resonance at 2.6 ppm caused the vinyl signals to simplify to two doublets (J = 11.3 Hz) at 6.13 and 6.11 ppm. In the ^{1}H NMR spectrum of the minor product, the vinyl protons give rise to a doublet of triplets (J = 15.8, =7.0 Hz) at 6.82 ppm, and a doublet (J = 15.8 Hz) at 6.09 ppm, as expected for a trans enone.[13a]

(13) (a) Sakaguchi, S.; Watase S.; Katayama, Y.; Sakata, Y.; Nishiyama, Y.; Ishii, Y. *J. Org. Chem.* **1994**, *59*, 5681−5686. (b) Taber, D. F.; Herr, R. J.; Pack, S. K.; Geremia, J. M. *J. Org. Chem.* **1996**, *61*, 2908−2910.

(14) Hydrogenation was carried out for 30 min at room temperature in 1 mL of methanol with 2.5 mg of 5% Pd on $BaSO_4$.

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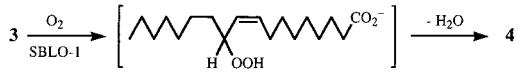
implied that the structure of the major product was 11-oxo-9(*Z*)-octadecenoic acid (**4**) and the minor product was 11-oxo-9(*E*)-octadecenoic acid (**8**).[15] The purified cis enone, **4**, isomerizes to the trans enone, **8**, and our observations suggest that most, and perhaps all, of the trans enone that we detect arises from isomerization of the initially formed cis isomer.

Likewise, incubation of 12(*Z*)-octadecenoic acid (**5**) under hyperbaric conditions gave two products in a 4.4:1 ratio as determined by HPLC analysis of the crude reaction mixture. The ^{1}H and ^{13}C NMR spectra of the purified minor product (6% yield) were consistent with a cis enone while the spectral data of the major product (41% isolated yield) were consistent with that of a trans enone. Methylation of the minor product followed by reduction of the olefin linkage gave a compound that was mass spectroscopically identical with the methyl 11-oxooctadecanoate obtained from the transformation of the oleic acid oxidation products. Hence, the minor product resulting from SBLO-mediated oxidation of 12(*Z*)-octadecenoic acid is 11-oxo-12(*Z*)-octadecenoic acid (**7**). With use of analogous methods, the major product was characterized as 13-oxo-11(*E*)-octadecenoic acid (**6**).

The rate of SBLO-mediated [1-^{14}C]oleic acid oxygenation is about 10^5 slower than the oxygenation of linoleic acid. Competition experiments were carried out to determine the relative rates of oleic acid (**3**) and 12(*Z*)-octadecenoic acid (**5**) oxygenation. In one experiment, a reaction mixture that was 75 μM in each of these substrates was incubated at 25 °C with 6.7 μM of lipoxygenase[4] plus 6.7 μM 13-HPOD. After 1 h, the reaction was quenched by acidification,[17] and the unreacted substrates were recovered by solid-phase extraction, converted to their methyl esters with diazomethane, and quantified by gas chromatography. It was found that about 9 μM **3** and 33 μM **5** had been consumed compared with controls from which enzyme was omitted. Analysis of the data from a series of such experiments[18] showed that k_{cat}/K_m for **5** is greater than that of **3** by a factor of 4.7 ± 0.8.

Scheme 2



These findings represent the first example of SBLO-mediated functionalization of unactivated monounsaturated fatty acids[21] and further demonstrate that the products of these reactions are enones and not allylic hydroperoxides.[22] This work also illustrates the value of hyperbaric conditions in facilitating preparative chemistry associated with lipoxygenase when very poor substrates are encountered. The scope and mechanism of this reaction are currently under investigation in our laboratories. We currently do not know if the oxygenation of monounsaturated fatty acids proceeds along the same mechanistic pathway as that of the traditional 1,4-pentadienyl-containing substrates, or if an additional or alternative mechanism is involved. One possibility is that a monoolefin substrate undergoes a typical lipoxygenation reaction to form an allylic hydroperoxide, only to be converted to the enone by loss of water (Scheme 2). It is noteworthy that the major product from oleic acid and both products from 12(*Z*)-octadecenoic acid result from an initial loss of hydrogen from C(11). This result implies that the positional specificity of SBLO for hydrogen abstraction from linoleic acid is retained even when one of the double bonds is absent. The relatively small difference in reactivity between oleic and 12(*Z*)-octadecenoic acids suggests that the 9- and 12-double bonds in linoleic acid contribute almost equally to the activation of the C(11)−H bond. This observation may help to distinguish among the different mechanistic possibilities suggested for C−H bond breaking by lipoxygenase,[1,9,23] provided it can be shown that the mechanistic pathway for the functionalization of the monoolefins is analogous to that of linoleic acid. Studies directed at this objective are in progress.

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Supporting Information Available: Representative procedure for the hyperbaric oxidation of monoolefins using SBLO-1; characterization of **6** and **7**; mass spectra of **4** and products from the methylation and reduction of **4** and **6**; experimental details and data for the competition experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) In our analytical experiments, carried out under atmospheric oxygen, two minor products were detected by HPLC. One of these, the one formed in smaller amounts, had the same retention time as **8**. The other minor product, corresponding to about 20% of the total product mixture formed in our analytical experiments, was not formed to an appreciable extent in our preparative hyperbaric runs. We have therefore not been able to obtain this material in sufficient quantity to permit definitive characterization. The EI mass spectrum of this substance gave major fragments at *m*/*z* 197, 171, 153, 125, which correspond to the published fragmentation pattern for 9-oxo-10-octadecenoic acid (16).

(16) Porter, N. A.; Wujek, J. S. *J. Org. Chem.* **1987**, *52*, 5085−5089.

(17) An internal standard (15(*Z*)-octadecenoic acid, Sigma) was added to a final concentration of 75 μM immediately before quenching.

(18) The analysis of the data is analogous to that used to determine competitive isotope effects.[19] For enzymatic reactions, the relative rates obtained in this fashion are relative values of k_{cat}/K_m.[20] In this case, $(k_{cat}/K_m)_5/(k_{cat}/K_m)_3 = \log(1 - f)/\log((1 - f)R_{60}/R_0)$ (see eq 4.8 in ref 19). R_{60} is the ratio of the GC peak areas for the methyl esters of **5** and **3** at 60 min, and R_0 is the same ratio determined at 0 min. The fraction of **5** remaining after 60 min ($1 - f$) was determined by measuring the ratios of the peak areas for the methyl ester of **5** and the methyl ester of the internal standard[17] at 0 and 60 min. The value of $1 - f$ was corrected for a small decrease in the concentration of **5** in control experiments from which enzyme was omitted. The concentration of **3** also decreased slightly in the nonenzymatic controls; the decrease was the same within error as that for **5**, and the ratio of **3** to **5** did not change significantly. Consequently, there was no need to correct the value of R_{60}. Since no products could be detected when [1-^{14}C]oleic acid was incubated under the reaction conditions but without enzyme, we suspect that the small drop in the concentrations of **3** and **5** in the controls for the competition experiments may have been due to adherence of the fatty acids to the sides of the plastic reaction tube rather than to a nonenzymatic reaction. Experimental details and data for the competition experiments are presented as Supporting Information.

(19) Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; R. E. Krieger Publishing: Malabar, FL, 1987; Chapter 4.

(20) Klinman, J. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *46*, 415−494.

(21) Kuhn and co-workers have reported that SBLO-1 will oxygenate methyl 12-oxo-9(*Z*)-octadecenoate, in which the hydrogens at C(11) are activated by the keto group on C(12). Interestingly, oxygenation was much slower for the free acid than for the methyl ester. The major product was methyl 9,12-dioxo-10(*E*)-octadecenoate: Kuhn, H.; Eggert, L.; Zabolosky, O. A.; Myagkova, G. T.; Shewe, T. *Biochemistry* **1991**, *30*, 10269−10273.

(22) Similar results have been reported by Veldink, Vliegenthart, and collaborators in their studies on acetylenic analogues of linoleic acid, which are suicide inactivators of lipoxygenase. These substances are converted by SBLO-1 to the corresponding 11-oxo derivatives, which were proposed to be formed from the initially produced 11-hydroperoxides: Nieuwenhuizen, W. F.; Schilstra, M. J.; Van der Kerk-Van Hoof, A.; Brandsma, L.; Veldink, G. A.; Vliegenthart, J. F. G. *Biochemistry* **1995**, *34*, 10538−10545. Nieuwenhuizen, W. F.; Van der Kerk-Van Hoof, A.; van Lenthe, J. H.; Van Schaik, R. C.; Versluis, K.; Veldink, G. A.; Vliegenthart, J. F. G. *Biochemistry* **1997**, *36*, 4480−4488.

(23) Nelson, M. J.; Cowling, R. A.; Seiz, S. P. *Biochemistry* **1994**, *33*, 4966−4973.