# Regiocontrol of Soybean Lipoxygenase Oxygenation. Application to the Chemoenzymatic Synthesis of Methyl 15(*S*)-HETE and Methyl 5(*S*),15(*S*)-diHETE

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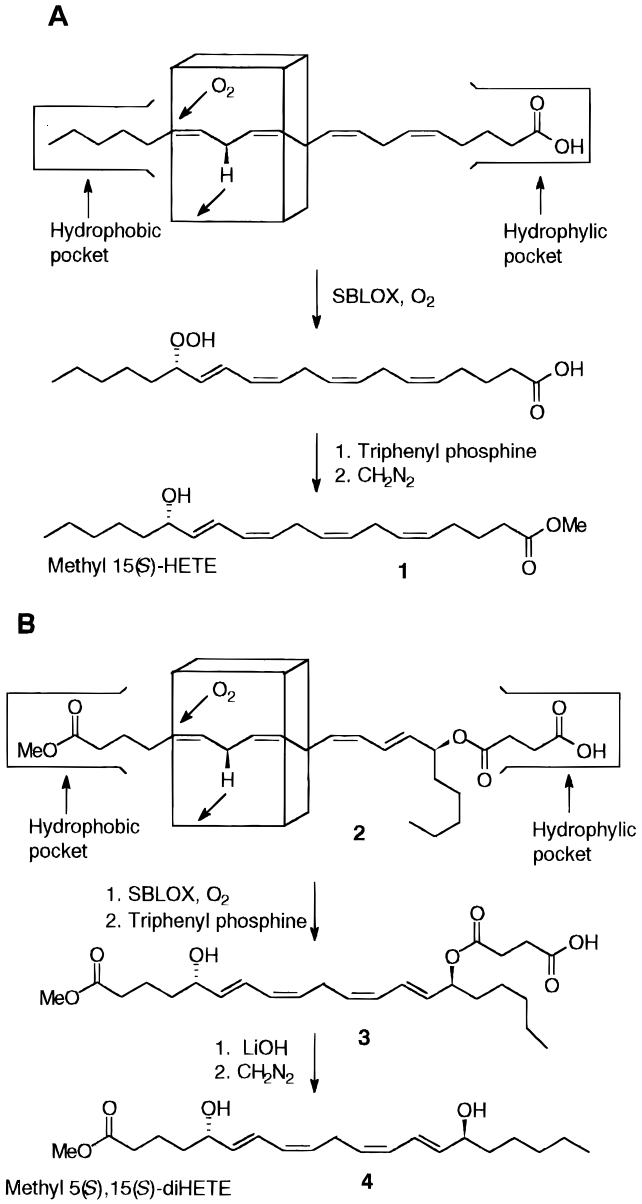
Lipoxygenases (LOX's) are dioxygenases that catalyze the stereospecific incorporation of molecular oxygen into the 1(*Z*),4(*Z*)-pentadienyl moiety of polyunsaturated fatty acids.[1] We have shown recently[2,3] that soybean LOX (SBLOX) can vigorously function at high linoleic acid concentration (0.1 M) under oxygen pressure. After reduction of the formed hydroperoxides (HPOD), coriolic acid [(9*Z*,11*E*,13*S*)-13-hydroxy-9,11-octadecadienoic acid] is obtained as a main product, in good yield (83%) and with high ee (98%). As pointed out by Zhang and Kyler,[4] SBLOX has attracted little attention from a synthetic point of view,[4,5] despite its extremely high selectivity. In an effort to extend its use, we have investigated the ability of this enzyme to functionalize arachidonic acid at the normal n-6 position but also on carbon number 5, a carbon normally not attacked by this LOX.[6] This approach is illustrated by the chemoenzymatic synthesis of methyl (5*S*,6*E*,8*Z*,11*Z*,13*E*,15*S*)-5,15-dihydroxy-6,8,-11,13-eicosatetraenoic acid [methyl 5(*S*),15(*S*)-diHETE] (**4**),[7] a naturally occuring eicosanoid with physiological properties.[8,9]

**Synthesis of Methyl 15(*S*)-HETE.** Arachidonic acid (0.1 M, 913.5 mg) was treated with SBLOX (120 mg, Fluka AG) under the optimized conditions determined previously for linoleic acid.[3] After 15 min, the reaction was stopped, and the formed hydroperoxides (∼99%, UV determination) were reduced with triphenylphosphine (TPP) and then esterified with diazomethane to afford methyl (5*Z*,8*Z*,11*Z*,13*E*,15*S*)-15-hydroxy-5,8,11,13-eicosatetraenoic acid [methyl 15(S)-HETE] (**1**) (see Scheme 1A) in 83% yield (798 mg), with high purity (>97%, normal-phase HPLC, $^1$H NMR) and very high ee (>99%, chiral phase HPLC).[10] This reaction is rapid and extremely stereoselective and proves the general ability of SBLOX to generate in high yields n-6(*S*)-HPOD of natural polyunsaturated fatty acids, when used at high substrate concentration under oxygen pressure.[2,3,11]

**Scheme 1. Substrate Recognition by SBLOX, Adapted from the Models Developped by Lehman and Hatanaka (A, Normal Orientation, 15 Oxygenation; B, Reverse Orientation, 5 Oxygenation)**




**Synthesis of Methyl 5(*S*),15(*S*)-diHETE.** We were then interested in the further functionalization of methyl 15(*S*)-HETE since this compound still bears two 1(*Z*),4-

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(1) For general reviews on LOX's, see: (a) Galliard, T.; Chan, H. W.-S. *Biochemistry of Plants*; Academic Press: New York, 1980; Vol. 4, p 131. (b) Veldink, G. A.; Vliegenthart, J. F. G. *Advances in organic biochemistry*; Elsevier Scientific Publishing Co.: Amsterdam, 1984; Vol. 6, p 139. (c) Corey, E. J. *Stereochemistry of organic and bioorganic transformations*; Bartmann, W., Sharpless, K. B., Eds.; VCH Publishers: Weiheim, 1986; p 1. (d) Vick, B. A.; Zimmerman, D. C. *Biochemistry of plants*; Academic Press: New York, 1987; Vol. 9, p 53. (e) Yamamoto, S. *Free Radical Biol. Med.* **1991**, *10*, 149.

(2) Iacazio, G.; Langrand, G.; Baratti, J.; Buono, G.; Triantaphylides, C. *J. Org. Chem.* **1990**, *55*, 1690.

(3) Martini, D.; Iacazio, G.; Ferrand, D.; Buono, G.; Triantaphylides, C. *Biocatalysis* **1994**, *11*, 47.

(4) Zhang, P.; Kyler, K. S. *J. Am. Chem. Soc.* **1989**, *111*, 9241.

(5) For other examples of the use of SBLOX in organic synthesis, see: (a) Corey, E. J.; Su, W. G.; Cleaver, M. B. *Tetrahedron Lett.* **1989**, *30*, 4181. (b) Maguire, N. M.; Mahon, M. F.; Molloy, K. C.; Read, G.; Roberts, S. M.; Sik, V. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2054. (c) Dussault, P.; Lee, I. Q. *J. Org. Chem.* **1995**, *60*, 218.

(6) Three groups have reported the bis oxygenation of arachidonic by SBLOX.[5a,15,18] In these three papers, after reduction of hydroperoxides, 8,15-diHETE is the main (65%,[5a] 60%[15]) or the exclusive[18] product of the reaction, the other product being, in the former two cases, 5,15-diHETE.

(7) For the chemical synthesis of 5(*S*),15(*S*)-diHETE see: Nicolaou, K. C; Weber, S. E. *J. Am. Chem. Soc.* **1984**, *106*, 5734.

(8) Maas, R. L.; Turk, J.; Oates, J. A.; Brash, A. R. *J. Biol. Chem.* **1982**, *257*, 7056.

(9) Morita, E.; Schröder, J.-M.; Christophers, E. *J. Immunol.* **1990**, *144*, 1893.

(10) Kühn, H.; Wieser, R.; Lankin, V. Z.; Nekrasov, A.; Alder, L.; Schewe, T. *Anal. Biochem.* **1987**, *160*, 24.

(11) The chemoenzymatic synthesis of **1** has already been reported but at a much lower substrate concentration ($1.6 \times 10^{-3}$ M) and in lower yield (50%); see: Baldwin, J. E.; Davies, D. I.; Hughes, L.; Gutteridge, N. J. A. *J. Chem. Soc., Perkin Trans. 1*, **1979**, 115.

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(*Z*)-pentadienyl systems, which could be enzymatically oxygenated. The challenging problem is to force SBLOX to recognize **1** as a substrate, but with a reverse orientation, in order to conduct a 5 lipoxygenation. Indeed, as shown in Scheme 1A, SBLOX is thought to position the reactive pentadienyl system of the substrate with the aid of two pockets, a hydrophobic and a hydrophylic one, receiving, respectively, the methyl ending and the carboxylic ending chains of the natural substrate. According to the models developed by Lehman[12] and Hatanaka,[13] methyl 15(*S*)-HETE should be orientated with the methyl ester chain in the hydrophobic pocket to be oxygenated at the 5 position. In order to secure this orientation, the second pocket should also be filled, something that could be done as proposed previously by Zhang and Kyler[4] by adding a carboxylic ending chain, such as a succinyl group, taking advantage in our case of the 15 hydroxy functionality as shown in Scheme 1B. As such, methyl 15-succinyl-15(*S*)-HETE (**2**) bears the two important features to be recognized and oxygenated at the 5 position by the enzyme. To test this hypothesis, **2** has been synthesized in 90% yield from **1** and succinic anhydride for 5 days in refluxing $CH_2Cl_2$ with DMAP as catalyst.

Preliminary experiments have proved that **2** is effectively a substrate of SBLOX. In order to isolate the product(s) of the reaction, oxygenation of **2** has been conducted in a 2 L fermentor vessel at a concentration of $10^{-3}$ M.[14] After 10 min, the substrate is totally consumed as judged by HPLC, and the formed hydroperoxides extracted and reduced with TPP, affording methyl 15-succinyl-5(*S*),15(*S*)-diHETE (**3**) as a main product in 78% yield. **3** is then hydrolyzed (LiOH, THF/$H_2O$, 1/1, 48 h) and methylated ($CH_2N_2$), affording methyl 5(*S*),15(*S*)-diHETE (**4**) in 59% yield from **2** and 44% from arachidonic acid. The structure of **4** has been established from its UV, IR, MS, and $^1H$ and $^{13}C$ NMR spectra, which are in full accordance with the previously reported ones.[15] The *S* absolute configuration at the newly formed asymmetric carbon was determined by a previously reported method,[16] based on the chromatography of diastereoisomeric (−)-menthoxy carbonate derivatives of dimethyl 2-hydroxyadipate. GC analysis of the mixture of diastereoisomers showed that the second oxygenation performed by SBLOX is essentially diastereoselective.[17]

**Conclusion.** We have shown that SBLOX could be used not only as a normal n-6 specific LOX, to generate in high yield and with high selectivity methyl 15(*S*)-HETE, but also to conduct a 5 lipoxygenation on the carbon skeleton of arachidonic acid. This second oxygenation involves a suitable structural modification of **1** in order to change the natural orientation of the substrate in the active site of the enzyme. Methyl 5(*S*),15(*S*)-diHETE is then easily accessible by very simple chemistry with a de of more than 98%.

## Experimental Section

**General Procedures.** Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. SBLOX was purchased from Fluka. Dichloromethane ($CH_2Cl_2$) was distilled from calcium hydride. $^1H$ NMR spectra were recorded at 200 MHz, and $^{13}C$ spectra were proton decoupled at 50 MHz. Chemical shifts are reported as ppm downfield of tetramethylsilane. *J* values are given in Hz. The synthesis of **1** was carried out in a high-pressure reactor,[3] and the synthesis of **4** was conducted in a fermentor.[3]

**Methyl 15(*S*)-HETE (1).** Exactly 913.5 mg ($3 \times 10^{-3}$ mol) of arachidonic acid was weighted in the reactor PTFE beaker, and then 30 mL of 0.1 M of borate buffer (referred to as dissolved $Na_2B_4O_7 \cdot 10H_2O$) pH 11 was added and allowed to reach a temperature of 5 °C. Then 0.12 g of soybean lipoxygenase was added, the vessel closed and pressurized at 2.5 bar of pure oxygen, and the stirring set at maximum speed (1600 rpm). After 15 min the reaction mixture was withdrawn from the reactor, diluted with 300 mL of brine, acidified to pH 3 with citric acid, and extracted with diethyl ether (3 × 500 mL). The combined organic phases were dried ($MgSO_4$, 0 °C) and the hydroperoxides reduced overnight (0 °C) with 0.866 g of TPP (3.3 mmol). The solvent volume was then reduced to approximatively 30 mL by evaporation and the product esterified with diazomethane. Purification of the crude product by silica gel chromatography eluting with 4:6 hexane/diethyl ether afforded 0.798 g (83%) of a colorless oil: $[\alpha]^{20}_D = +24.2$ ($c = 0.67$, MeOH), UV (EtOH) $\lambda_{max} = 237$ nm, $\epsilon = 29\,900$ mol$^{-1}$·cm$^{-1}$·L; IR (thin film) 3428, 3013, 2931, 2859, 1740, 1438, 986, 952, 913 cm$^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ 0.89 (t, 3H), 1.20−1.90 (bm, 11H), 2.12 (dt, 2H, $J = 6, 6$), 2.33 (t, 2H, $J = 7$), 2.81 (dd, 2H, $J = 6, 6$), 2.96 (dd, 2H, $J = 6, 6$), 3.67 (s, 3H), 4.17 (dt, 1H, $J = 7, 7$), 5.40 (m, 5H), 5.70 (dd, 1H, $J = 7, 15$), 6.00 (dd, 1H, $J = 11, 11$), 6.54 (dd, 1H, $J = 11, 15$); $^{13}C$ NMR (50 MHz) $\delta$ 14.1, 22.7, 24.8, 25.2, 25.6, 26.1, 26.6, 31.8, 33.4, 37.4, 51.6, 72.8, 125.2, 127.6, 128.1, 128.6, 128.8, 129.0, 130.1, 136.8, 174.2. Anal. Calcd for $C_{21}H_{34}O_3$: C, 75.40; H, 10.25. Found: C, 75.28; H, 10.18.

**Methyl 15-Succinyl-15(*S*)-HETE (2).** To a solution of 0.5 g (1.49 mmol) of compound **1** and 0.05 g (0.4 mmol) of dimethylaminopyridine in 15 mL of dichloromethane was added a solution of 0.449 g (4.49 mmol) of succinic anhydride in 15 mL of the same solvent. The reaction mixture was then heated to reflux for 5 days under nitrogen. The crude product was concentrated and purified by silica gel chromatography, eluting with 6:4:0.5 diethyl ether/hexane/methanol, to afford 0.585 g (90%) of a colorless oil: IR (thin film) 3012, 2930, 2859, 1737, 1713, 1436, 1170, 987, 953, 726 cm$^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ 0.80 (t, 3H), 1.20 (bs, 6H), 1.4−1.7 (m, 4H), 2.02 (dt, 2H, $J = 6$), 2.24 (t, 2H, $J = 7$), 2.55 (t, 4H, $J = 5$), 2.71 (dd, 2H, $J = 6, 6$), 2.86 (dd, 2H, $J = 6, 6$), 3.59 (s, 3H), 5.1−5.4 (m, 6H), 5.50 (dd, 1H, $J = 7, 15$), 5.87 (dd, 1H, $J = 11, 11$), 6.45 (dd, 1H, $J = 11, 15$); $^{13}C$ NMR (50 MHz) $\delta$ 14.0, 22.5, 24.7, 24.7, 25.6, 26.1, 26.5, 29.0, 29.2, 31.5, 33.5, 34.5, 51.5, 75.3, 127.4, 127.7, 127.8, 128.7, 128.7, 129.1, 131.2, 131.4, 171.5, 174.2, 178.0. Anal. calcd for $C_{25}H_{38}O_6$: C, 69.09; H, 8.81. Found: C, 69.01; H, 8.75.

**Methyl 15-Succinyl-5(*S*),15(*S*)-diHETE (3).** A solution of **2** (0.276 g, 0.64 mmol) in 5 mL of dioxane and 635 mL of borate buffer 0.1 M, pH 9 were placed in the fermentor and allowed to reach a temperature of 5 °C. Then 0.162 g of soybean lipoxy-

(12) Hatanaka, A. *Phytochemistry* **1993**, *34*, 1201.

(13) Lehman, W. D. *Free Radical Biol. Chem.* **1994**, *16*, 241.

(14) At higher substrate concentrations ($3 \times 10^{-3}$ or $10^{-2}$ M), the formed hydroperoxide is unstable in the reaction medium. This phenomenon could be related to the known ability of lipoxygenase to transform 5-HPETE in leucotriene $LTA_4$ when activated at a lipidic interface (see: Riendeau, D.; Falgueyret, J.-P.; Meisner, D.; Sherman, M. M.; Laliberté, F.; Street, I. P. *J. Lipid Med.* **1993**, *6*, 23). Indeed, as shown by UV spectroscopy, this unstability (disparition of the UV band at 235 nm) is accompanied by the formation of lipoxin-like compounds (appearance of three bands at 280, 302, and 320 nm characteristic of conjugated tetraenic structure). An explanation of this phenomenon could be the general ability of LOX's to transform 5-HPOD of arachidonic acid or derivatives via an epoxidation/hydrolysis process, similar to the one involved in the biosynthesis of leucotriene $LTA_4$ and lipoxins. In our case, an increase in the concentration of **2** could create a water/oil interface, at which SBLOX activates to generate the 15-succinyl equivalent of lipoxins. It should be noted that the same phenomenon has been observed when ethyl (15-succinyl)-15(*S*)-HETE has been used instead of **2**, but at a much lower concentration ($5 \times 10^{-5}$ M).

(15) Van Os, C. P. A.; Rijke-Schilder, G. P. M.; Van Halbeek, H.; Verhagen, J.; Vliegenthart, J. F. G. *Biochim. Biophys. Acta* **1981**, *663*, 177.

(16) Hamberg, M. *Anal. Biochem.* **1971**, *43*, 515.

(17) Authentic racemic samples were obtained through oxidative ozonolysis of the (−)-menthoxy carbonate of 1-cyclohexenol followed by methylation and preparation of the authentic *S* sample by the same procedure, but starting from enzymatically generated optically pure 1(*S*)-cyclohexenol (see: Gupta, A. K.; Kazlauskas, R. J. *Tetrahedron: Asymmetry* **1993**, *4*, 879).

(18) Bild, G. S.; Ramadoss, C. S.; Lim, S.; Axelrod, B. *Biochem. Biophys. Res. Commun.* **1977**, *74*, 949.

genase was added, the vessel closed and pressurized at 1 bar of pure oxygen, and the stirring set at maximum speed (1000 rpm). After 10 min, the reaction mixture was withdrawn from the reactor, saturated with NaCl, acidified to pH 3 with citric acid, and extracted with diethyl ether (3 × 600 mL). The combined organic phases were dried ($MgSO_4$, 0 °C) and the hydroperoxides reduced overnight (0 °C) with 0.183 g of TPP (0.7 mmol). The crude product was concentrated and purified by silica gel chromatography, eluting with 7:3:0.25 diethyl ether/hexane/methanol, to afford 0.223 g (78%) of a colorless oil: IR (thin film) 3217, 3013, 2932, 2862, 1734, 1718, 1437, 1169, 986, 955, 911, 734 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ 0.88 (t, 3H), 1.28 (bs, 6H), 1.5−1.8 (bm, 6H), 2.34 (t, 2H, $J$ = 7), 2.64 (bs, 4H), 3.08 (dd, 2H, $J$ = 7, 7), 3.67 (s, 3H), 4.23 (dt, 2H, $J$ = 7, 7), 5.2−5.8 (bm, 2H), 5.98 (dd, 2H, $J$ = 7, 15), 6.5−6.7 (bm, 2H), 7.4−7.7 (bm, 2H); $^{13}C$ NMR $\delta$ 14.0, 20.9, 22.5, 24.8, 26.6, 28.8, 29.3, 31.5, 33.8, 34.5, 36.4, 51.6, 72.0, 75.2, 125.2, 127.6, 128.0, 128.4, 129.4, 130.5, 131.5, 136.2, 171.8 174.3, 176.4. Anal. Calcd for $C_{25}H_{38}O_7$: C, 66.64; H, 8.50. Found: C, 66.58; H, 8.46.

**Methyl 5(*S*),15(*S*)-diHETE (4).** To a solution of 0.223 g (0.49 mmol) of **3** in 20 mL of THF/$H_2O$ 1:1 was added 0.082 g of $LiOH·H_2O$ (1.96 mmol). The solution was then stirred at 20 °C for 48 h. The reaction mixture was diluted with 30 mL of brine, acidified to pH 3 with citric acid, and extracted with diethyl ether (3 × 50 mL). The combined organic phases were dried ($MgSO_4$, 0 °C), the solvent volume was then reduced to approximatively 30 mL by evaporation, and the product was methylated with diazomethane. Purification of the crude product by silica gel chromatography eluting with 3:7 hexane/diethyl ether afforded 0.132 g (76%) of a colorless oil: $[\alpha]^{20}_D = +18.4$ ($c$ = 0.66, MeOH); UV (EtOH) $\lambda_{max}$ = 243 nm, $\epsilon$ = 34 000 $mol^{-1}·cm^{-1}·L$; IR (thin film) 3392, 3010, 2931, 2859, 1738, 1438, 986, 955, 913, 734 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ 0.90 (t, 3H), 1.30 (bs, 6H), 1.5−1.8 (m, 6H), 2.36 (t, 2H, $J$ = 7), 3.08 (dd, 2H, $J$ = 8, 8), 3.67 (s, 3H), 4.19 (dt, 2H, $J$ = 6,6), 5.43 (dt, 2H, $J$ = 7, 11), 5.6−5.8 (m, 2H), 6.01 (dd, 2H, $J$ = 11, 11), 6.58 (dd, 2H, $J$ = 11, 15); $^{13}C$ NMR (50 MHz) $\delta$ 14.0, 20.7, 22.6, 25.1, 26.5, 31.7, 33.7, 36.5, 37.3, 51.5, 72.0, 72.5, 124.9, 125.3, 128.1, 128.3, 129.2, 129.6, 136.2, 136.8, 174.2. Anal. calcd for $C_{21}H_{34}O_4$: C, 71.96; H, 9.78. Found: C, 71.96; H, 9.75.

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