

Scaling-up the Production of 13*S*-Hydroxy-9*Z*,11*E*-octadecadienoic Acid (13*S*-HODE) through Chemoenzymatic Technique

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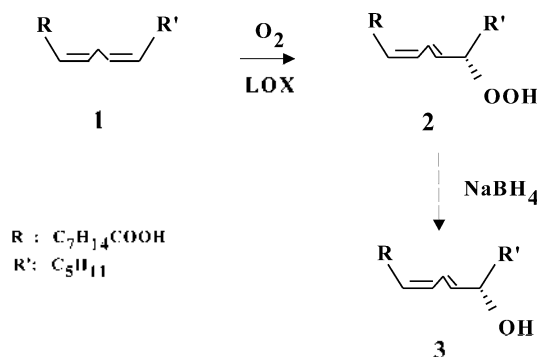
In spite of greater use of oils and fats in the food industry, the other applications have not been explored very much. Beside the oleochemical industry, the other areas that attract greater interest nowadays are the utilization of fatty acids and their derivatives in pharmaceutical applications. While most fatty acids are achiral molecules, this lack of chirality becomes a great challenge to the chemists to look into possibilities of transforming them into highly value-added chiral molecules which exhibit a vast variety of biological roles in plants and mammals. The most popular technique to transform achiral fatty acids into their corresponding chiral metabolites is through biotechnology processes.¹ With the increasing demands for microbial and enzymatic biotransformation, the search for new biologically active substances has been given great attention. In the case of linoleic acid **1**, the enzyme which can transform the acid into a chiral hydroperoxide, *i.e.* 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HIPOD, **2**) is called lipoxygenase (LOX). 13*S*-HIPOD is a precursor for the formation of 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HODE, **3**) (Scheme 1).

13*S*-HODE **3** is an important chiral molecule because it shows a vast variety of biological effects in plants and mammals. In mammals, 13*S*-HODE exhibits calcium ionophoric properties,² decreases platelet adhesion to endothelial cell and maintains the thromboresistance of vascular endothelium.³ Meanwhile, in plants, 13*S*-HODE exhibits anti-

fungal activities⁴ and serves as precursor in the formation of cutin,⁵ the protective layer of plants against pathogenic attacks. Beside that, 13*S*-HODE is also utilized as a starting material for the production of an unsaturated macrocyclic C-13 lactone⁶ and other biologically active molecules. Due to ever increasing demand for 13*S*-HODE **3** as the starting material for organic syntheses, the scaling-up production of this compound from milligram to gram scale would become our main synthetic target.

Several steps have been taken to increase the production of **2** and **3** at milligram scale.⁶⁻¹¹ Gardner and Newton⁷ have successfully produced 600 mg of 13*S*-HIPOD **2** from 800 mg of linoleic acid **1** using 10 mg soybean LOX, cooled at 0 °C and bubbled with pure oxygen at pH 10. To improve solubility of oxygen, Iacazio *et al.*⁸ have used oxygen under 4 atm pressure in an aqueous buffer solution which was cooled at 0-4 °C. Meanwhile, Srinivasulu and Rao^{9,10} have utilized detergent and alcohol in order to facilitate the solubility of the substrate. Organic solvents such as isooctane and dimethyl sulfoxide (DMSO) have also been used by Emken and Dutton¹¹ to improve oxygen solubility as well as to increase substrate concentration. Recently, immobilized lipoxygenase (LOX) on various supports has been employed with the aim of increasing the yield and for multiple uses of the enzyme. Maguire *et al.*⁶ have obtained the hydroperoxide in high yield by immobilizing LOX on oxirane acrylic beads in phosphate buffer at pH 7.5 and then storing at 4 °C for 60 hrs prior to use. In our work, the conversion of **1** at milligram scale (800 mg/L) using immobilized soy LOX-1B followed the same procedure as reported earlier⁶ with a slight modification. It was found that the yield of **3** (75.8%) was comparable to the result obtained by previous researcher (*i.e.* 77%).⁶ The spectroscopic data (IR, ¹H NMR, and MS) supported by specific rotation [α] confirmed the presence of **3**. However, the yield of **3** decreased when the concentration of **1** was increased to 5 g/L during the scaling-up process even the immobilized LOX concentration was increased from 70 mg/L to 100 mg/L. Although with the addition of DMSO, the yield was still below the expected value (*i.e.* 75.8%). It showed that the higher the concentration of **1** might retard the activity of LOX even though the LOX concentration was increased.

The scaling-up production of 13*S*-HODE **3** using linoleic acid (**1**, 5 g/L) and incubating with soy lipoxygenase LOX-



Scheme 1

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Table 1. Scaling-up the production of 13S-HODE **3**

Experiment	Yield (%)
LOX	48.3
LOX and DMSO	56.2
Immobilized LOX	59.6
Immobilized LOX and DMSO	68.5

IB gave **3** in 48.3% yield, while the incubation with the addition of DMSO improved the yield of **3** to 56.2% (Table 1). It showed that the addition of DMSO increased the solubility of oxygen and the concentration of **1** in buffer solution.¹¹ As a result, more conversion of **1** to **2** would take place and hence improved the yield of **3**. Although the use of immobilized LOX without the addition DMSO gave a better yield (59.6%) than the combined LOX and DMSO, the best yield (*i.e.* 68.5%) was obtained when the combined immobilized LOX and DMSO were used. However, the concentration of DMSO should be kept between 10 to 20% in order to avoid the inhibition of the activity of the enzyme⁹⁻¹¹ due to the excessive use of the solvent.⁹⁻¹¹ In conclusion, the scaling-up procedure using the immobilized LOX-IB with added DMSO afforded a sufficient amount of **3** which could be used as an important chiral source for further transformation into the other biologically active molecules.¹²

Experimental Section

Optical rotation was recorded on Model D Polarimeter (*Bellingham & Stanley, UK*) and the sample was dissolved in chloroform. TLC was performed on pre-coated silica gel G60 (0.2 mm, *Merck*) with solvent system of diethyl ether in *n*-hexane containing 1% glacial acetic acid. The plate was developed by dipping into a solution of *p*-anisaldehyde (1 mL, *Sigma*) and sulphuric acid (2 mL) in methanol (100 mL) and heated at 110 °C for 1 min. Silica gel column chromatography was performed using silica gel G60 (70-230 mesh, *Merck*) and the sample was eluted with diethyl ether in hexane with the addition of glacial acetic acid. The fractions collected were evaporated under N₂ and monitored by thin-layer chromatography (TLC) and gas chromatography (GC). The GC analyses were performed on a Philips PU4600 gas chromatograph using capillary column (DB1, 30 m × 0.32 mm, i.d. × 1 μm; *J&W Scientific, UK*) and equipped with flame ionization detector (FID). The hydrogen was used as carrier gas and flowed under 60 kPa head column pressure. The detector and injector temperatures were 300 °C and 280 °C respectively and the column temperature was programmed from 90 °C to 300 °C at 4 °C min⁻¹. The sample was converted into trimethylsilyl ether by treating the sample with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), heated at 60 °C for 15 min prior to GC analysis. The chromatograms and results were recorded using a Spectra Physics SP4600 integrator. IR spectra were analysed on a Perkin-Elmer 1600 series FTIR grating spectrophotometer. ¹H NMR spectra were recorded on a JEOL JNM-FX90Q FT-NMR spectrometer operating at 90 MHz. Samples were

dissolved in CDCl₃ and chemical shifts (δ_H) were reported in ppm downfield from tetramethylsilane (TMS). The coupling constants (*J*) were quoted in Hz. Mass spectra were provided by the EPSR National Mass Spectrometry Centre, The Department of Chemistry, University of Swansea.

13S-HODE **3** was prepared in milligram scale according to the previous method⁶ with a slight modification. Briefly, the immobilized soybean lipoxygenase type 1B (70 mg, *Sigma*) on oxirane acrylic beads (3 g, 150 μ, *Sigma*) was suspended in 0.1 M borate buffer (800 mL, pH 9.5) and cooled at 0 °C. While stirring, the suspension was added portionwise the emulsion of **1** (3.10 mmol in 0.1 M borate buffer). Then, the emulsion mixture was bubbled with oxygen and the reaction was monitored by gas chromatographic analysis (GC) at every 15 min incubation time. After one and half-hour the mixture was acidified to pH 3, saturated with sodium chloride and extracted with diethyl ether (200 mL). The ethereal layer was dried and evaporated *in vacuo* to afford the crude hydroperoxide **2**. The crude product was dissolved in methanol (30 mL), stirred and reduced with NaBH₄ (25 mmol) at 0 °C for 20 min. Then, the reaction product was acidified to pH 3, diluted with water (200 mL) and extracted with diethyl ether (100 mL). The combined ethereal layer was dried and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using 30% diethyl ether in *n*-hexane containing 1% glacial acetic acid to afford 13S-HODE (**3**) (2.35 mmol, 75.8 % yield) as a colourless oil [R_f = 0.61 (TLC, 40% Et₂O)]; [α]_D²⁰ +9.6° (*c* 0.6, CHCl₃); IR (neat) cm⁻¹: 3443, 2997, 2920, 2845, 1707, 896; δ_H ppm: 0.91 (3H, t, CH₃), 1.26-1.68 (18H, m, CH₂), 2.20 (2H, m, 8-CH₂), 2.36 (2H, t, 2-CH₂), 4.10 (1H, m, H-13), 5.41 (1H, t, *J* = 10.7 Hz, H-9), 5.70 (1H, m, H-12), 5.80 (2H, s, OH and CO₂H), 5.93 (1H, d, H-10), 6.43 (2H, m, *J* = 15.3 Hz, H-11); MS(EI) *m/z* 73, 74, 173, 225, 293, 295, 311, 367, 382, 383.

Scaling-up the production of 13S-HODE

Production of 13S-HODE using LOX. Soybean lipoxygenase type 1B (100 mg) was suspended in 0.1 M borate buffer (800 mL, pH 9.5), stirred and cooled at 0 °C. The cooled suspension was added portionwise the emulsion of **1** (17.8 mmol) in 0.1 M borate buffer (200 mL, pH 9.5) and the mixture was bubbled with oxygen. The conversion of **1** was monitored by gas chromatographic analysis (GC) at every 15 min incubation time. After 2 h, the reaction mixture was acidified to pH 3, saturated with NaCl and extracted with diethyl ether (250 mL). The ethereal layer was dried and concentrated *in vacuo*. The residue was dissolved in methanol (100 mL), cooled at 0 °C and added portionwise NaBH₄ (50 mmol). Then, the reaction product was acidified to pH 3, diluted with water (400 mL) and extracted with diethyl ether (250 mL). The combined ethereal layer was washed with brine solution (200 mL), dried and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using 30% diethyl ether in *n*-hexane containing 1% glacial acetic acid to afford **3** (8.6 mmol, 48.3% yield) as a colourless oil.

Production of 13S-HODE using LOX and DMSO. A

borate buffer solution (900 mL, 0.1 M) containing **1** (17.8 mmol) and a lipoxygenase enzyme (LOX-1B) (100 mg) was stirred and bubbled with oxygen. The mixture was then cooled at 0 °C and was then added portionwise DMSO (100 mL). The reaction product was monitored by GC. After incubation, the suspension was acidified to pH 3 and extracted with diethyl ether (250 mL). The combined ethereal layer was dried and evaporated under reduced pressure to afford the crude hydroperoxide **2**. The hydroperoxide was dissolved in methanol (100 mL), cooled at 0 °C then reduced with NaBH₄ (0.05 mol). The methanolic solution was acidified to pH 3, diluted with distilled water (100 mL) and extracted with diethyl ether (100 mL). The solvent extract was dried and then evaporated under reduced pressure. Purification using silica gel column chromatography with 30% diethyl ether in hexane containing 1% glacial acetic acid afforded 13S-HODE **3** as a colourless oil (10.0 mmol, 56.2%).

Production of 13S-HODE using immobilized LOX. The immobilized soybean lipoxygenase type 1B (100 mg) on acrylic oxirane beads (5 g) was prepared according to the method previously reported.⁶ The enzyme was suspended in 0.1 M borate buffer (800 mL) which was stirred and cooled at 0 °C. While stirring, the enzyme suspension was bubbled with oxygen and the emulsion of **1** (17.8 mmol) in 0.1 M borate buffer (200 mL) was added portionwise. The reaction product was monitored by GC. After 2 h the reaction mixture was acidified to pH 3, saturated with sodium chloride and extracted with diethyl ether (250 mL). The ethereal layer was dried and concentrated *in vacuo*. The residue was dissolved in methanol (100 mL), cooled at 0 °C and reduced with NaBH₄ (50 mmol). The reaction product was acidified to pH 3, diluted with water (400 mL) and extracted with diethyl ether (250 mL). The combined ethereal layer was dried and then concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using 30% diethyl ether in *n*-hexane containing 1% glacial acetic acid to afford **3** (10.6 mmol, 59.6% yield) as a colourless oil.

Production of 13S-HODE using immobilized LOX and

DMSO. The soybean lipoxygenase type 1B was immobilized on oxirane acrylic beads as described earlier.⁶ The filtered immobilized LOX 1B (100 mg) on the oxirane acrylic beads (3 g) was suspended in 0.1 M borate buffer (1000 mL, pH 9.5), cooled at 0 °C and oxygen was bubbled into the mixture. The cooled suspension was stirred and added portionwise the emulsion of **1** (17.8 mmol in 200 mL of 0.1 M borate buffer) followed by the addition of DMSO (100 mL). The stirring was continued for 2 h and the conversion of linoleic acid **1** to **2** was monitored by GC. After incubation, the suspension was acidified to pH 3 and extracted with diethyl ether (250 mL). The combined ethereal layer was dried and concentrated under reduced pressure. The residue was dissolved in methanol (100 mL), cooled at 0 °C and reduced with sodium borohydride (0.05 mol). The methanolic solution was acidified to pH 3, diluted with distilled water (100 mL) and extracted with diethyl ether (100 mL). The ethereal layer was dried and concentrated *in vacuo*. Purification using silica gel column chromatography with 30% diethyl ether in hexane containing 1% glacial acetic acid afforded **3** as a colourless oil (12.2 mmol, 68.5%).

References

1. Kilara, A. *Process Biochem.* **1985**, *20*, 35.
2. Blondin, G. A. *Ann. N. Y. Acad. Sci.* **1975**, *264*, 98.
3. Buchanan, M. R.; Haas, T. A.; Lagarde, M.; Guichardant, M. *J. Biol. Chem.* **1985**, *260*, 16056.
4. Kato, T.; Yamaguchi, Y.; Hirano, T.; Yokoyama, T.; Uychara, T.; Namai, T.; Yamanaka, S.; Harada, N. *Chem. Lett.* **1984**, 409.
5. Kolattukudy, P. E.; Walton, T. J.; Kushwaha, R. P. S. *Biochem. Biophys. Res. Commun.* **1971**, *4*, 739.
6. Maguire, N. M.; Mahon, M. F.; Molloy, K. C.; Read, G.; Roberts, S. M.; Sik, V. *J. Chem. Soc. Perkin Trans. 1* **1991**, 2054.
7. Gardner, H. W.; Newton, J. W. *Phytochemistry* **1987**, *26*, 621.
8. Iacazio, G.; Langrand, G.; Baratti, J.; Buono, G.; Triantaphylides, C. *J. Org. Chem.* **1990**, *55*, 1690.
9. Srinivasulu, S.; Rao, A. G. A. *J. Agric. Food Chem.* **1993**, *41*, 366.
10. Srinivasulu, S.; Rao, A. G. A. *J. Agric. Food Chem.* **1995**, *43*, 562.
11. Emken, E. A.; Dutton, H. J. *JAOCS* **1971**, *48*, 324.
12. Omar, M. N. *PhD Thesis*; Liverpool John Moores University: UK, 2000.