Pergamon

Tetrahedron: Asymmetry, Vol. 7, No. 5, pp. 1489-1492, 1996
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0957-4166/96 $15.00 + 0.00

S0957-4166(96)00167-X

Chemo-Enzymatic Synthesis of Methyl 9(*S*)-HODE (Dimorphecolic Acid Methyl Ester) and Methyl 9(*S*)-HOTE Catalysed by Barley Seed Lipoxygenase.

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Abstract: The straightforward chemo-enzymatic synthesis of methyl 9(*S*)-HODE (dimorphecolic acid methyl ester) and methyl 9(*S*)-HOTE, from linoleic and α-linolenic acids, using barley seeds lipoxygenase under oxygen pressure and at high substrate concentration, is described.
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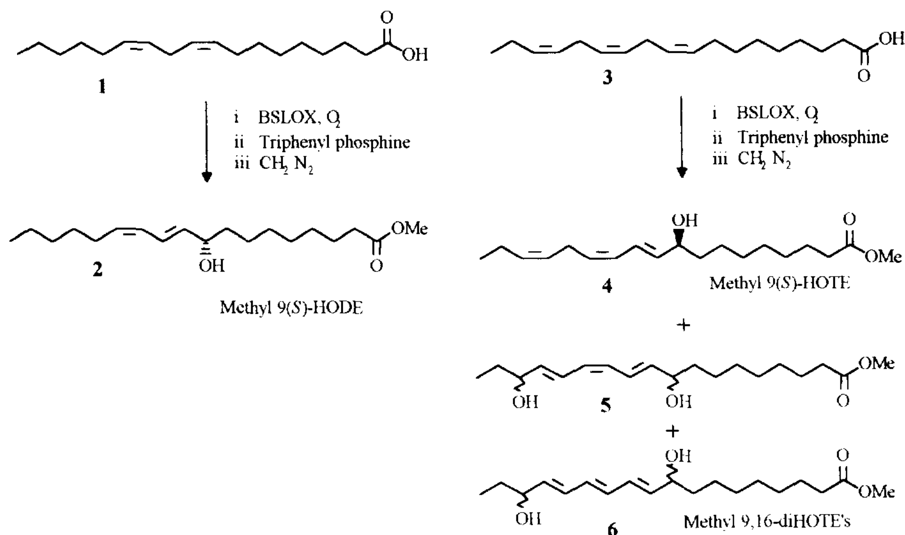
Lipoxygenases (LOX's) (EC 1.13.11.12) are non heme iron-containing dioxygenases which catalyse the stereoselective incorporation of molecular oxygen into the 1-*Z*,4-*Z* pentadienyl system of polyunsaturated fatty acids (PUFA's)[1]. The product of the reaction is a dienic (*E*,*Z*) conjugated hydroperoxide (HPOD) with mainly the *S* absolute configuration. We have recently shown that soybean LOX-1, a 13-specific LOX, could be used at very high linoleic acid **1** concentrations (up to 0.1 M), under oxygen pressure in an aqueous medium, to generate in high yield (80-98 % UV determination), the corresponding 13(*S*)-HPODE.[2,3] In an effort to generalize the preparative use of LOX's[4], we have examined in this work the ability of 9-LOX to transform on a large scale, linoleic and α-linolenic **3** acids into their corresponding 9 HPOD. For the former, the reduction of the HPOD leads to the synthesis of 9-HODE, also called dimorphecolic acid, a natural product found in rice suffering from rice blast disease[5] (see scheme 1).

The first choice to be made in this study was the one of the enzyme since, in contrast to soybean LOX-1, no 9-LOX is commercially available at a reasonable price. If the commonly used 9-LOX in plant biochemical studies is the LOX from potato tuber, its optimum pH (5.5-6) was judged to be too low to conduct high substrate concentration lipoxygenation. We turn then to another 9-LOX, barley seed LOX (BSLOX), which has many advantages:-Barley seeds are an easily accessible raw material. -LOX activity is present in great quantities in the ungerminated seeds.[6,7]-The enzyme is 9 specific towards linoleic acid.[7]-Its optimum pH is around 7-7.5.[6,7] The purification of BSLOX was conducted following in part the procedure of Van Aarle *et al.*.[7] Indeed after

ion exchange chromatography, the enzyme was dialysed against borate buffer 0.1 M, pH 8, to generate a ready to use enzyme preparation (Enzymatic activity : 4-4.5 U / mg of protein).



Scheme 1. Biotransformation of linoleic and α-linolenic acids by barley seed lipoxygenase.

In a preliminary study (data not shown), we determined the optimum values for different physico-chemical parameters of the oxygenation of linoleic acid catalysed by BSLOX. They were as follows: borate buffer ($Na_2B_4O_7,10H_2O$) 0.1M, pH 8, substrate concentration 5. 10^{-2} M (14 g/L), temperature 5°C, oxygen pressure 2.5 bar, enzyme activity 400 U. Using all these values in a single experiment, on a 30 mL scale, we were able to obtain after 90 minutes, a maximum yield in HPOD of 51 % (UV determination, ε=24130 $cm^{-1}.mol^{-1}.L$)[8]. After reduction of the HPOD (with triphenyl phosphine TPP), methylation and purification on silica (hexane/diethyl ether, 50/50), methyl (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoate **2** (methyl 9(*S*)-HODE) was recovered in 44 % yield (195 mg) with a purity of 98.5 % (normal phase HPLC)[2] and more than 99 % e.e. (chiral phase HPLC).[9] The *S* absolute configuration was attributed by comparaison of the optical sign of the specific rotation of 9-HODE ($[\alpha]^{20}_D$=+13.2, c=0.5 (MeOH)) with that of known *S* configuration described in the literature[10] ($[\alpha]^{25}_D$=+14.0, c=26.1 (MeOH)). Methyl 13(*S*)-HODE is the only other product detected in the reaction (1.5 %, e.e. 70%). Unreacted linoleic acid is also recovered as its methyl ester in 31% yield (137 mg).

The reaction was then extended to α-linolenic acid, under the same experimental conditions. After 90 minutes of reaction (no more variation in OD), the recorded UV spectrum of the reaction mixture indicated the formation of a conjugated dienic hydroperoxyde (λ=235 nm) and the probable formation of a conjugated trienic compound (λ= 260, 270 nm). After reduction and methylation, the presence of at least two products was confirmed by CCM. After purification on silica (hexane/diethyl ether, 50/50), the main compound (39% yield,

180 mg) was shown to be methyl (9*S*,10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoate **4** methyl 9(*S*)-HOTE with a purity (normal phase HPLC)[2] and an e.e. (GC analysis)[11] of more than 99%. The second product (5% yield, 24 mg) was shown to be a mixture of approximatively equimolar amounts of methyl (10*E*,12*Z*,14*E*)-9,16-diHOTE **5** and methyl (10*E*,12*E*,14-*E*)-9,16-diHOTE **6** by ^{1}H and ^{13}C NMR, MS and normal phase HPLC. Unreacted α-linolenic acid was also recovered as its methyl ester in 36% yield (158 mg).

In conclusion, we have shown that BSLOX is of interest for the preparative synthesis of both 9(*S*)-HPOD's of linoleic and α-linolenic acids and of their corresponding hydroxy methyl esters. Under the used conditions, the enzyme showed a very high regio, stereo and enantioselectivity, allowing the chemo-enzymatic synthesis of dimorphecolic acid methyl ester. This work together with our previous reports[2,3] demonstrates the general ability of LOX's to functionalize PUFA's at high substrate concentration, providing an optimum pH of the enzyme in the neutral-basic range.

EXPERIMENTAL

The ^{1}H NMR and ^{13}C NMR spectra were recorded with a Brucker AC 200 (200 MHz) spectrometer using TMS as internal standard. Optical rotations were measured on a Perkin Elmer 241 MC instrument. Elemental analyses were performed in the Microanalytical Department in Faculty of Sciences of St Jérôme. Linoleic and α-linolenic acids were from Fluka, other chemicals were from Aldrich. The vessel used for enzymatic oxygenation was a Stainless steel Sotelem MU 4004 laboratory micro unit (100 mL). The reactor was equipped with a thermocouple, a manometer, a magnetically-driven stirrer, the possibility to withdraw liquid samples and a specially fitted PTFE beaker.

General procedure for enzymatic synthesis, hydroperoxide reduction and fatty acid alcohol purification: Exactly 1.5 10^{-3} mole of fatty acid were weighed in a PTFE beaker and then 30 mL of the BSLOX enzymatic preparation (5°C) were added, the vessel closed, pressurized at 2.5 bar of pure oxygen and the stirring set at maximum speed (1600 rpm). The temperature of 5°C was maintained during the reaction by external cooling. Aliquots were withdrawn from time to time and the reaction progress was followed by reading the O.D. at 234 nm (see text) after appropriate dilution. At the end of the reaction, the mixture was acidified to pH 3 with citric acid, diluted with 300 mL of brine and extracted with diethyl ether (3X500mL). The combined organic phases were dried over $MgSO_4$ and the hydroperoxydes reduced overnight (0°C) with TPP (1.5 10^{-3} mole). The solvent was then removed under vacuum to a final volume of approximatively 30 mL and the hydroxy acids methylated with CH_2N_2. Methyl 9(*S*)-HODE and Methyl 9(*S*)-HOTE were then purified by column chromatography over silica using hexane/diethyl ether: 70/30 as developing system.

Methyl 9(*S*)-HODE: colorless oil, $[\alpha]^{20}_D$= +13.8 (c=1, EtOH); UV: λ_{max} = 234 nm, ε=26000 $cm^{-1}.mol^{-1}$. L; IR (thin film): 3441, 2928, 2857, 1741, 1461, 1437, 1199, 1173, 985, 950 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ

0.89 (t, 3H), 1.2-1.5 (bs, 14H), 1.5-1.8 (bm, 4H), 2.18 (dt, 2H, *J*=7Hz, *J'*=7Hz), 2.30 (t, 2H, *J*=7Hz), 3.67 (s, 3H), 4.15 (dt, 1H, *J*=7Hz, *J'*=7Hz), 5.45 (dt, 1H, *J*=8Hz, *J'*=11Hz), 5.66 (dd, 1H, *J*=7Hz, *J'*=15Hz), 5.98 (dd, 1H, *J*=11Hz, *J'*=11Hz), 6.48 (dd, 1H, *J*=11Hz, *J'*=15Hz); ^{13}C NMR (50 MHz) δ 14.0, 22.5, 24.9, 25.3, 27.7, 29.0, 29.1, 29.3, 29.3, 31.4, 34.0, 37.2, 51.4, 72.8, 125.8, 127.6, 133.0, 135.7, 174.3; EI MS (after hydrogenation and silylation) m/z 229, 259; Elem. anal. calculated for $C_{19}O_3H_{34}$: C 73.50, H 11.04, found C 73.52, H 11.04.

Methyl 9(*S*)-HOTE: colorless oil, $[\alpha]^{20}_D$= +15.2 (c=1, EtOH); UV: λ_{max} = 236 nm, ε=27400 cm^{-1}.mol^{-1}.L; IR (thin film): 3435, 2932, 2856, 1741, 1461, 1438, 1200, 1173, 985, 951 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.98 (t, 3H), 1.2-1.5 (bs, 8H), 1.5-1.8 (bm, 4H), 2.08 (dq, 2H, *J*=7Hz, *J'*=7Hz), 2.30 (t, 2H, *J*=7Hz), 2.93 (dd, 2H, *J*=7Hz, *J'*=7Hz), 3.67 (s, 3H), 4.15 (dt, 1H, *J*=7Hz, *J'*=7Hz), 5.2-5.5 (bm, 3H), 5.68 (dd, 1H, *J*=7Hz, *J'*=15Hz), 5.99 (dd, 1H, *J*=11Hz, J'=11Hz), 6.52 (dd, 1H, *J*=11Hz, *J'*=15Hz); ^{13}C NMR (50 MHz) δ 14.3, 20.6, 25.0, 25.4, 26.1, 29.1, 29.2, 29.4, 34.1, 37.3, 51.5, 72.9, 125.5, 126.6, 127.9, 130.8, 132.5, 136.4, 174.4; EI MS (after hydrogenation and silylation) m/z 229, 259; Elem. anal. calculated for $C_{19}O_3H_{32}$: C 73.98, H 10.46, found C 74.06, H 10.41.

ACKNOWLEDGMENT

This work was supported by a grant of the « *Ministère de la Recherche et de l'Enseignement* » n° 90320 attributed to Martini D.

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(Received in UK 6 *March* 1996)