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Manufacture of (5*Z*,8*Z*,11*Z*,13*E*)(15*S*)-15-Hydroxyeicosa-5,8,11,13-tetraenoic Acid Sodium Salt for Clinical Trials

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Abstract:

A robust synthesis of (5*Z*,8*Z*,11*Z*,13*E*)(15*S*)-15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15(*S*)-HETE) sodium salt was established, utilising a biooxidation process. Treatment of arachidonic acid with soybean lipoxidase in 0.1 M sodium tetraborate buffer under oxygen pressure resulted in formation of the hydroperoxide, 15(*S*)-HPETE. Addition of sodium borohydride to the reaction mixture reduced the hydroperoxide to 15(*S*)-HETE, which was then purified by column chromatography. 15(*S*)-HETE sodium salt was prepared by treatment of an ethanol solution of HETE with aqueous sodium hydrogen carbonate. Multiple 10-g batches of 15(*S*)-HETE sodium salt with >98% enantiomeric excess and >98% chemical purity were prepared to support clinical trials.

Introduction

(5*Z*,8*Z*,11*Z*,13*E*)(15*S*)-15-Hydroxyeicosa-5,8,11,13-tetraenoic acid (15(*S*)-HETE, Icomucret) has been in clinical trials for the treatment of dry-eye syndrome.[1] Chirotech was required to develop a robust synthesis and manufacture several 10 g batches of 15(*S*)-HETE sodium salt to support these clinical trials. Due to the high potency of this compound, only small volumes of the active ingredient were required. The sodium salt was preferred to the acid due to improved stability in solution.

Our initial literature survey showed that 15(*S*)-HETE can be prepared by a variety of synthetic organic routes. For example, Nicolaou has reported a synthesis where the correct stereochemistry is introduced by the coupling of a single enantiomer vinyl bromide with a terminal acetylene.[2] The overall yield is a very respectable 25% but due to the number of steps, 15(*S*)-HETE is most conveniently prepared biosynthetically from arachidonic acid. In the procedure described by Baldwin, arachidonic acid was treated with soybean lipoxidase under one atmosphere of oxygen.[3] The intermediate hydroperoxide was reduced by treatment with sodium borohydride in ethanol. Alternatively, sodium borohydride can be added to the reaction mixture at the start, allowing *in situ* reduction of the hydroperoxide and access to 15(*S*)-HETE directly. The major problem of this process is the low volume efficiency (0.5 g/L). The oxidation can be achieved much more rapidly and with greater volume efficiency if it is carried out under increased oxygen pressure, typically 35 psi.[4] However, in this procedure the isolation of the hydroperoxide is very dilute (<1 g/L). The hydroperoxide was reduced using triphenylphosphine, and 15(*S*)-HETE was isolated as the methyl ester derivative after treatment with diazomethane. From our literature assessment we decided that a biooxidation process would be best, but none of these methods could be used directly without further development. Therefore, we set out to devise a modified biooxidation process to produce batches of 15(*S*)-HETE sodium salt.

Results and Discussion

We decided to follow the biooxidation process of Iacazio,[4] using 35 psi oxygen pressure. After the biooxidation we investigated methods to improve the reduction of 15(*S*)-HPETE and isolation of 15(*S*)-HETE. Scheme 1 shows the biooxidation of arachidonic acid and preparation of 15(*S*)-HETE sodium salt.

The first small-scale experiments were carried out in a 50-mL pressure vessel equipped with a glass liner, allowing the input of 1 mmol of substrate in 10 mL of solvent. The following variables were investigated to ensure a robust process, capable of manufacturing several 10-g batches to the same high quality.

Temperature. Temperature was found to be a critical factor, with more side products observed at higher temperature (10 °C) and a slight drop in enantiomeric excess observed. To ensure high enantiomeric excess the reaction mixture was cooled to 0−3 °C before commencing the oxidation and maintained below 5 °C throughout the reaction.

Concentration. An attempt was made to increase the volume efficiency of the reaction, but the reaction at 50 g/L was slower and gave less pure product. At 20 g/L a relatively clean reaction was observed. Further dilution would be expected to give an even cleaner reaction; however, 20 g/L was chosen so we could manufacture 10-g batches in a 2-L pressure vessel. This still represented a significant improvement on what had been achieved previously.

Reaction Time. The yield was lower when the reaction mixture was left under oxygen pressure for an extended period. Polar impurities resulting from further reaction of the hydro-

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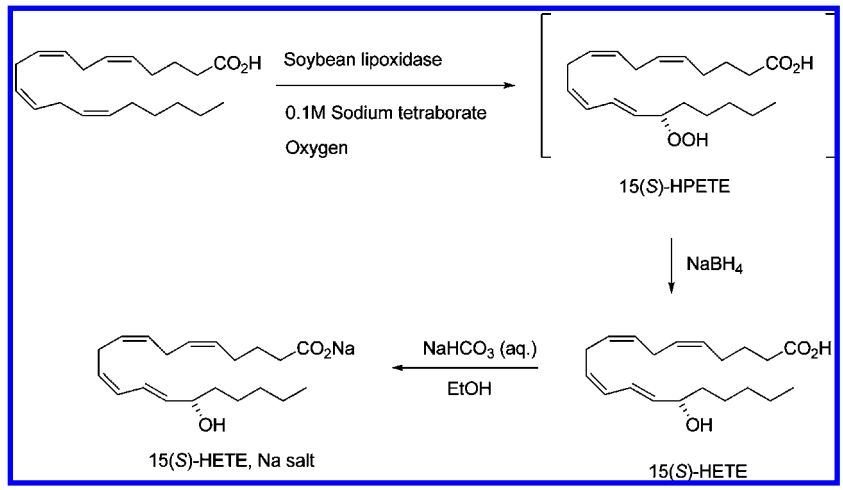
Scheme 1. **Biooxidation of arachidonic acid**



peroxide were observed. In order to maximize the yield, it was necessary to achieve a rapid reaction and a release of the pressure as soon as the reaction was complete (oxygen uptake ceased). However, it was important for the reaction to reach completion since residual arachidonic acid made chromatographic purification of the product difficult, whereas any polar impurities were easily removed by silica gel chromatography. As the reaction was scaled up, vigorous stirring was found to be important in promoting a fast reaction and ensuring all arachidonic acid was consumed. Carrying out the reaction with 13 wt % soybean lipoxidase at 35 psi oxygen enabled complete conversion in approximately 25 min. Further increase in pressure did not have a significant effect.

Reduction of 15(*S*)-HPETE. Various ways of carrying out the reduction of 15(*S*)-HPETE were investigated. The *in situ* reduction with sodium borohydride is not suitable in a pressure reactor even though this appears to be the method of choice when carrying out the reaction under one atmosphere of oxygen. Workup of the hydroperoxide and then treatment with sodium borohydride in ethanol as described by Baldwin was successful, but there is a potential risk associated with concentrating the unstable hydroperoxide, especially as the reaction is scaled up. Use of triphenylphosphine instead of sodium borohydride resulted in slower reaction. This made it difficult to reliably determine the end point of the reaction since a faint positive test for peroxide could be observed after 2−3 h, whereas in the case of the borohydride reduction a negative peroxide test was achieved within 30 min. In addition, isolation of the product from phosphorus impurities was more troublesome. This method appears more suitable for preparation of the corresponding ester, methyl 15(*S*)-HETE since the chromatographic separation is less difficult. We found that reduction of the hydroperoxide in aqueous solution directly after the biooxidation gave the most convenient procedure. Use of an excess of sodium borohydride (3.7 equiv), as described in the original *in situ* method, was successful, but considerable foaming occurred during workup. By using one equivalent of sodium borohydride a significantly easier workup with reduced foaming was developed. Conveniently, the reaction could be followed by TLC (MTBE/heptane, 1:1, containing 0.25% acetic acid) and was found to be complete in 60−90 min. Another benefit of this procedure is that fewer nonpolar impurities were observed during chromatography.

15(*S*)-HETE was obtained in approximately 50% yield after silica gel chromatography, eluting with MTBE/heptanes. The sodium salt was prepared by treatment with aqueous sodium bicarbonate in ethanol followed by evaporation to dryness to give the product as a white powder. To improve stability this was conveniently stored as a 1% wt/v solution in ethanol. Due to concerns over stability of the product towards exposure to light and heat, initial reactions were carried out in amber glassware, and rotary evaporation was limited to 20 °C. However, a series of stressing experiments indicated that processing could be carried out in standard laboratory glassware and evaporation temperatures raised to 30 °C without detriment to the product. This facilitated manufacture of the clinical batches.

Identification of Process-Related Impurities. Samples of 15(*S*)-HETE sodium salt were analysed by liquid chromatography (HPLC) for related substances (see Experimental Section, Table 1, for the HPLC method). Figure 1 shows relative retention times of potential impurities and lists the amount present in each of the first five batches manufactured. These were identified by comparison with appropriate standards. (Note 15-KETE was detected at 260 nm). The main impurities in batches of 15(*S*)-HETE produced by this process were found to be the *trans*-isomer, triene, and 11-HETE. The 15(*R*)-isomer was quantified using chiral SFC (see Experimental Section for the method).

To conclude, we have demonstrated a robust and reproducible synthesis of 15(*S*)-HETE sodium salt that was used to produce multiple batches of high-quality material, with purity and enantiomeric excess >98%, to support clinical trials.

Experimental Section

General Procedures. ^{1}H NMR spectra were recorded at 400 MHz (Bruker DPX 400). ^{13}C NMR spectra were recorded at 100 MHz. Chemical shifts (δ) are quoted in ppm, and coupling constants (J) are given in hertz (Hz). Analytical thin layer chromatography was performed on Merck silica gel precoated plates and visualised using ceric ammonium molybdate. Soybean lipoxidase was obtained from Fluka and had an activity of 150,000 U/mg (where 1U corresponds to the amount of enzyme that causes an increase in absorbance at 234 nm of 0.001 min $^{-1}$ at 25 °C and pH 9.0 on linoleic acid substrate).

		Impurity Levels (%)				
Impurity	RRT	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
(5*Z*,8*Z*,11*E*,13*E*)-15-Hydroxyeicosa-5,8,11,13-tetraenoic acid (15-HETE *trans* isomer)	1.07	0.1	0.1	0.1	0.1	0.1
(5*Z*,8*Z*,11*Z*,13*E*)-15-oxoeicosa-5,8,11,13-tetraenoic acid (15-KETE, ketone)	1.12	-	-	-	-	-
(5*Z*,8*Z*,12*E*,14*Z*)-11-Hydroxyeicosa-5,8,12,14-tetraenoic acid (11-HETE)	1.22	0.4	0.4	0.4	0.4	0.4
(8*Z*,11*Z*,13*E*)-15-Hydroxyeicosa-8,11,13-trienoic acid (15-HETRE, triene)	1.38	0.6	0.65	0.6	0.6	0.7
(5*Z*,8*Z*,11*Z*,13*E*)-15-Hydroperoxyeicosa-5,8,11,13-tetraenoic acid (15-HPETE, hydroperoxide)	1.42	-	-	-	-	-
Total impurities		1.42	1.52	1.36	1.74	1.36
(5*Z*,8*Z*,11*Z*,13*E*)-15(*R*)-Hydroxyeicosa-5,8,11,13-tetraenoic acid (15(*R*)-HETE)		0.9	0.7	0.7	0.7	0.8

Figure 1. **Impurity profile for five batches of 15(*S*)-HETE.**

(5*Z*,8*Z*,11*Z*,13*E*)(15*S*)-15-Hydroxyeicosa-5,8,11,13-tetraenoic Acid. Sodium tetraborate decahydrate (41.32 g, 108 mmol) and water (1065 mL) were charged to the 2-L vessel. The mixture was stirred until a clear solution was obtained. The solution was cooled to 0−3 °C. Arachidonic acid (21.55 g, 70.8 mmol) and soybean lipoxidase (2.83 g) were charged to the vessel. The vessel was closed, and the mixture was stirred vigorously. The vessel was charged to 35 psi with oxygen (vented and refilled three times to obtain an atmosphere of pure oxygen). The mixture was stirred until uptake of oxygen ceased (pressure was recharged to 35 psi every 3−5 min and temperature was maintained below 5 °C). Disappearance of arachidonic acid was confirmed by TLC. The vessel was flushed with nitrogen and then charged with sodium borohydride (2.70 g, 71.4 mmol). The mixture was stirred at 0−5 °C until TLC indicated disappearance of the hydroperoxide. The solution was acidified to pH 3 by charging 2 N sulfuric acid (185 mL). The vessel contents were transferred to a separating funnel and extracted with ice-cold MTBE (720 mL and 2 × 360 mL). The combined organic phases were carefully decanted, and the residual emulsion was filtered through a pad of Kieselguhr (28.6 g). The product was washed through with MTBE (300 mL). The combined MTBE solutions were washed with water (2 × 360 mL) and brine (360 mL). The organic solution was dried over sodium sulfate (100 g) and then filtered. The filter cake was washed with MTBE (250 mL). The MTBE solution was evaporated under reduced pressure (20 °C, final pressure 7 mbar) to give the crude product (28.46 g). The crude product was applied to a silica column (714 g) and eluted with MTBE/heptane (1:1 v/v). Fractions containing pure 15(*S*)-HETE were combined and concentrated under reduced pressure (20 °C, final pressure 0.4 mbar). The vacuum was released under nitrogen to give 15(*S*)-HETE (11.79 g, 36.8 mmol, 52%) as a colourless oil.

ee 98.6% (Chiralpak AD, 90/10 CO_2/MeOH, 3.0 mL/min, 3000 psi, 35 °C, 220 nm, retention times *R* 4.6 min, *S* 7.7 min).

$[\alpha]^{25}_D$ +23.5° (c = 1.0, EtOH).

ν_{max} (film) 3500−2500 (br), 1707, 1457, 1410, and 1237 cm^{-1}.

^{1}H NMR (400 MHz, CD_3OD) δ ppm 6.44 (1 H, dd, *J* 15, 11), 5.89 (1 H, t, *J* 11), 5.56 (1 H, dd, *J* 15.5, 7), 5.35−5.20 (5 H, m), 4.00 (1 H, q, *J* 6), 2.93−2.86 (2 H, m), 2.77−2.73 (2 H, m), 2.20 (2 H, t, *J* 7.5), 2.06−2.01 (2 H, m), 1.57 (2 H, quintet, *J* 7.5), 1.45−1.17 (9 H, m), and 0.81 (3 H, t, *J* 7).

^{13}C NMR (100 MHz, CD_3OD) δ 182.07, 142.60, 135.22, 134.78, 134.47, 134.20, 134.02, 133.33, 130.86, 77.98, 43.07, 38.93, 37.64, 32.20, 31.63, 31.19, 30.95, 30.61, 28.37, and 19.09.

(5*Z*,8*Z*,11*Z*,13*E*)(15*S*)-15-Hydroxyeicosa-5,8,11,13-tetraenoic Acid, Sodium Salt. 15(*S*)-HETE (11.79 g, 36.8 mmol) was dissolved in absolute ethanol (352 mL). Sodium hydrogen carbonate (3.08 g, 36.7 mmol) was dissolved in water (79 mL) and added to the ethanolic 15(*S*)-HETE solution. The mixture was stirred until carbon dioxide evolution ceased. The solution was filtered through a Whatman No. 3 filter paper. The reaction flask was rinsed, and the filter was washed through with absolute ethanol (100 mL). The solution was evaporated under reduced pressure (20 °C, final pressure 0.8 mbar). The vacuum was released under nitrogen. 15(*S*)-HETE sodium salt was dissolved in absolute ethanol (390 mL). The solution was evaporated under reduced pressure (20 °C, final pressure 0.8 mbar). The vacuum was released under nitrogen. The solution and evaporation sequence was repeated twice more. 15(*S*)-HETE sodium salt was dried under high vacuum (20 °C, 0.7 mbar) until weight loss was less than 100 mg/h. 15(*S*)-HETE sodium salt (12.4 g, 36.1 mmol, 98%) was obtained as a white solid. The sodium salt was dissolved in absolute ethanol (1227 mL) and filtered through a Whatman No. 3 filter paper. The solution was transferred to an amber bottle, flushed with nitrogen, and sealed with a Sure/Seal cap. The product was stored at −20 °C.

ee 98.6% (Chiralpak AD, 90/10 CO_2/MeOH, 3.0 mL/min, 3000 psi, 35 °C, 220 nm, retention times *R* 4.6 min, *S* 7.7 min).

Purity 98.5% (Luna phenyl hexyl column, acetonitrile/ammonium acetate/methanol gradient).

$[\alpha]^{25}_D$ +13.3° (c = 1.0, EtOH).

ν_{max} (Nujol) 3283, 1563, 1455, 1418, and 1377 cm^{-1}.

^{1}H NMR (400 MHz, CD_3OD) δ 6.56 (1 H, dd, *J* 15, 11), 6.01 (1 H, t, *J* 11), 5.66 (1 H, dd, *J* 15, 7), 5.45−5.32 (5 H, m),

Table 1. **HPLC Method for Related Substances in 15(*S*)-HETE**

ThermoFinnigan P4000 pump	
UV6000 photodiode array	
AS3000 autosampler	
column	LUNA phenylhexyl, 150 mm × 3.0 mm, 3 μm
temperature	40 °C
eluent A	acetonitrile/0.1 M ammonium acetate/methanol (270:650:80)
eluent B	acetonitrile/0.1 M ammonium acetate/methanol (695:100:205)
flow rate	0.6 mL/min
detection	235 nm (200−300 nm scanning)
injection	10 μL
retention	15-HETE typically 30−35 min

	pump events					
time (min)	0	60	60.1	70	70.1	80
eluent A%	100	100	0	0	100	100
eluent B%	0	0	100	100	0	0

4.11 (1 H, q, *J* 6), 3.04−2.93 (2 H, m), 2.87 (2 H, t, *J* 6), 2.20 (2 H, t, *J* 7.5), 2.15 (2 H, q, J 7.5), 1.68 (2 H, quintet, *J* 7.5), 1.55−1.25 (9 H, m), and 0.93 (3 H, t, *J* 7).

^{13}C NMR (100 MHz, CD_3OD) δ ppm 183.23, 138.38, 131.38, 131.00, 130.16, 129.78, 129.44, 128.98, 126.63, 73.70, 39.28, 38.82, 33.40, 28.72, 28.23, 27.37, 26.97, 26.71, 24.12, 14.83.

See Table 1:

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