Chemoenzymatic halocyclization of 4-pentenoic acid at preparative scale

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Number of tables: 4

Preparation of the biocatalyst

E. coli cultivation

Two 50 mL pre-pre-cultures of LB medium containing 100 µg/mL of ampicillin were inoculated with 5 µL *E. coli* TOP10 pBADgIIIB VCPO glycerol stock and incubated overnight at 37°C and 180 rpm. Subsequently, two preculture of 200 mL and 500 mL were inoculated with pre-preculture. Overexpression was carried out in 15 L stirred tank reactor with 13 L of TB medium supplemented with 100 µg/mL of ampicillin and cultures were inoculated with 200 mL of pre-culture (OD_{600} =4.08) to an OD of approx. 0.05 and grown at 37°C and 180 rpm. When an OD_{600} of 1.0 was reached (approx. 3 h), 15 mL 20% L-arabinose was added to reach a final inducer concentration of 0.02%. After induction, cultures were incubated for additional 24 h at 25°C and 180 rpm.

Purification

The bacterial pellets obtained after centrifugation were re-suspended in 50 mM Tris/H_2SO_4 buffer (pH 8.2). The re-suspended bacterial pellets (0.5 g cells per mL) were stored at -20°C until processed as described below. 0.1 mM PMSF (100 mM stock in isopropanol) was added to the re-suspended cells, which were ruptured with a French press (1.5 Bar) in two cycles. The samples were then centrifuged (10 000 rpm for 20 minutes) and the supernatant was incubated for 0.5 h after reaching 70 °C. The supernatant was again centrifuged for 10 min at 10000 RPM to remove denaturized protein. Subsequently, the supernatant was concentrated using Amicon membrane filters (30 kDa cut-off).The solution was desalted by exchanging 5 times with 50 mM Tris/H_2SO_4 buffer pH8.2 and 1 mM *ortho* vanadate. After centrifugation, the clear supernatant can be used or stored at -20°C until further purification. Purity of the preparation was accessed by SDS page comparing the band intensities (Figure S1).

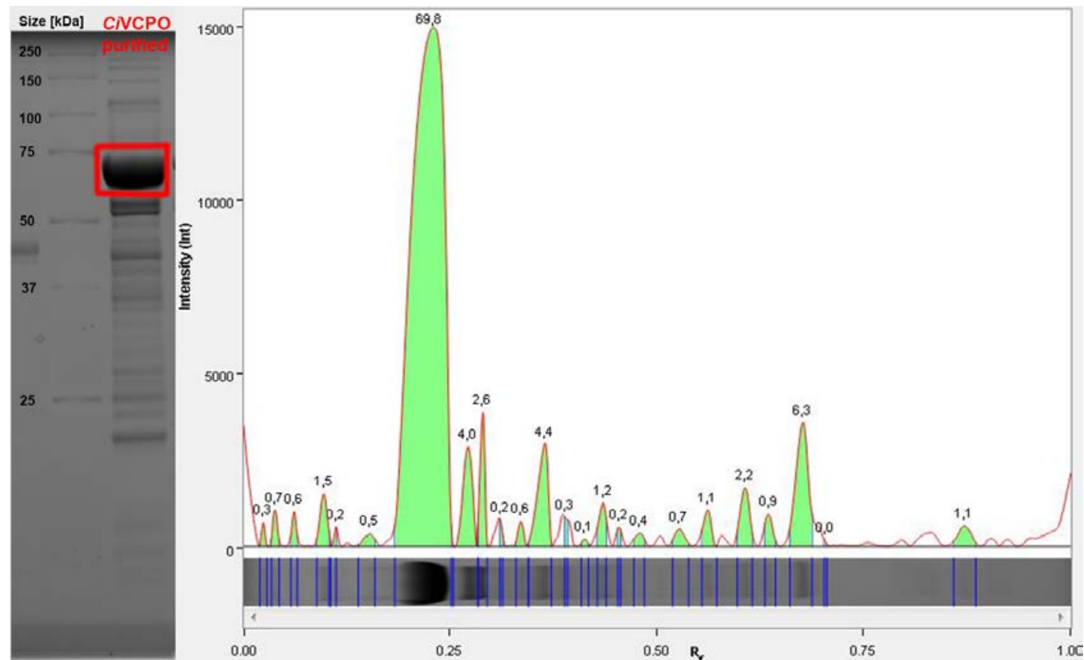


Figure S1. SDS-gel and lane intensity analysis of *Ci*VCPO purification

Activity assay for *Ci*VCPO

*Ci*VCPO activity can be qualitatively tested using the MCD assay by incubating aliquots of cell extracts or purified enzyme in 50 mM citrate buffer(pH 5) containing monochlorodimedone (MCD), *o*-vanadate, KBr and H_2O_2. Reaction is started by the addition of *Ci*VCPO and absorption is followed at 290 nm.

100 µL	MCD (0.5 mM)	50 µM
5 µL	KBr (1M)	5 mM
100 µL	*o*-vanadate (Na_3VO_4 , 1 mM)	0.1 mM
100 µL	1 M sodium citrate buffer, pH 5.0	0.1 M
5 µL	H_2O_2 (1 M)	5 mM
20 µL	enzyme solution (diluted)	
670 µL H_2O		

General procedures

Preparative scale - 500 mL reaction

The preparative scale reaction was performed in a 500 mL three-neck round bottom flask, containing 200 nM *Ci*VCPO (15nmol) in 75 mL water pH 5 and stirred with a magnetic stirrer (Figure S1). As a second layer 200 mL ethyl acetate were applied. The reaction was fed with 2 separate feeds: 1. 0.6 M 4-pentenoic acid and 0.6 mM KBr (pH5) and 2.Hydrogen peroxide (1M) (pH4). Both were fed with a rate of 3.75 mL/h for 20 h. The pH was controlled by a pH STAT titration system (Metrohm, pH 5.0, 25°C) using 1 M acetic acid solution. The reaction was followed online monitoring the amount of acidic acid added. Substrate and product concentration of both phases were measured *via* gas chromatography. An additional aliquot of 15 nmol *Ci*VCPO was added after 8 h and 25 h. In total 68.5 mL 1 M acetic acid were added. The end volume reached was ca. 494 mL. The amount of enzyme applied in the final reaction volume was 45 nmol.

Pilot scale - 10 L reaction

The pilot scale reaction was performed in a 15 L stirred tank reactor (autoclavable bioreactor from Applikon©) equipped with a pH sensor, condenser at 5 degree centigrade, stirring 100 rpm. The head space of the reactor was flushed with a soft nitrogen flow. Starting with 5 L of ethyl acetate phase and 1.5 L of aqueous phase. Feeding pure substrate and 2 M acetic acid from the top into the ethyl acetate phase. H_2O_2 (2 M) and KBr (1.2 M) were fed into the aqueous phase through the gas inlet of the reactor with a feeding rate of 37.5 mL/h. The reaction was started with a *Ci*VCPO aliquot of 0.3 µmol. Substrate and product concentration of both phases were measured by gas chromatography. After the reaction the ethyl acetate phase was dried with magnesium sulfate (1 kg) and the solvent was removed by rotary evaporation at 30 degree with 200 mbar pressure. Residual acetic acid was removed by washing with water and potassium carbonate. Additionally, the aqueous phase was extracted with 2.5 L of DCM in 2 steps. Solvent was removed by rotary evaporation.

After noticing substantial loss in the downstream the 100 mL of remaining aqueous solution was acidified with $HClO_4$ (pH 1.0). There was a white precipitate formed and the solution turned yellow. The mixture was extracted using dichloromethane (3X100 mL), dried over $MgSO_4$, filtered, and evaporate the solvent.

Analytical procedures

GC-analytics

Samples of 0.02 mL aqueous reaction mixture were extracted with 0.2 mL ethyl acetate and dried with magnesium sulfate. Samples of 0.02 mL from the ethyl acetate phase were diluted with 0.18 mL ethyl acetate dried with magnesium sulfate. As internal standard 5 mM acetophenone was used.

Table S1.GC analysis specifications for concentration determination

[Compounds] (retention time)	GC column	GC method
4-pentenoic acid (5.1 min)/ 5-(hydroxy)dihydro-2(3H)-furanone (9.5 min)/ 5-(bromomethyl)dihydro-2(3H)-furanone (10.5 min)/ acetophenone (8 min)	Cp-sil 5 CB GC column (25 m x 0.25 mm x 1.2 µm)	70 °C for 1 min. 30 °C/min to 140 °C hold for 2 min. 30 °C/min to 195°C hold for 2 min. 30 °C/min to 225 °C hold for 1 min. 30 °C/min to 345 °C hold for 1 min.

GC-MS analytics

Gas chromatography-mass spectrometry was performed with the Shimadzu GC-2010 system which is connected to the GCMS-QP2010s mass detector from Shimadzu.

Table S2.GC-MS analysis specifications for structure determination

[Compounds] (retention time)	GC column	GC method
4-pentenoic acid (5.1 min)/ 5-(hydroxy)dihydro-2(3H)-furanone (9.5 min)/ 5-(bromomethyl)dihydro-2(3H)-furanone (10.5 min)/ acetophenone (8 min)	Cp-sil 5 CB GC column (25 m x 0.25 mm x 0.4 µm)	70 °C for 1 min. 30 °C/min to 140 °C hold for 2 min. 30 °C/min to 195°C hold for 2 min. 30 °C/min to 225 °C hold for 1 min. 30 °C/min to 345 °C hold for 1 min.

Supporting Figures

Time courses of smaller-scale bromolactonization reactions

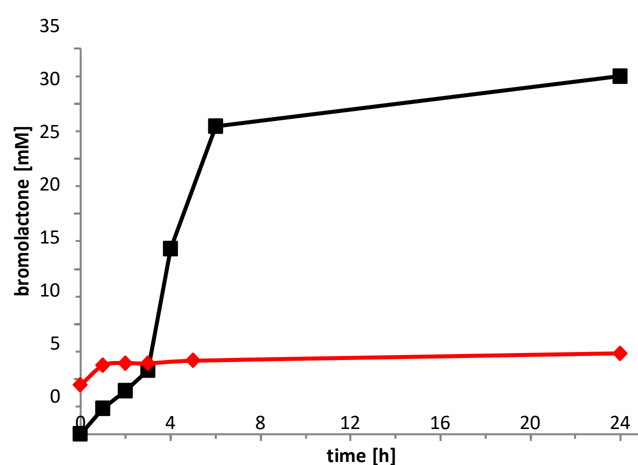
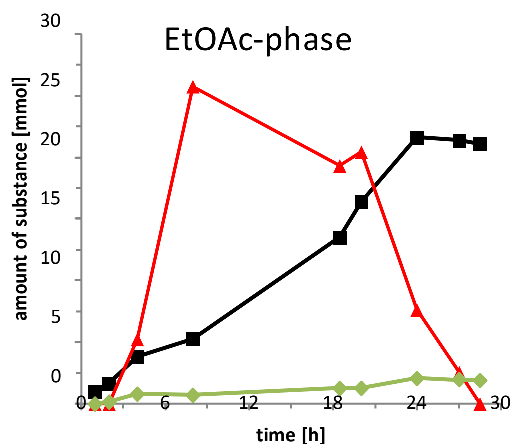
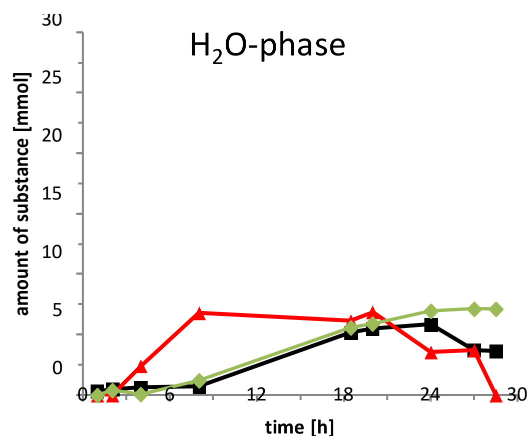


Figure S2. Chemoenzymatic bromolactonization on mL-scale using 40 mM (■) and 500 mM (♦) starting material.
General conditions: 100 mM citrate buffer (pH5), T = 25ºC, [*Ci*VCPO] = 100 nM, 40 mM experiment: [KBr] = [H_2O_2] = 160; 500 mM experiment: [KBr] = [H_2O_2] = 500 mM.





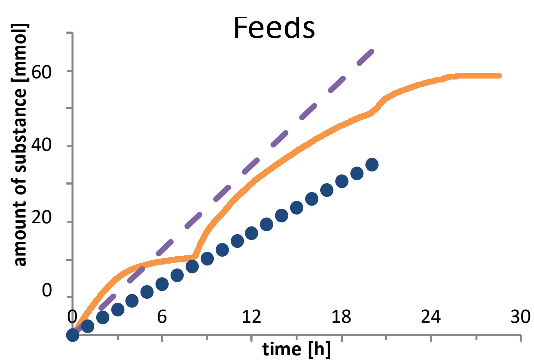


Figure S3. Chemoenzymatic bromolactonization in a biphasic reaction on 500 mL-scale. Preparative scale bromolactonization biphasic 200 mL EtOAc total end volume 500 mL
(■): 5-(bromomethyl)dihydro-2(3H)-furanone; (◆): 5-(hydroxymethyl)dihydro-2(3H)-furanone; (▲): 4-pentenoic acid;
Conditions: v(EtOAc) = 200 mL, v(H_2O)$_{initial}$ = 200 mL (100 mM citrate buffer pH 5); n(*Ci*VCPO)$_{initial}$ = 15 nmol (further additions of 15 nmol at 8 and 25h); Feeds: (●) 4-pentenoic acid 0.6 M and KBr 0.6 M with 3.75 mL/h; H_2O_2 1 M with 3.75 mL/h (**solid line**)
General conditions: T = 25°C, pH 5 controlled by pH stat with 1 M acetic acid (**solid line**).

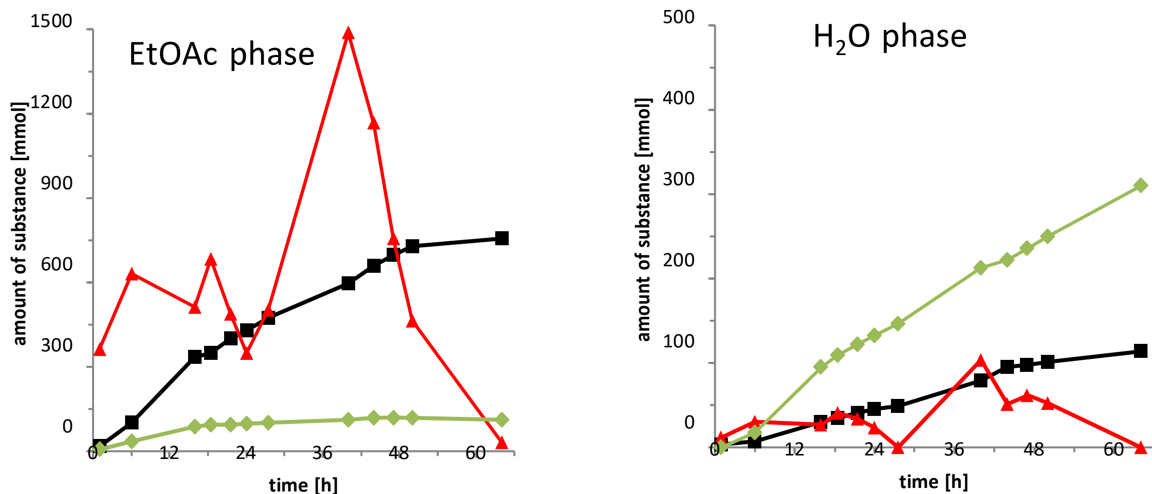


Figure S4. Chemoenzymatic bromolactonization at 10 L-scale (■): 5-(bromomethyl)dihydro-2(3H)-furanone; (◆): 5-(hydroxymethyl)dihydro-2(3H)-furanone; (▲): 4-pentenoic acid;
General conditions: T = 25°C, pH 5 controlled by pH stat with 2 M acetic acid 50 rpm after 24 h to 75 rpm; *Ci*VCPO aliquots added (0.3 µmol) 0 h; 6 h; 21.5 h; 27.5 h; 41.5 ; Feeds: pure 4-pentenoic acid 9.798 M; 4.04 mL/h; KBr 1.2 M 37.5 mL/h; H_2O_2 2M 37.5 mL/h; Feeds started 2h prior to first *Ci*VCPO addition; starting volume of 1.63 L with 49 mM 4-pentenoic acid, 55 mM KBr and 92 mM H_2O_2; 50 h

NMR spectra

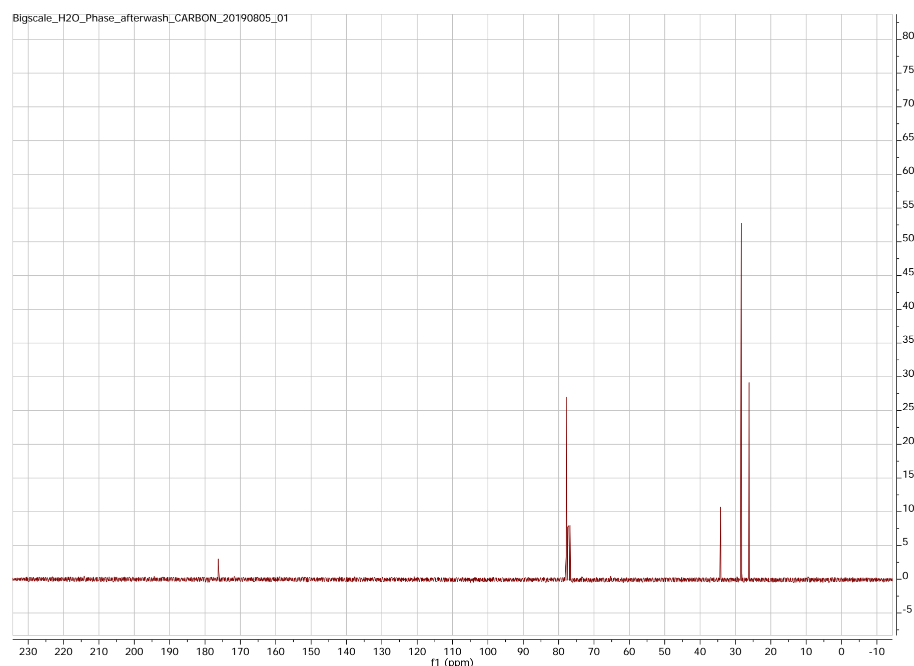


Figure S5. ^{13}C NMR spectrum

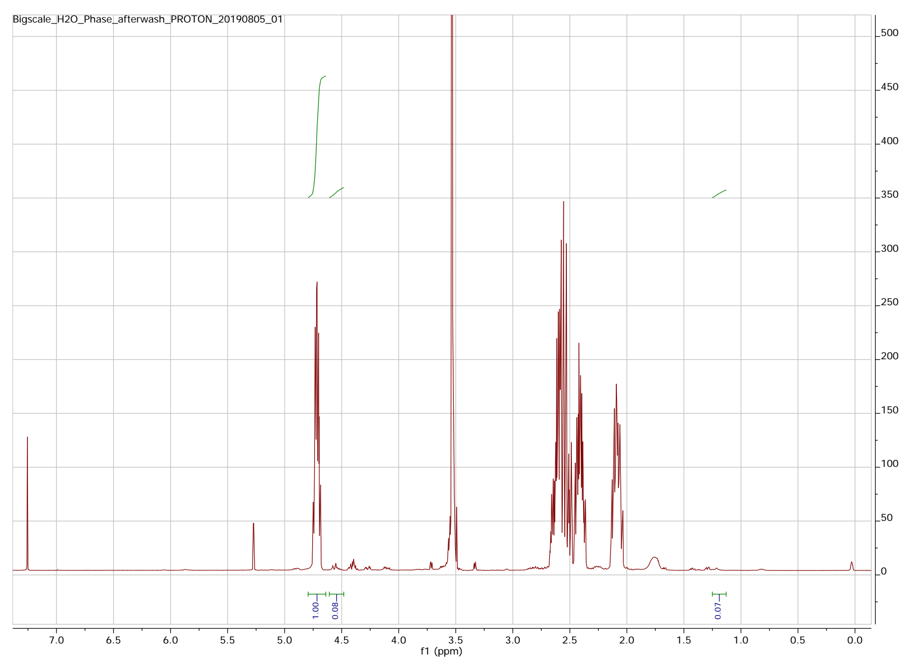


Figure S6. ^{1}H NMR- spectrum

5-(hydroxymethyl)dihydro-2(3H)-furanone): ^{1}H NMR: d=2.06–2.15 (m, 1 H), 2.18–2.27 (m, 1 H), 2.45–2.63 (m, 2 H), 3.60 (dd, J=4.6, 12.5 Hz, 1 H), 3.85 (dd, J=2.9, 12.5 Hz, 1 H), 4.57–4.62 (m, 1H);

(5-(bromomethyl)dihydro-2(3H)-furanone): ^{1} H NMR (400 MHz, $CDCl_3$) δ 2.14 (m, 1H: CHHCHO), 2.46 (m, 1H: CHHCHO), 2.53-2.73 (m, 2H, CH_2CO_2), 3.55 (dd, J = 5.9, 10.8 Hz, 1H: CHHBr), 3.59 (dd, J = 4.4, 10.8 Hz, 1H: CHHBr), 4.76 (p, J = 6.7, 1H: CHO); ^{13}C NMR (75 Hz, $CDCl_3$) δ 26.6 (CH_2CHO), 28.8 (CH_2Br) 34.5

Mass spectra

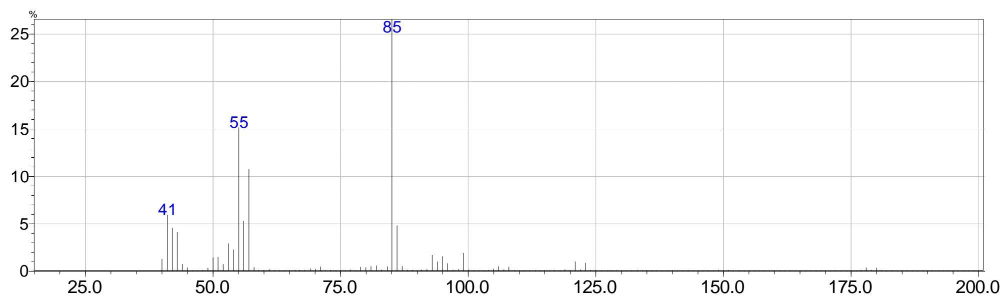


Figure S7. Mass spectrum 5-(bromomethyl)dihydro-2(3H)-furanone

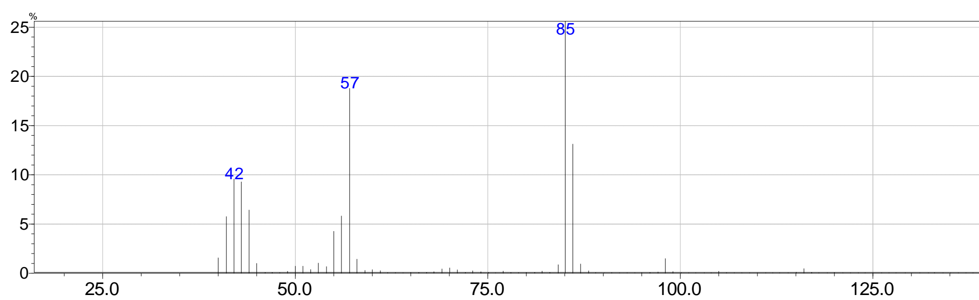


Figure S8. Mass spectrum 5-(hydroxymethyl)dihydro-2(3H)-furanone

Photographs of the reaction setup

Figure S9. Pilot scale experimental setup.

Figure S10. Amperometer used to determine the electricity consumption.

Values for the E- and E+-factor calculation

Table S3. E-factor and E^+-factor with OECD average (2015) value of 404 g_{CO2} kWh^{-1} for 15 L scale fermentation and purification of *Ci*VCPO

Fermentation	Energy contribution [kWh]	[kg]	CO_2 [kg]
LB (0.8 L)		0.020	
TB (13L)		0.778	
20% L-arabinose (0.015 L)		0.0003	
Wash buffer 50 mM Tris/H_2SO_4 (1L)		0.007	
Water (14 L)		14.000	
Cryostat	25.622		10.351
Stirring and heating	5.679		2.294
Autoclave before	2.256		0.911
Autoclave after	2.256		0.911
Centrifuge harvest	8.000		3.232
Centrifuge wash	8.000		3.232
Fermentation total	51.813	14.806	

Purification	[kWh]	[kg]	CO_2 [kg]
Resuspension b. 50 mM Tris/ H_2SO_4 (0.95 L)		0.007	
Desalting b. 50 mM Tris/H_2SO_4 1mM VO_4^{3-} (2.4 L)		0.018	
Water (3.3 L)		3.320	
Cell lysis	14.300		5.777
Centrifuge	0.506		0.204
Cryostat	5.562		2.247
Centrifuge	0.506		0.204
Purification total	20.874	3.345	8.433
***Ci*VCPO production including:** **Fermentation & Purification**			
Total for *Ci*VCPO production			
*Ci*VCPO produced [kg]		0.0006775	

Table S4. E⁺-factor for the pilot scale bromolactonization, reaction, downstream and total process

Reaction	Energy contribution kWh]	[kg]	CO_2 [kg]
EtOAc		4.470	
Water		4.839	
*Ci*VCPO		0.0001	
H_2O_2		0.102	
KBr		0.123	
4-Pentenoic acid		0.013	
Acetic acid		0.117	
Cryostat	29.342		11.854
Stirring	0.452		0.182
pH stat	0.398		0.161
Reaction total	30.191	9.665	12.197
Downstream	[kWh]	[kg]	CO_2 [kg]
$MgSO_4$		1	
Potassium carbonate		0.1	
Water		0.5	
Rotary evaporation	3.918		1.583
Downstream total	3.918	1.6	1.583
Total process including:			
Reaction			
Downstream			
Fermentation			
Purification			
Total process			
Bromolactone produced [kg]		0.081426	