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Supporting Information Animation S1. Determination of the ratio of DhaA31, HheC and EchA required to achieve 95 % conversion of 2 mM 1,2,3-trichloropropane into glycerol within 300 min while minimizing the loading of each enzyme.

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Reagents. 1,2,3-trichloropropane (TCP), (*R*,*S*)-2,3-dichloropropane-1-ol (DCP), (*R*,*S*)-epichlorohydrin (ECH), (*R*,*S*)-3-chloropropane-1,2-diol (CPD), (*R*,*S*)-glycidol (GDL), glycerol (GLY), acetone, ethyl acetate, and hexanol were purchased from Sigma-Aldrich (USA). All chemicals used in this study were of analytical grade. The Free Glycerol Assay Kit was purchased from BioVision (USA).

DNA manipulations. The nucleotide sequences of genes encoding wild-type haloalcohol dehalogenase HheC (accession number AF397296.1) and epoxide hydrolase EchA (Y12804.1) from *Agrobacterium radiobacter* AD1 were downloaded from the GenBank database. The genes of HheC and EchA together with those for the wild-type and mutant haloalkane dehalogenases DhaA, DhaA31 and DhaA90R were synthesized commercially (Geneart, Germany). Restriction sites for cloning into pET21b (*Nde*I, *Bam*HI) (Merck, Germany) or pET28b (*Nco*I, *Hin*dIII) were attached to all sequences. Due to the creation of an *Nco*I restriction site, the threonine-encoding second codon of the *echA* gene (ACT) was replaced with a GCA codon encoding alanine. A tag sequence of six histidine codons was attached downstream of the *dhaAwt*, *dhaA31*, *dhaA90R* and *echA* genes and the sequences of all four genes were optimized for expression in *Escherichia coli* during their synthesis. The synthetic genes (Supporting Information Table S1) were subcloned into the *Nde*I and *Bam*HI restriction sites of pET21b (*dhaA*, *dhaA31*, *dhaA90R* and *hheC*) or *Nco*I and *Hin*dIII restriction sites of pET28b (*echA*). Competent cells of strain *E. coli* DH5α were transformed with the resulting constructs (pET21b::*dhaA*, pET21b::*dhaA31*, pET21b::*dhaA90R*, pET21b::*hheC* and pET28b::*echA*) using the heat-shock method for plasmid propagation.

Cultivation conditions and enzyme purification. Competent cells of *E. coli* strain BL21(DE3) were transformed with plasmid constructs using the heat-shock method and plated on LB agar plates with ampicillin (100 $\mu g.ml^{-1}$) or kanamycin (50 $\mu g.ml^{-1}$). Plates were incubated overnight at 37 °C. Single colonies were used to inoculate 10 ml of LB medium containing the appropriate antibiotic, and cells were grown overnight at 37 °C. The overnight cultures were used to inoculate 1 l of LB medium containing the appropriate antibiotic. The cells were then cultivated at 37 °C with shaking until an OD_{600} of 0.4 – 0.6 was achieved, at which point expression was induced with 0.5 mM IPTG and the cells were cultivated overnight at 20°C. Biomass was harvested by centrifugation. Cells were washed and

resuspended in purification buffer A (20 mM K_2HPO_4 and KH_2PO_4, 0.5 M NaCl, 10 mM imidazole, pH 7.5 for DhaA, DhaA31 and EchA or 20 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.5 for HheC). 1 U of DNaseI (New England Biolabs, USA) per 1 ml of cell suspension was added. Cells were disrupted by sonication using a Hielscher UP200S ultrasonic processor (Teltow, Germany) with 0.3 s pulses and 85 % amplitude. The cell lysate was centrifuged for 1 h at 21,000 *g* at 4 °C, after which the resulting cell-free extract was decanted. DhaA, DhaA31, DhaA90R and EchA were purified by single-step nickel affinity chromatography. The crude extract was applied to a 5 ml Ni-NTA Superflow column (Qiagen, Germany) attached to a BioLogic Duo Flow chromatography system (Bio-Rad, USA). The buffer system consisted of buffer A and buffer B (20 mM K_2HPO_4 and KH_2PO_4, 0.5 M NaCl, 500 mM imidazole, pH 7.5). Recombinant enzymes were eluted in 60 % of buffer B using a two-step gradient sequence, with the proportion of B in the eluent increasing from 0 – 10 % in 5 column volumes and then from 10 – 60 % in 10 column volumes. Fractions containing DhaA, DhaA31 or EchA were pooled and the proteins were concentrated using a stirred ultrafiltration cell (Millipore, USA). Enzymes were dialyzed against 50 mM phosphate buffer (pH 7.5). HheC was purified using anion-exchange chromatography:[1] the crude extract was applied to a 35 ml glass Econo-Column (Bio-Rad, USA) that was packed with 25 ml of Q Sepharose Fast Flow (GE Healthcare, USA) and attached to a BioLogic Duo Flow chromatography system (Bio-Rad, USA). The buffer system consisted of buffer A and buffer B (20 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.45 M $(NH_4)_2SO_4$, pH 7.5). HheC eluted in 20 – 25 % of buffer B during the following two-step linear gradient: 0 – 45 % buffer B in 20 column volumes followed by 45 – 100 % buffer B in 5 column volumes. Fractions containing HheC were pooled and the protein was concentrated. The enzyme was dialyzed against 50 mM phosphate buffer (pH 7.5) containing 2 mM dithiothreitol. Protein concentrations were determined using the Bradford Reagent (Sigma-Aldrich, USA). Enzyme purity was determined by SDS-PAGE analysis. Purified enzymes were stored at 4°C until further use.

Analytical techniques. A gas chromatograph (GC) 6890N with a flame ionisation detector (Agilent Technologies, USA) and a ZB-FFAP 30 m x 0.25 mm x 0.25 μm capillary column (Phenomenex, USA) was used for routine analysis of samples containing TCP, DCP, ECH, CPD, and GDL (Figure S1). A GC 7890A and 5975C MSD Mass Spectrometer (MS) (Agilent Technologies, USA) equipped with a ZB-FFAP 30 m x 0.25 mm x 0.25 μm capillary column (Phenomenex, USA) were used to verify the presence of individual TCP pathway metabolites

in selected samples. Samples were injected into the GC at an inlet temperature 250°C and a split ratio of 20:1. The column was operated using helium as the carrier gas with an initial flow of 0.6 ml.min^{-1} for 1 min, followed by a flow increase from 0.6 to 1.8 ml.min^{-1} (ramp 0.2 ml.min^{-1}). The column temperature was initially maintained at 50°C for 1 min after which it was increased from 50 to 220°C (ramping at 25°C.min-^{1}) and held at 220 °C for 2 min. The temperature of the detector was set to 250°C and the MS scan speed was 6.9 s^{-1}. To facilitate data analysis, calibration curves for TCP, DCP, ECH, CPD and GDL were constructed covering concentrations of 0 – 5 mM and using hexanol as an internal standard. Detection limits for the target analytes were calculated using OriginPro v8 (OriginLab Corporation, USA) and were 3, 5, 6, 186 and 22 μM for TCP, DCP, ECH, CPD and GDL, respectively.

Production of (*S*)-DCP by preparative kinetic resolution of (*R*,*S*)-DCP. Kinetics of HheC with (*S*)-DCP was determined with the substrate prepared by kinetic resolution of commercially available racemate using (*R*)-selective HheC. Kinetic resolution of (*R*,*S*)-DCP was performed in 4 l of 50 mM Tris-SO_4 buffer (pH 8.5) at 30ºC using a 10 l screw cap bottle. Substrate of purity ≥ 97 % (Sigma Aldrich, USA) was added to a final concentration of 22 mM. The enzymatic reaction was initiated by adding approximately 115 mg of HheC as a crude extract and 125 mg of purified EchA. The excess of EchA prevented the reverse reaction during the conversion of DCP and CPD by pushing the equilibrium toward the product.[2] The reaction was periodically monitored for 8 h and samples were analysed using a Network GC System 6890N (Agilent Technologies, USA) equipped with a flame ionisation detector and an Astec CHIRALDEX B-TA 30 m x 0.25 mm x 0.12 μm capillary column (Sigma-Aldrich, USA). Samples (0.5 ml) were collected from the reaction mixture, extracted with 1 ml of ethyl acetate, and dried with anhydrous sodium sulphate. Aliquots (1 μl) were injected into the GC at an injector temperature of 200°C and a split ratio of 83:1. The operating column temperature was held at 100°C for 15 min. Helium was used as the carrier gas at a continuous flow rate of 0.8 ml.min^{-1}. The flame ionisation detector was operated at 250°C. Reaction was terminated and unconverted (*S*) enantiomer was extracted twice with 0.5 and 1 l of ethyl acetate. The organic phase was dried with sodium sulphate. Ethyl acetate was removed at 40 °C (water bath temperature) and 240 mbar and then at 60 °C (bath temperature) and 60 mbar using a Vacuum Rotavapor R-215 fitted with a vacuum controller, vacuum pump and heating bath (Büchi, Switzerland). After the evaporation of most of the ethyl acetate, the chemical composition of the residue was determined by NMR after dissolution in $CDCl_3$. NMR spectra were acquired using a 600 MHz AVANCE spectrometer (Bruker, Germany)

with a double resonance TCI probe (^{1}H, ^{13}C). The resulting NMR spectra were compared to predicted NMR data calculated using Advanced Chemistry Development Software v11.01 (ACD/Labs, Canada). The purity (85 %) of the synthesized (*S*)-DCP with residual ethyl acetate was determined by GC using (*R*,*S*)-DCP as a reference material. The (S)-DCP was obtained obtained with e.e. ≥ 99 %.

Enzyme kinetics. Steady-state kinetic parameters for DhaA, DhaA31 and DhaA90R with TCP and for HheC with (*R*,*S*)-DCP, (*S*)-DCP and (*R*,*S*)-CPD were determined in 25 ml micro-flasks sealed with Mininert valves (Alltech, USA) in 10 ml of 50 mM Tris-SO_4 buffer (pH 8.5) at 37°C. Because HheC is strongly (*R*)-selective, the kinetic constants for (*R*)-DCP, which was commercially unavailable, were determined by measuring the initial velocity of racemic DCP conversion. The initial velocity measurements were carried out at various substrate concentrations that were verified by GC. The reaction was initiated by adding the purified enzyme and terminated by mixing samples of the reaction mixture with 35% (v/v) nitric acid. The concentration of the reaction product (chloride ions) was measured using a Sunrise spectrophotometer (Tecan, Switzerland) at 460 nm after the addition of mercuric thiocyanate and ferric ammonium sulfate.[3] Specific activities were quantified by measuring the slope of the initially-linear region in a plot of halide concentration against time. The kinetic constants were calculated by non-linear fitting using Origin 7.5 (OriginLab Corporation, USA) or DynaFit 4 (BioKin, USA)[4] based on the Michaelis-Menten equation (1) where v is the reaction velocity, E_0 is the enzyme concentration, k_{cat} is the turnover number, S is substrate concentration and K_m is the Michaelis constant. Steady-state kinetic parameters for EchA with (*R*,*S*)-ECH and (*R*,*S*)-GDL were determined by monitoring the depletion of the substrate (initial concentration: 5 mM) in 10 ml of 50 mM Tris-SO_4 buffer (pH 8.5) at 37°C. The substrate concentration was verified by GC. The reaction was initiated by adding the purified enzyme. Samples of the reaction mixture (0.5 ml) were mixed with 0.5 ml of acetone containing the internal standard hexanol and analyzed by GC. The numerical integration program Scientist 1.0 (MicroMath, USA) was used to fit the ECH concentration progress curve to equation (1). The GDL concentration progress curve was fitted to equation (2), where P is the concentration of the reaction product and K_i is a product inhibition constant. The inhibition constant K_c was determined by measuring the inhibitory effects of TCP at various concentrations (0, 0.5, 1.0 and 2.5 mM) on the conversion of 5 mM GDL by EchA in 15 ml of 50 mM Tris-SO_4 buffer (pH 8.5) at 37°C (Figure S3). Experimental concentrations of GDL and TCP were verified by GC. The numerical integration software Scientist 1.0 (MicroMath,

USA) was used to fit the GDL concentration progress curves for each separate inhibitor concentration to equation (3), where I is the inhibitor concentration. The resulting expressions were compared to obtain a consensus value for the equilibrium inhibition constant K_c.

$$\frac{v}{E_0} = \frac{k_{cat} \times S}{K_m + S} \quad (1)$$

$$\frac{v}{E_0} = \frac{k_{cat} \times S}{K_m + S \times (1 + \frac{P}{K_i})} \quad (2)$$

$$\frac{v}{E_0} = \frac{k_{cat} \times S}{K_m + S \times (1 + \frac{P}{K_i} + \frac{I}{K_c})} \quad (3)$$

Enzyme enantioselectivity. The formation of the (*R*) and (*S*) enantiomers of DCP during the conversion of TCP (2 mM) by DhaA, DhaA31 or DhaA90R was measured in 15 ml of Tris-SO_4 buffer (pH 8.5) at 37°C. Samples (0.5 ml) were taken periodically, mixed with 1 ml of ethyl acetate and vortexed for 30 s. The organic phase was then dried over sodium sulphate and analysed by chiral GC as described previously.

Kinetic model development. The kinetic model of the TCP pathway was assembled based on the reaction scheme shown in Figure 1 using the kinetic parameters listed in Table 1. All five reaction steps in the multi-enzyme conversion of TCP exhibited Michaelis-Menten kinetics. The conversion of the prochiral TCP into either (*R*)-DCP or (*S*)-DCP by DhaA, DhaA31 or DhaA90R was described using equation (4). The rate constants $k_{cat,TCP,(R)\text{-}DCP}$ and $k_{cat,TCP,(S)\text{-}DCP}$ were determined from the overall k_{cat} for the appropriate DhaA variant with TCP and the ratio of (*R*)- and (*S*)-DCP produced from the prochiral TCP by DhaA, DhaA31 or Dha90R. The formation of (*R*)-DCP and (*S*)-DCP and the subsequent conversion of both enantiomers into (*R*,*S*)-ECH was described using equations (5) and (6). The remaining reaction steps were assumed to be non-selective. The consecutive conversions of (*R*,*S*)-ECH, (*R*,*S*)-CPD and (*R*,*S*)-GDL by HheC and EchA were described using equations (7), (8) and (9), respectively. The final reaction step, i.e. the conversion of (*R*,*S*)-GDL into the final product GLY by EchA, was described using Michaelis-Menten equations (9) and (10). These expression feature the equilibrium constants K_i and K_c, which describe the competitive inhibition caused by GLY and TCP, respectively.

$$\frac{dc_{TCP}}{dt} = -\frac{k_{cat,TCP,(R)-DCP} \times c_{DhaA} \times c_{TCP}}{(c_{TCP} + K_{m,TCP})} - \frac{k_{cat,TCP,(S)-DCP} \times c_{DhaA} \times c_{TCP}}{(c_{TCP} + K_{m,TCP})} \quad (4)$$

$$\frac{dc_{(R)-DCP}}{dt} = \frac{k_{cat,TCP,(R)-DCP} \times c_{DhaA} \times c_{TCP}}{c_{TCP} + K_{m,TCP}} - \frac{k_{cat,(R)-DCP} \times c_{HheC} \times c_{(R)-DCP}}{c_{(R)-DCP} + K_{m,(R)-DCP}} \quad (5)$$

$$\frac{dc_{(S)-DCP}}{dt} = \frac{k_{cat,TCP,(S)-DCP} \times c_{DhaA} \times c_{TCP}}{c_{TCP} + K_{m,TCP}} - \frac{k_{cat,(S)-DCP} \times c_{HheC} \times c_{(S)-DCP}}{C_{(S)-DCP} + K_{m,(S)-DCP}} \quad (6)$$

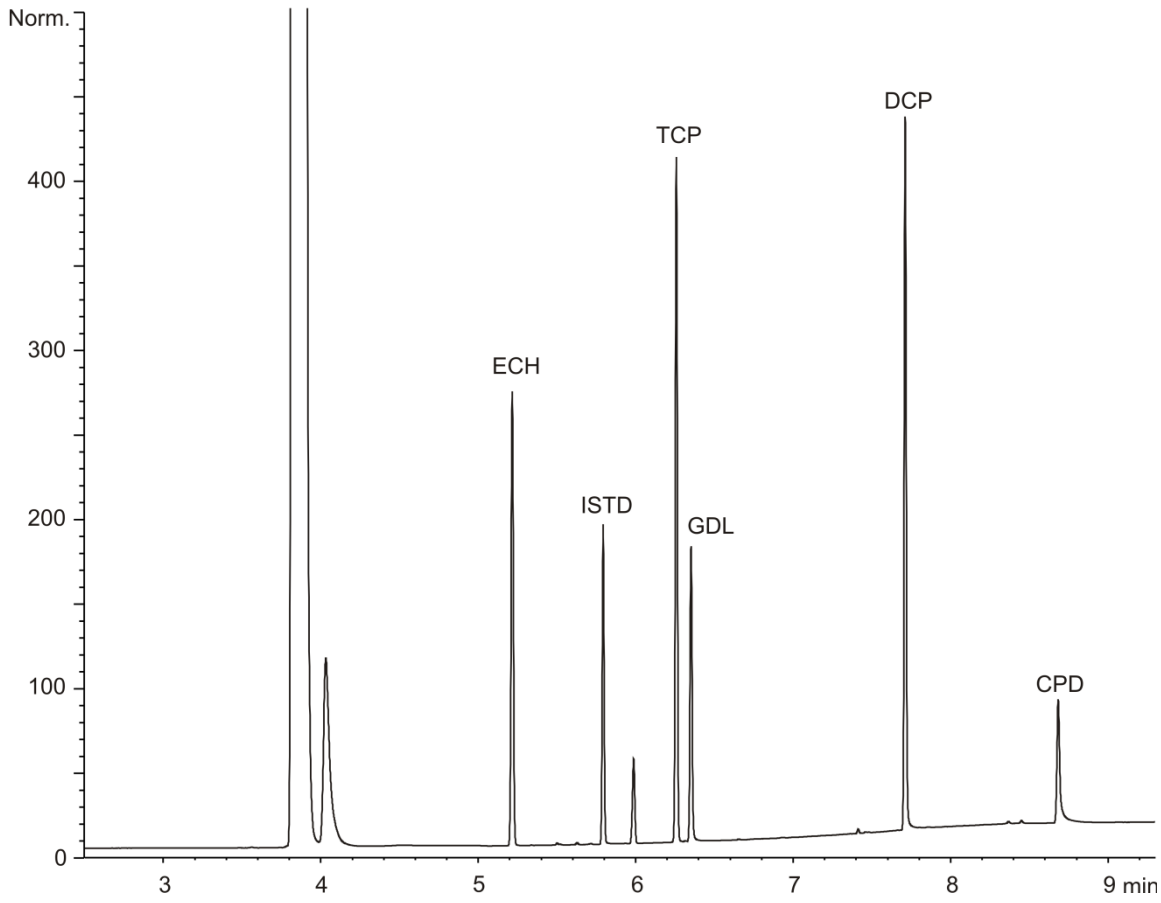
$$\frac{dc_{ECH}}{dt} = \frac{k_{cat,(R)-DCP} \times c_{HheC} \times c_{(R)-DCP}}{c_{(R)-DCP} + K_{m,(R)-DCP}} + \frac{k_{cat,(S)-DCP} \times c_{HheC} \times c_{(S)-DCP}}{c_{(S)-DCP} + K_{m,(S)-DCP}} - \frac{k_{cat,ECH} \times c_{EchA} \times c_{ECH}}{c_{ECH} + K_{m,ECH}} \quad (7)$$

$$\frac{dc_{CPD}}{dt} = \frac{k_{cat,ECH} \times c_{EchA} \times c_{ECH}}{c_{ECH} + K_{m,ECH}} - \frac{k_{cat,CPD} \times c_{HheC} \times c_{CPD}}{c_{CPD} + K_{m,CPD}} \quad (8)$$

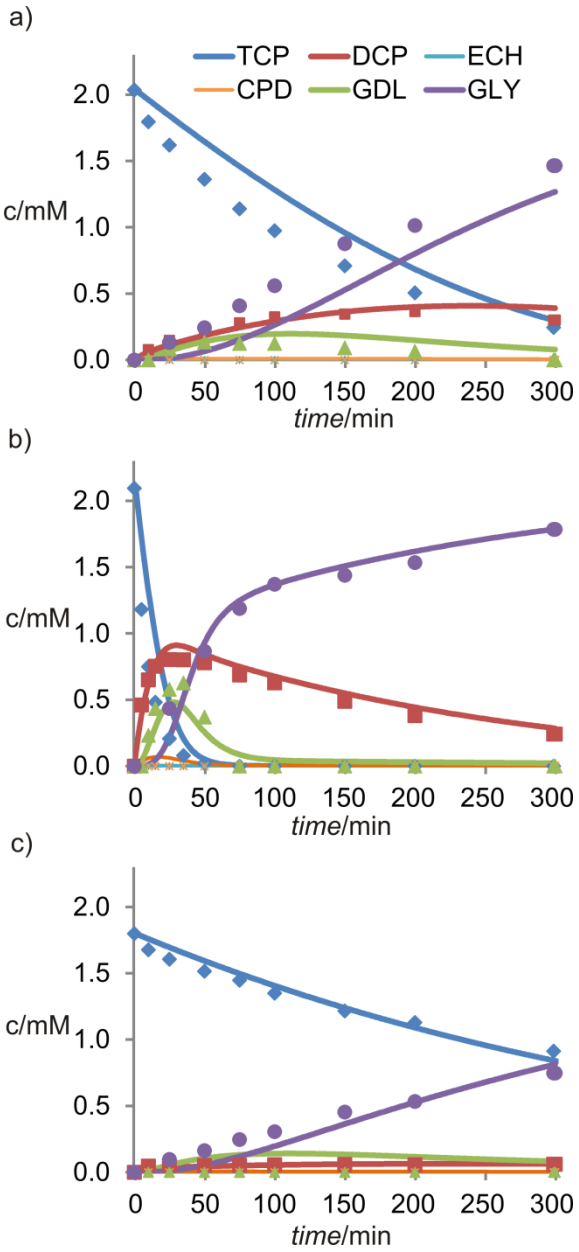
$$\frac{dc_{GDL}}{dt} = \frac{k_{cat,CPD} \times c_{HheC} \times c_{CPD}}{c_{CPD} + K_{m,CPD}} - \frac{k_{cat,GDL} \times c_{EchA} \times c_{GDL}}{c_{GDL} + K_{m,GDL} \times \left(1 + \frac{c_{GLY}}{K_i} + \frac{c_{TCP}}{K_c}\right)} \quad (9)$$

$$\frac{dc_{GLY}}{dt} = \frac{k_{cat,GDL} \times c_{EchA} \times c_{GDL}}{c_{GDL} + K_{m,GDL} \times (1 + \frac{c_{GLY}}{K_i} + \frac{c_{TCP}}{K_c})} \quad (10)$$

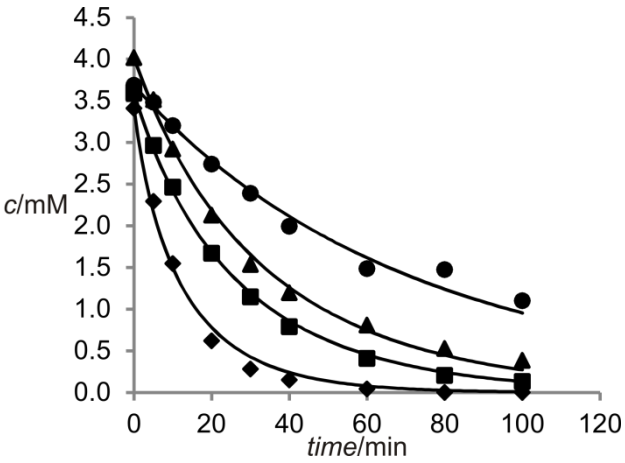
In vitro **multi-enzyme conversion of TCP in batch experiments.** The multi-enzyme conversion of TCP into the final product GLY was assayed in 10 ml of 50 mM Tris-SO_4 buffer (pH 8.5) in 25 ml Micro-flasks sealed with Mininert valves (Alltech, USA) and incubated in water bath with shaking at 37°C. The reaction was initiated by adding a specific amount of purified DhaA variant, HheC and EchA into the reaction mixture along with TCP at an initial concentration of 2 mM. Samples were periodically taken from the reaction mixture, mixed with acetone (1:1) containing hexanol as an internal standard, and analysed by GC. Calibration curves for TCP, DCP, ECH, CPD and GDL concentrations of 0 – 5 mM were constructed to facilitate data analysis. Selected samples were analysed by GC-MS to verify the identities of the metabolites; in other cases, metabolites were identified based on flame ionisation detection. The concentration of GLY in the reaction mixture was determined spectrophotometrically using the Free Glycerol Assay Kit. Samples of the reaction mixture (0.1 ml) were heated at 95°C for 5 min to terminate the enzymatic reaction and then centrifuged at 18,000 *g* for 1 min, after which they were diluted in an assay buffer and analysed according to the manufacturer's protocol. The GLY concentration was calculated from the sample's absorbance at 570 nm. A calibration curve for GLY concentrations of 10 – 80 µM was constructed by analyzing samples prepared from a 1 mM GLY standard solution.



Supporting Information Figure S1. Chromatogram from a gas chromatography analysis of 5 mM standards of 1,2,3-trichloropropane (TCP), 2,3-dichloropropane-1-ol (DCP), epichlorohydrin (ECH), 3-chloropropane-1,2-diol (CPD), and glycidol (GDL). Hexan-1-ol of 4 mM concentration was used as an internal standard (ISTD).



Supporting Information Figure S2. Three-enzyme conversion of 1,2,3-trichloropropane (TCP) to glycerol (GLY) catalyzed by the pathway employing (a) wild-type DhaA, (b) engineered DhaA31 and (c) engineered DhaA90R, respectively. Experimental and simulated metabolite concentrations are indicated by symbols and solid lines, respectively. The reaction's intermediates are 2,3-dichloropropane-1-ol (DCP), epichlorohydrin (ECH), 3-chloropropane-1,2-diol (CPD), and glycidol (GDL). The following parameters were constrained during the *in vitro* and *in silico* experiments: reaction volume (10 mL), initial TCP concentration (~2 mM; experimental concentrations are listed in Tables S2-S4), reaction time interval (300 min), the concentrations of each enzyme (DhaA variant, HheC and EchA; 0.1 $mg.ml^{-1}$), and the total loading of all three enzymes in the reaction mixture (3 mg). Data points represent mean values from three independent measurements. Error bars are omitted for clarity; standard deviations are provided in Supporting Information Tables S2-S4.



Supporting Information Figure S3. Determination of inhibition constant K_c by measuring the inhibitory effect of TCP on the conversion of 5 mM GDL. Symbols represent different TCP concentrations: Diamonds, 0 mM TCP; squares, 0.5 mM TCP; triangles, 1 mM TCP; spheres, 2.5 mM TCP.

Supporting Information Table S1. Nucleotide sequences of genes used in this study.[a]

Synthetic gene *dhaA* with 6xHis tag, codon optimized for expression in *E. coli*
CATATGAGCGAAATTGGCACCGGTTTTCCGTTTGATCCGCATTATGTTGAAGTTCTGGGTGAACG TATGCATTATGTGGATGTTGGTCCGCGTGATGGTACACCGGTTCTGTTTCTGCATGGTAATCCGA CCAGCAGCTATCTGTGGCGTAACATTATTCCGCATGTTGCACCGAGCCATCGTTGTATTGCACCG GATCTGATTGGTATGGGTAAAAGCGATAAACCTGATCTGGATTATTTCTTCGATGATCATGTGCGT TATCTGGATGCATTTATTGAAGCACTGGGTCTGGAAGAAGTTGTGCTGGTTATTCATGATTGGGG TAGCGCACTGGGTTTTCATTGGGCAAAACGTAATCCGGAACGTGTTAAAGGTATTGCCTGCATGG AATTTATTCGTCCGATTCCGACCTGGGATGAATGGCCTGAATTTGCACGTGAAACCTTTCAGGCA TTTCGTACCGCAGATGTGGGTCGTGAACTGATTATTGATCAGAACGCATTTATCGAAGGTGCACT GCCGAAATGTGTTGTTCGTCCGCTGACCGAAGTTGAAATGGATCATTATCGTGAACCGTTTCTGA AACCGGTTGATCGCGAACCGCTGTGGCGTTTTCCGAATGAACTGCCGATTGCCGGTGAACCTGC AAATATTGTTGCACTGGTTGAAGCCTATATGAATTGGCTGCATCAGAGTCCGGTTCCGAAACTGC TGTTTTGGGGCACACCGGGTGTTCTGATTCCGCCTGCAGAAGCAGCACGTCTGGCAGAAAGCCT GCCGAATTGTAAAACCGTTGATATTGGTCCGGGTCTGCATTATCTGCAAGAAGATAATCCGGACC TGATCGGTAGTGAAATTGCACGTTGGCTGCCTGCACTG**CATCATCATCACCATCAT**TAAGGATCC
Synthetic gene *dhaA31* with 6xHis tag, codon optimized for expression in *E. coli*
CATATGTCCGAAATTGGCACCGGCTTCCCGTTTGATCCGCACTATGTTGAAGTTCTGGGCGAACG TATGCACTATGTTGATGTTGGTCCGCGTGATGGCACCCCGGTTCTGTTTCTGCACGGTAACCCGA CGAGCTCTTATCTGTGGCGTAATATTATCCCGCATGTCGCCCCGAGTCACCGCTGCATTGCACCG GATCTGATCGGCATGGGTAAATCCGACAAACCGGATCTGGACTATTTCTTTGATGACCATGTCCG CTACCTGGATGCATTTATTGAAGCTCTGGGCCTGGAAGAAGTGGTTCTGGTGATCCATGACTGGG GCTCTGCGCTGGGTTTCCACTGGGCCAAACGTAATCCGGAACGCGTGAAAGGTATTGCGTGTAT GGAATTTATCCGTCCGTTCCCGACCTGGGATGAATGGCCGGAATTTGCCCGCGAAACCTTTCAG GCGTTCCGTACGGCCGATGTTGGCCGCGAACTGATTATCGACCAAAACGCATTCATTGAAGGTG CTCTGCCGAAATATGTCGTGCGTCCGCTGACGGAAGTGGAAATGGATCATTACCGCGAACCGTTT CTGAAACCGGTTGACCGTGAACCGCTGTGGCGCTTCCCGAACGAACTGCCGATTGCAGGCGAAC CGGCTAATATCGTTGCGCTGGTCGAAGCCTACATGAACTGGCTGCACCAGTCACCGGTGCCGAA ACTGCTGTTTTGGGGCACCCCGGGTTTCATTATCCCGCCGGCAGAAGCAGCACGTCTGGCTGAA TCGCTGCCGAATTGCAAAACGGTTGATATCGGCCCGGGTCTGCATTTTCTGCAAGAAGATAACCC GGACCTGATTGGCTCTGAAATTGCCCGCTGGCTGCCGGCTCTG**CACCACCACCACCACCAC**TAA GGATCC
Synthetic gene *dhaA90R* with 6xHis tag, codon optimized for expression in *E. coli*
CATATGAGCGAAATTGGCACCGGTTTTCCGTTTGATCCGCATTATGTTGAAGTTCTGGGTGAACG TATGCATTATGTGGATGTTGGTCCGCGTGATGGTACACCGGTTCTGTTTCTGCATGGTAATCCGA CCAGCAGCTATCTGTGGCGTAACATTATTCCGCATGTTGCACCGAGCCATCGTTGTATTGCACCG GATCTGATTGGTATGGGTAAAAGCGATAAACCTGATCTGGATTATTTCTTCGATGATCATGTGCGT TATCTGGATGCATTTATTGAAGCACTGGGTCTGGAAGAAGTTGTGCTGGTTATTCATGATTGGGG TAGCGCACTGGGTTTTCATTGGGCAAAACGTAATCCGGAACGTGTTAAAGGTATTGCCTGCATGG AATTTATTCGTCCGCTGACCACCTGGGATGAATGGCCTGAATTTGCACGTGAAACCTTTCAGGCA TTTCGTACCGCAGATGTGGGTCGTGAACTGATTATTGATCAGAACATGTGGATTGAAGGTCTGAT TCCGGCAGGCGTGATTCGCCCTCTGACCGAAGTTGAAATGGATCATTATCGTGAACCGTTTCTGA AACCGGTTGATCGCGAACCGCTGTGGCGTTTTCCGAATGAACTGCCGATTGCCGGTGAACCTGC AAATATTGTTGCACTGGTTGAAGCCTATATGAATTGGCTGCATCAGAGTCCGGTTCCGAAACTGC TGTTTTGGGGTAATCCGGGTTATCTGATTACACCGGCAGAAGCAGCACGTCTGGCAGAAAGCCT GCCGAATTGTAAAACCGTTGATATTGGTCCGGGTCTGCATTTTCTGCAAGAAGATAATCCGGACC TGATCGGTAGTGAAATTGCACGTTGGCTGCCTGCACTG**CATCATCATCACCATCAT**TAAGGATCC
Wild-type gene *hheC*[b]
CATATGTCAACCGCAATTGTAACAAACGTTAAGCATTTTGGGGGAATGGGGTCTGCACTTCGTCT

CTCGGAAGCAGGACATACAGTGGCTTGCCACGATGAAAGCTTCAAACAAAAGGACGAACTTGAA
GCCTTTGCCGAAACCTATCCACAACTCAAACCAATGTCGGAACAAGAACCAGCGGAACTCATCGA
GGCAGTTACCTCCGCTTATGGTCAAGTTGATGTACTTGTGAGCAACGACATATTCGCACCAGAGT
TCCAACCCATAGATAAATACGCTGTAGAGGACTATCGCGGTGCGGTCGAGGCGCTACAAATTAGA
CCATTTGCACTGGTCAACGCCGTTGCAAGTCAAATGAAGAAGCGCAAAAGCGGACATATTATCTT
TATTACCTCTGCAACGCCCTTCGGGCCTTGGAAGGAACTTTCTACCTACACGTCAGCCCGAGCAG
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ATAGGACCCAATTATCTTCACAGTGAAGATAGTCCCTACTTCTACCCCACAGAACCGTGGAAAAC
GAATCCAGAACACGTTGCCCATGTCAAAAAAGTCACTGCGCTCCAGCGGTTAGGTACACAGAAAG
AATTGGGAGAACTCGTCGCGTTTCTCGCGTCTGGTAGTTGTGACTATCTGACCGGCCAGGTGTTC
TGGTTGGCCGGCGGATTCCCAATGATCGAGCGTTGGCCTGGTATGCCCGAGTAGGGATCC

Synthetic gene *echA* with 6xHis tag, codon optimized for expression in *E. coli*

CCATGGCAATCCGTCGTCCTGAAGATTTCAAACACTATGAGGTCCAGCTGCCTGATGTTAAAATT
CATTATGTCCGTGAGGGAGCCGGTCCGACACTGCTGCTGCTGCATGGTTGGCCTGGTTTTTGGT
GGGAATGGTCGAAAGTCATCGGTCCACTGGCCGAGCACTATGATGTTATTGTGCCTGATCTGCG
CGGTTTTGGTGATAGCGAGAAACCTGACCTGAACGATCTGAGCAAATATAGCCTGGATAAAGCCG
CTGATGATCAAGCTGCCCTGCTGGATGCTCTGGGTATCGAAAAAGCCTATGTCGTGGGCCATGAT
TTTGCCGCTATTGTGCTGCACAAATTCATCCGTAAATATTCCGACCGTGTCATCAAAGCAGCCATC
TTTGACCCGATTCAACCAGACTTTGGGCCGGTGTATTTTGGACTGGGCCATGTTCATGAAAGCTG
GTATAGCCAGTTTCACCAACTGGACATGGCTGTTGAAGTGGTAGGCTCTTCACGTGAAGTGTGTA
AAAAATATTTCAAACATTTCTTCGATCACTGGTCCTATCGTGACGAACTGCTGACAGAGGAGGAAC
TGGAAGTCCACGTGGACAATTGTATGAAACCGGATAATATCCACGGCGGGTTCAACTATTATCGT
GCCAACATTCGTCCTGATGCTGCTCTGTGGACAGACCTGGATCATACCATGAGTGACCTGCCGG
TTACTATGATTTGGGGTCTGGGCGACACATGTGTTCCTTATGCCCCACTGATTGAGTTTGTTCCG
AAATATTATAGCAACTATACGATGGAAACCATCGAGGATTGTGGCCATTTTCTGATGGTGGAGAAA
CCGGAAATCGCCATCGACCGTATTAAAACGGCCTTCCGT**CACCACCACCACCACCAT**TAAAAGC
TT

[a] Restriction sites used for cloning are underlined and the 6xHis tag is shown in bold. [b] GenBank accession number AF397296.1.

Supporting Information Table S2. Concentrations of metabolites from time courses of 1,2,3-trichloropropane conversion catalyzed by wild-type DhaA, HheC and EchA.[a]

1 : 1 : 1 (SUM 3 mg)[b]; productivity 72 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	2.03±0.06	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.03
10	1.80±0.01	0.08±0.02	0.00±0.00	0.00±0.00	0.00±0.00	n.d.	n.d.
25	1.62±0.02	0.15±0.01	0.00±0.00	0.00±0.00	0.08±0.01	0.13±0.01	1.98
50	1.36±0.02	0.23±0.01	0.00±0.00	0.00±0.00	0.13±0.01	0.24±0.01	1.96
75	1.14±0.02	0.28±0.01	0.00±0.00	0.00±0.00	0.12±0.02	0.41±0.01	1.95
100	0.97±0.02	0.32±0.02	0.00±0.00	0.00±0.00	0.12±0.02	0.56±0.03	1.97
150	0.71±0.02	0.35±0.03	0.00±0.00	0.00±0.00	0.09±0.01	0.88±0.00	2.03
200	0.51±0.03	0.36±0.03	0.00±0.00	0.00±0.00	0.06±0.02	1.01±0.08	1.94
300	0.25±0.02	0.30±0.03	0.00±0.00	0.00±0.00	0.00±0.00	1.47±0.06	2.02
2.4 : 2.4 : 2.4 (SUM 7.2 mg)[b]; productivity 94 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	1.95±0.06	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.95
10	1.54±0.06	0.09±0.01	0.00±0.00	0.00±0.00	0.06±0.01	n.d.	n.d.
25	1.29±0.05	0.19±0.01	0.00±0.00	0.00±0.00	0.13±0.00	0.14±0.01	1.75
50	0.96±0.03	0.29±0.02	0.00±0.00	0.00±0.00	0.14±0.01	0.40±0.01	1.79
75	0.72±0.02	0.33±0.03	0.00±0.00	0.00±0.00	0.10±0.02	0.70±0.06	1.85
100	0.53±0.01	0.35±0.03	0.00±0.00	0.00±0.00	0.07±0.01	1.07±0.03	2.02
150	0.26±0.00	0.32±0.02	0.00±0.00	0.00±0.00	0.04±0.01	1.29±0.02	1.91
200	0.11±0.01	0.25±0.03	0.00±0.00	0.00±0.00	0.00±0.00	1.54±0.01	1.90
300	0.01±0.01	0.12±0.02	0.00±0.00	0.00±0.00	0.00±0.00	1.83±0.03	1.96
2.3 : 2.7 : 0.7 (SUM 5.7 mg)[b]; productivity 98 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	1.95±0.11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.95
10	1.62±0.10	0.08±0.02	0.00±0.00	0.00±0.00	0.06±0.00	n.d.	n.d.
25	1.33±0.09	0.17±0.01	0.00±0.00	0.00±0.00	0.21±0.01	0.07±0.01	1.78
50	1.00±0.06	0.27±0.02	0.00±0.00	0.00±0.00	0.33±0.02	0.26±0.02	1.86
75	0.75±0.04	0.31±0.03	0.00±0.00	0.00±0.00	0.36±0.04	0.52±0.02	1.94
100	0.54±0.05	0.31±0.02	0.00±0.00	0.00±0.00	0.30±0.01	0.86±0.10	2.01
150	0.27±0.03	0.28±0.02	0.00±0.00	0.00±0.00	0.18±0.01	1.18±0.10	1.91
200	0.11±0.01	0.20±0.03	0.00±0.00	0.00±0.00	0.08±0.01	1.56±0.08	1.95
300	0.00±0.00	0.09±0.01	0.00±0.00	0.00±0.00	0.00±0.00	1.92±0.12	2.01

[a] Data represent mean values ± standard deviation calculated from three independent experiments.

[b] Mass ratio of DhaA : HheC : EchA in mg of enzyme dissolved in 10 ml of reaction mixture.

n.d., not determined

[c] Productivity was calculated using the formula: $\frac{[GLY]_{800\ min}}{[TCP]_{0\ min}} \times 100$

Supporting Information Table S3. Concentrations of metabolites from time courses of 1,2,3-trichloropropane conversion catalyzed by DhaA31, HheC and EchA.[a]

1 : 1 : 1 (SUM 3 mg)[b]; productivity 85 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	2.09±0.10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.09
5	1.18±0.03	0.46±0.02	0.00±0.00	0.00±0.00	0.00±0.00	n.d.	n.d.
10	0.75±0.03	0.65±0.01	0.00±0.00	0.00±0.00	0.23±0.05	n.d.	n.d.
15	0.48±0.03	0.75±0.01	0.00±0.00	0.00±0.00	0.43±0.04	n.d.	n.d.
25	0.21±0.00	0.80±0.03	0.00±0.00	0.00±0.00	0.58±0.10	0.43±0.04	2.02
35	0.08±0.01	0.82±0.01	0.00±0.00	0.00±0.00	0.63±0.06	n.d.	n.d.
50	0.02±0.02	0.78±0.02	0.00±0.00	0.00±0.00	0.37±0.05	0.87±0.05	2.04
75	0.00±0.00	0.69±0.01	0.00±0.00	0.00±0.00	0.00±0.00	1.19±0.05	1.88
100	0.00±0.00	0.63±0.02	0.00±0.00	0.00±0.00	0.00±0.00	1.37±0.18	2.00
150	0.00±0.00	0.49±0.01	0.00±0.00	0.00±0.00	0.00±0.00	1.44±0.00	1.93
200	0.00±0.00	0.38±0.01	0.00±0.00	0.00±0.00	0.00±0.00	1.53±0.09	1.91
300	0.00±0.00	0.24±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.78±0.09	2.02
1.8 : 1.8 : 1.8 (SUM 5.4 mg)[b]; productivity 96 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	2.06±0.22	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.06
5	0.96±0.12	0.64±0.06	0.00±0.00	0.00±0.00	0.15±0.03	n.d.	n.d.
10	0.45±0.06	0.79±0.06	0.00±0.00	0.00±0.00	0.45±0.09	n.d.	n.d.
15	0.21±0.03	0.84±0.08	0.00±0.00	0.00±0.00	0.56±0.02	n.d.	n.d.
25	0.05±0.02	0.79±0.08	0.00±0.00	0.00±0.00	0.53±0.02	0.67±0.02	2.04
35	0.01±0.01	0.77±0.06	0.00±0.00	0.00±0.00	0.35±0.01	n.d.	n.d.
50	0.00±0.00	0.69±0.07	0.00±0.00	0.00±0.00	0.14±0.02	1.27±0.05	2.10
75	0.00±0.00	0.59±0.03	0.00±0.00	0.00±0.00	0.00±0.00	1.60±0.08	2.19
100	0.00±0.00	0.50±0.03	0.00±0.00	0.00±0.00	0.00±0.00	1.68±0.03	2.18
150	0.00±0.00	0.37±0.02	0.00±0.00	0.00±0.00	0.00±0.00	1.79±0.04	2.16
200	0.00±0.00	0.26±0.02	0.00±0.00	0.00±0.00	0.00±0.00	1.88±0.02	2.14
300	0.00±0.00	0.13±0.02	0.00±0.00	0.00±0.00	0.00±0.00	1.98±0.08	2.11
0.4 : 2.3 : 0.5 (SUM 3.2 mg)[b]; productivity 98 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	2.02±0.12	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.02
10	1.39±0.11	0.27±0.02	0.00±0.00	0.00±0.00	0.16±0.01	n.d.	n.d.
25	0.85±0.07	0.45±0.03	0.00±0.00	0.00±0.00	0.48±0.01	0.21±0.01	1.99
50	0.37±0.03	0.56±0.04	0.00±0.00	0.00±0.00	0.65±0.02	0.47±0.01	2.05
75	0.14±0.03	0.54±0.03	0.00±0.00	0.00±0.00	0.57±0.01	0.85±0.10	2.10
100	0.05±0.01	0.47±0.02	0.00±0.00	0.00±0.00	0.45±0.05	1.08±0.03	2.05
150	0.00±0.00	0.33±0.02	0.00±0.00	0.00±0.00	0.19±0.02	1.53±0.02	2.05
200	0.00±0.00	0.22±0.01	0.00±0.00	0.00±0.00	0.08±0.02	1.82±0.04	2.12
300	0.00±0.00	0.09±0.02	0.00±0.00	0.00±0.00	0.00±0.00	1.98±0.05	2.08

[a] Data represent mean values ± standard deviation calculated from three independent experiments.

[b] Mass ratio of DhaA31 : HheC : EchA in mg of enzyme dissolved in 10 ml of reaction mixture.

n.d., not determined

[c] Productivity was calculated using the formula: $\frac{[\mathrm{GLY}]_{300\ \mathrm{min}}}{[\mathrm{TCP}]_{0\ \mathrm{min}}} \times 100$

Supporting Information Table S4. Concentrations of metabolites from time courses of 1,2,3-trichloropropane conversion catalyzed by DhaA90R, HheC and EchA.[a]

1 : 1 : 1 (SUM 3 mg)[b]; productivity 42 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	1.80±0.07	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.80
10	1.68±0.04	0.05±0.01	0.00±0.00	0.00±0.00	0.00±0.00	n.d.	n.d.
25	1.60±0.04	0.05±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.01	1.75
50	1.51±0.01	0.05±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.16±0.01	1.82
75	1.45±0.01	0.05±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.25±0.00	1.75
100	1.35±0.01	0.06±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.31±0.02	1.72
150	1.22±0.02	0.06±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.45±0.00	1.73
200	1.13±0.03	0.06±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.53±0.06	1.72
300	0.91±0.02	0.06±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.75±0.03	1.72
4 : 4 : 4 (SUM 12 mg)[b]; productivity 85 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	2.06±0.04	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.06
10	1.69±0.07	0.03±0.00	0.00±0.00	0.00±0.00	0.10±0.01	n.d.	n.d.
25	1.40±0.02	0.05±0.02	0.00±0.00	0.00±0.00	0.13±0.05	0.21±0.01	1.79
50	1.27±0.09	0.04±0.01	0.00±0.00	0.00±0.00	0.13±0.02	0.51±0.01	1.95
75	1.06±0.08	0.06±0.01	0.00±0.00	0.00±0.00	0.09±0.04	0.82±0.04	2.03
100	0.88±0.04	0.04±0.01	0.00±0.00	0.00±0.00	0.05±0.05	0.97±0.04	1.94
150	0.67±0.03	0.04±0.01	0.00±0.00	0.00±0.00	0.00±0.00	1.39±0.10	2.10
200	0.44±0.04	0.04±0.02	0.00±0.00	0.00±0.00	0.00±0.00	1.56±0.03	2.04
300	0.28±0.02	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	1.75±0.06	2.04
5.2 : 1.5 : 0.8 (SUM 7.5 mg)[b]; productivity 88 %[c]							
time (min)	TCP	DCP	ECH	CPD	GDL	GLY	SUM
0	1.96±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.96
10	1.56±0.07	0.10±0.01	0.00±0.00	0.00±0.00	0.00±0.00	n.d.	n.d.
25	1.30±0.05	0.10±0.01	0.00±0.00	0.00±0.00	0.24±0.05	0.09±0.02	1.73
50	1.00±0.04	0.10±0.00	0.00±0.00	0.00±0.00	0.47±0.04	0.32±0.02	1.89
75	0.83±0.02	0.10±0.01	0.00±0.00	0.00±0.00	0.50±0.04	0.62±0.02	2.05
100	0.66±0.03	0.09±0.01	0.00±0.00	0.00±0.00	0.34±0.05	0.84±0.03	1.93
150	0.46±0.03	0.09±0.01	0.00±0.00	0.00±0.00	0.17±0.02	1.31±0.09	2.03
200	0.32±0.01	0.08±0.03	0.00±0.00	0.00±0.00	0.09±0.02	1.56±0.07	2.05
300	0.19±0.01	0.06±0.01	0.00±0.00	0.00±0.00	0.00±0.00	1.72±0.10	1.97

[a] Data represent mean values ± standard deviation calculated from three independent experiments.

[b] Mass ratio of DhaA31 : HheC : EchA in mg of enzyme dissolved in 10 ml of reaction mixture.

n.d., not determined

[c] Productivity was calculated using the formula: $\frac{[\mathrm{GLY}]_{300\ \mathrm{min}}}{[\mathrm{TCP}]_{0\ \mathrm{min}}} \times 100$

Supporting Information References:

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Supporting Information Animation S1. Identification of the ratio of DhaA31, HheC and EchA providing 95 % conversion of 2 mM 1,2,3-trichloropropane to glycerol within 300 min while minimizing the total enzyme loading . The algorithm searches for the optimal ratio of three enzymes, starting with a total enzyme loading of 3 mg.

Supporting Information Software S1. Computer code for optimizing the stoichiometry of a three-enzyme system. The package contains following files: README, optimizer.py, config_dhaa31.txt, config_dhaa90r.txt and config_dhaawt.txt.