Supporting Information

Alteration of the Route to Menaquinone towards Isochorismate-Derived Metabolites

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I. Supporting data

**Figure S1.** LC-MRM scans of the transition ions corresponding to 2,3-trans-CHD (4, \( m/z \) 154.9 → 93.0, grey), SDHCHC (8 and iso-8, \( m/z \) 256.8 → 133.1, green), and SEPHCHC (7, \( m/z \) 327.1 → 133.2, black).
Figure S2. Relative activity of MenD variants in comparison to wt-MenD (89.6 ±5.2 mU/mg). Substrate concentration 1.6 mM 2,3-trans-CHD (4); time: 300 s.\textsuperscript{[20]}

Figure S3. Relative activity of MenD variants in comparison to wt-MenD. Substrate concentration 6 mM 2,3-trans-CHA (5); time: 24 h.\textsuperscript{[20]}
Protein sequence network and sequence alignment

The protein sequence network (Figure 4) was based on pairwise sequence identities derived from Needleman–Wunsch alignments implemented in the EMBOSS software suite (gap opening and extension penalties of 10 and 0.5, respectively).[1,2] The Backbone layout in Visone (version 2.17) was used to visualize the network, setting a constraint that all sequences (nodes) remained connected to each other.[3,4] Sequence pairs were ranked in order of decreasing sequence identity. A cut-off of 2.5% was applied to select the highest ranked sequence identities as part of the 'backbone' of the network. Edges were coloured in greyscale with darker edges representing higher sequence identities.

A multiple sequence alignment of eleven selected protein sequences from Figure 4, sharing pairwise sequence identities between 31 and 65%. Standard position 102 for ThDP-dependent decarboxylases is marked in red.

NCGI-GI-429491348|Bacteroidales
NCGI-GI-212673002|NCGI-GI-492852583|Desulfovibrio_piger
NCGI-GI-546348585|Allistipes_sp_CAG_514
NCGI-GI-912593283|Herbinix_hemicellulosilytica
NCGI-GI-1134248926|Alkalispirochaeta_americana
NCGI-GI-763248296|Prevotella_brevis
NCGI-GI-727805511|Bacteroides_sartorii
NCGI-GI-518076626|Allistipes_ihumii_AP11
NCGI-GI-1124925647|Bacteroidales_bacterium
NCGI-GI-524327904|Bacteroides_sp_CAG_754
NCGI-GI-1106688415|Enterococcus_devriesei

| GI       | Organism                                      | Sequence                                                                 |
|----------|-----------------------------------------------|-------------------------------------------------------------------------|
| GI_1106688415| Enterococcus_devriesei                     | LPGLTEAYYSKIPVIAITSL                                                    |
| GI_1124925647| Bacteroidales_bacterium                   | LPGLTEAYYRKLPVLVVTFA                                                     |
| GI_518076626| Alistipes_ihumii_AP11                       | MPGLTEAYYRKLPVLAITSI                                                     |
| GI_763240296| Prevotella_brevis                           | LVGSMQHDSFFEMYSCVDERAAAYMAIGMAEKSNEPVVLSCTGATASRNY                      |
| GI_1106688415| Enterococcus_devriesei                     | LVGSIQYDPWFEIFSAVDERSAAYMACGLAAEESGEPVVLTCTGATASRNY                     |

S3
NCBI-GI 49249134|Bacteroidales

NCBI-GI 2112637002|NCBI-GI 492852583|Desulfovibrio piger

NCBI-GI 546348585|Alistipes_sp_CAG_514

NCBI-GI 518076626|Alistipes_ihumii_AP11

NCBI-GI 518076626|Alistipes_ihumii_AP11

NCBI-GI 49249134|Bacteroidales

GI_524327904|Bacteroides_sp_CAG_754

GI_1124925647|Bacteroidales_bacterium

GI_727808551|Bacteroides_sartorii

GI_763240296|Prevotella_brevis

NCBI-GI 518076626|Alistipes_ihumii_AP11

GI_763240296|Prevotella_brevis

GI_524327904|Bacteroides_sp_CAG_754

GI_518076626|Alistipes_ihumii_AP11

GI_524327904|Bacteroides_sp_CAG_754

GI_492491340|Bacteroidales

GI_492491340|Bacteroidales

GI_212673002,NCBI

GI_546348585|Alistipes_sp_CAG_514

GI_212673002,NCBI

NCBI-GI 524327904|Bacteroides_sp_CAG_754

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NCBI-GI 1124925647|Bacteroidales_bacterium

NCBI-GI 727808551|Bacteroides_sartorii

NCBI-GI 763240296|Prevotella_brevis

GI_212673002,NCBI

GI_524327904|Bacteroides_sp_CAG_754

GI_1106688415|Enterococcus_devriesei

GI_524327904|Bacteroides_sp_CAG_754

GI_518076626|Alistipes_ihumii_AP11

GI_492491340|Bacteroidales

GI_492491340|Bacteroidales

GI_546348585|Alistipes_sp_CAG_514

GI_212673002,NCBI

GI_518076626|Alistipes_ihumii_AP11

GI_524327904|Bacteroides_sp_CAG_754

GI_1106688415|Enterococcus_devriesei

GI_524327904|Bacteroides_sp_CAG_754
II. Bacterial strains and culture conditions

**Strains.** *E. coli* BL21(DE3) Gold (Agilent Technologies) was used for the production of N-terminal His-tagged MenD. The modified strains *E. coli* F97 [genotype LJ110 lac::(P_{lac-} aroFBL+) Δ(pheA tyrA aroF) Δ(EntCEBA) ΔmenC]^{[5]} and *E. coli* F68 [genotype LJ110 Δ(trpE) Δ(pheA tyrA aroF):kan]^{[6]} were used for *in vivo* conversions. The plasmids used for the transformations are described below (Section IV. Molecular biology methods).

**Expression of MenD for in vitro assays.** *E. coli* BL21(DE3) Gold (Agilent Technologies) was transformed with the plasmid pETM coding for N-terminal His-tagged MenD (1). The expression of MenD was performed according to Studier et al.\(^7\) in the ZYM-5052 autoinduction medium (24–25 °C, 160–180 rpm). After 15–18 h cultivation, the cells were centrifuged (1 h, 4350 × g, 4 °C) and stored at −80 °C until processing.

**In vivo conversions.** The media were prepared according to Horn et al.\(^8\) and modified taking into account the auxotrophies of the producing strains.

The following stock solutions were used:

(a) Salt solution I: Composition (g/L): Na₂HPO₄·2H₂O 8.6, KH₂PO₄ 3.0, NaCl 0.5, NH₄Cl 1.0, Fe(III) citrate hydrate tribasic 60.0 × 10⁻³, H₃BO₃ 3.0 × 10⁻³, MnCl₂·4H₂O 15.0 × 10⁻³, EDTA·2H₂O 8.4 × 10⁻³, CuCl₂·2H₂O 1.5 × 10⁻³, Na₂MoO₄·2H₂O 2.5 × 10⁻³, CoCl₂·6H₂O 2.5 × 10⁻³. The solution was autoclaved.

(b) Salt solution II: Composition (g/L): KH₂PO₄ 16.6, (NH₄)₂HPO₄ 4.0, citric acid 2.1, Fe(III) citrate hydrate tribasic 75.0 × 10⁻³, H₃BO₃ 3.8 × 10⁻³, MnCl₂·4H₂O 18.8 × 10⁻³, EDTA·2H₂O 10.5 × 10⁻³, CuCl₂·2H₂O 1.9 × 10⁻³, Na₂MoO₄·2H₂O 3.1 × 10⁻³, CoCl₂·6H₂O 3.1 × 10⁻³, Zn(CH₃COO)₂·2H₂O 10 × 10⁻³. The pH was adjusted to 6.8 with 25% (vol/vol) NH₃ and the solution was autoclaved.

(c) MgSO₄·7H₂O was prepared as a 246 g/L (1 M) stock, and autoclaved.

(d) The Fe(II) stock solution was prepared as 7.5 g/L FeSO₄·7H₂O with 100 g/L sodium citrate(-2H₂O), and autoclaved.

(e) Thiamine diphosphate was prepared as a 10 g/L stock and sterile-filtered.

(f) The amino acids L-tyrosine, L-phenylalanine and L-tryptophan were prepared as 12 mg/mL stocks with 2 M NaOH addition for autosterility, and added according to the auxotrophies of the strains: F97 (L-tyrosine, L-phenylalanine), F68 (L-tyrosine, L-phenylalanine, L-tryptophan).

(g) Glucose, glycerol and lactose were prepared as 2.5% or 50%, 25% and 10% (wt/vol) stocks, respectively, and autoclaved.

The producing *E. coli* strains F97 and F68 [3% (vol/vol) inoculum] were grown in 250-mL baffled flasks.
with membrane caps containing 50 mL of medium (37 °C, 350 rpm). When an OD₆₀₀ of 10 was reached, the membrane caps were replaced by perforated aluminum/plastic caps to increase the oxygen exchange. The antibiotic ampicillin was used at a final concentration of 100 µg/mL and the induction of the expression was performed with 0.1–0.5 mM IPTG.

**Screening of the variants for the conversion of 2,3-trans-CHD (4).** Salt solution I was used with a dilution of 0.75-fold. The other components of the medium were used at the following final concentrations (g/L): 0.57 MgSO₄·7H₂O, 0.11 FeSO₄·7H₂O, 1.5 sodium citrate·2H₂O, 10 × 10⁻³ thiamine diphosphate, 0.16 L-tyrosine, 0.25 L-phenylalanine, 10.0 glucose. The cultures were fed with 1.1% (vol/vol) glycerol after 39, 64 and 87 h.

**High-cell-density fermentation of selected variants for the conversion of 2,3-trans-CHD (4) and 2,3-trans-CHA (5).** Salt solution II was used with a dilution of 0.75-fold. The other components of the medium were used at the following final concentrations (g/L): 0.57 MgSO₄·7H₂O, 0.11 FeSO₄·7H₂O, 1.5 sodium citrate·2H₂O, 10 × 10⁻³ thiamine diphosphate, 0.16 L-tyrosine, 0.25 L-phenylalanine, 0.24 L-tryptophan (only for the conversion of 2,3-trans-CHA), 0.5 glucose, 31 glycerol, 7.6 lactose. The cultures were fed with 1.1% (vol/vol) glycerol and the pH was adjusted to 6.8 with either 25% (vol/vol) NH₃ or 2 M NaOH after 15, 22, 25, 38, 42, 46 and 64 h.

**In vivo conversions for the structural characterization of iso-SDHCHC (iso-8).** The strain *E. coli* F97 was cultivated according to Pan et al.⁹ (pH 7.2, 100 h, 28 °C).

**Whole-cell biotransformation of 2,3-trans-CHA (5) for the structural elucidation of the product.** *E. coli* BL21(DE3) Gold transformed with pETM (MenD variants R107K and R107Y) was grown in 100 mL LB medium at 28 °C and 160 rpm. Expression of MenD was started with 1 mM IPTG at an OD₆₀₀ of 0.6 and the culture further incubated at 24 °C for 17 h. The cells were centrifuged (10 min, 4650 × g, 24 °C) and suspended in 50 mL buffer pH 7 (8 g/L NaCl, 0.2 g/L KCl, 1.81 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, 0.56 g/L MgSO₄·7H₂O, 0.3% glucose, 10 mg/L thiamine diphosphate) containing 1.23 g/L 2,3-trans-CHA (5), and incubated at 24 °C and 160 rpm for 30 h.
III. Enzymatic assays

**Purification of MenD for in vitro assays.** The cells were suspended in buffer A (50 mM phosphate, 250 mM NaCl, 1 mM MgCl\(_2\)-6H\(_2\)O, 50 μM thiamine diphosphate, 20 mM imidazole, pH 7.4; 10 mL buffer/g pellet) and disrupted three times for 30 s with an ultrasonic homogenizer (Branson Sonifier II W-250) with a duty cycle of 60% and a power output of 6. The homogenate was centrifuged (1 h, 15500 × g, 4 °C) and filtered (Puradisc 25, 0.45 μm, polyethersulfone membrane, Whatman). The filtrate was loaded on to a 5 mL HisTrap HP column (GE Healthcare), connected to an NGC Quest 10 Plus chromatography system (BioRad). After washing with four column volumes (CV) of 74:26 buffer A:buffer B (50 mM phosphate, 250 mM NaCl, 1 mM MgCl\(_2\), 50 μM thiamine diphosphate, 500 mM imidazole, pH 7.4), MenD was eluted with 100% buffer B. The fractions with MenD were pooled together and the buffer was exchanged by passage through either 4 × 5 mL Hitrap (5 mL/min) or Hiprep 26/10 desalting columns (17 mL/min, GE Healthcare) using 50 mM sodium phosphate buffer pH 8 or Tris-HCl buffer pH 7.4, containing 200 mM NaCl, 1 mM MgCl\(_2\) and 50 μM thiamine diphosphate.

**Determination of kinetic parameters with 2,3-trans-CHD (4).** The reactions were performed at 25 °C with 0.8 mM α-ketoglutarate, 0.025–1.60 mM 2,3-trans-CHD and 0.25 mg/mL EcMenD using 50 mM Tris-HCl buffer pH 7.4, containing 200 mM NaCl, 1 mM MgCl\(_2\) and 50 μM thiamine diphosphate. The decrease in the absorbance of the substrate (260 nm) was measured for 300 s in 96-well plates (pureGrade UV, Brand GmbH) with a SpectraMax 190 microplate reader. The curves were adjusted according to the Michaelis–Menten kinetics with the software OriginPro 8.5 (OriginLab Corporation).

**Enzymatic reactions with 2,3-trans-CHD (4) and 2,3-trans-CHA (5).** The reactions (0.4 mL volume) were performed at 28 °C with 6 mM α-ketoglutarate, 6 mM substrate (2,3-trans-CHD or 2,3-trans-CHA) and 1 mg/mL EcMenD using 50 mM sodium phosphate or Tris-HCl buffer, containing 200 mM NaCl, 1 mM MgCl\(_2\) and 50 μM thiamine diphosphate, at different pH values. The reaction mixtures were analyzed and quantified by LC-MS.
IV. Molecular biology methods

The genes of interest were cloned by standard PCR and cloning techniques\[10,11\] using the plasmid pC20 (pJF119EH1-aroF-entC-entB-aroB-aroL) as backbone.\[6,12\] EcMenD was amplified from pETM\[5\] including the ribosome-binding site and the His tag from the pET vector. The genes phzD and phzE of the phenazine biosynthesis pathway from Pseudomonas aeruginosa were amplified from pC30 and pET16:phzE. The description of the plasmids used is summarized in Table S1.

Table S1. Plasmids used for transformation.

| Plasmid         | Description                          | Strain, use                                      |
|-----------------|--------------------------------------|-------------------------------------------------|
| pJF119EH1\[12\] | Amp\(^R\), lac\(^I\), P\(_{lac}\), ori col\(E1\) |                                                 |
| pC30\[13\]     | pJF119EH1-trpE\(^{H398M}\)-phzD      | Amplification of phzD from Pseudomonas aeruginosa PAO1 |
| pET16-phzE\[13\] |                                       | Amplification of phzE from Pseudomonas aeruginosa PAO1 |
| pETM\[5\]      | pET-19b-menD                          | BL21, production of his-tagged MenD              |
| pC20\[6\]      | pJF119EH1-aroF-entC-entB-aroB-aroL  | F97, production of 2,3-trans-CHD (4) as negative control. |
| pC20-menD      | pJF119EH1-aroF-entC-entB-aroB-aroL-menD | F97, production of SDHCHC (8) and iso-SDHCHC (iso-8) |
| pC35/2         | pJF119EH1-aroF-phzD-phzE-aroB-aroL  | F68, production of 2,3-trans-CHA (5) as negative control. |
| pC35/2-menD    | pJF119EH1-aroF-phzD-phzE-aroB-aroL-menD | F68, production of SAHCHC (10) and iso-SAHCHC (iso-10) |

Site-directed mutagenesis. To select hotspots, the docking of isochorismate to the crystal structure of EcMenD (PDB 3HWX) was performed manually using the software PyMOL for the visualization.\[14\] The surface electrostatic potential was calculated with the Adaptive Poisson-Boltzmann Solver (APBS) and the PQR data were generated with the PDB2PQR server.\[15,16\] The homology models were developed with the “SWISS-MODEL workspace”.\[17,18\] The codons were chosen taking into account the occurrence in E. coli and the mutations were introduced with the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The mutation N117R was introduced according to Weiner and Costa.\[19\] PfuUltra High-Fidelity DNA and Phusion Flash polymerases were used for plasmids up to 8 kb and >8 kb, respectively.
V. Purification of the products

Purification of iso-SDHCHC (iso-8). The cultures (75 mL) were centrifuged (50 min, 15500 × g, 4 °C), the supernatant was filtered (Millipore Express Plus, PES, 0.45 μm) and the pH was adjusted to 9.4 with 12.5% NH₃. The filtrate was loaded (flow rate: 3 mL/min) onto an anion-exchange column (Dowex 1X8, 66 mL, 200 mesh). As mobile phase, a linear gradient of eluent A [30 mM NH₄CHO (pH 10.8)] and eluent B [30 mM NH₄CHO (pH 3.0)] was applied (flow rate: 15 mL/min); gradient program: eluent B: 0% (equilibration); 0 → 100%, 0–90 min; 100%, 90–120 min. The fractions containing SDHCHC (8) and iso-SDHCHC (iso-8) were identified by MS, pooled together, freeze-dried and dissolved in 12 mL H₂O. iso-SDHCHC (iso-8) was further purified by reversed-phase flash chromatography using an Isolera flash chromatography system (Biotage) with a SNAP KP-C18-HS column (30 g, Biotage). The separation was carried out using an elution gradient of 0.1% formic acid and acetonitrile (flow rate: 25 mL/min); gradient program: acetonitrile: 0%, 5 CV; 0 → 50%, 8 CV; 50 → 80%, 1 CV). The fractions containing SDHCHC (8) and iso-SDHCHC (iso-8) were pooled together, acetonitrile was removed under reduced pressure at 25 °C and the remainder was freeze-dried. The residues were dissolved in 600 μL H₂O and further purified by semipreparative hydrophilic interaction chromatography (HILIC) using an Agilent 1200 HPLC system with a preparative XBridge Amide column (5 μm, 150 × 10 mm, Waters). For elution, a linear gradient of 10 mM NH₄CHO (pH 6.8) and acetonitrile was used (flow rate: 3.4 mL/min); gradient program: acetonitrile: 95%, 0–6 min; 95% → 40%, 6–36 min; 40%, 36–52.5 min. The fractions containing SDHCHC (8) and iso-SDHCHC (iso-8) were pooled, acetonitrile was removed under reduced pressure and the remainder was freeze-dried (yield: 16 mg per 75 mL culture).

Purification of iso-SAHCHC (iso-10). The cultures (100 mL) were centrifuged (50 min, 15500 × g, 4 °C), the supernatant was filtered (Millipore Express Plus, PES, 0.45 μm) and the pH was adjusted to 3.0–3.2 with formic acid. The filtrate was loaded (flow rate: 7 mL/min) onto an cation-exchange column (Dowex 50WX8, 33 mL, 200 mesh). As mobile phase, a linear gradient of eluent B [30 mM NH₄CHO (pH 3.0)] and eluent C [20 mM NH₄CHO (pH 10.8)] was applied (flow rate: 10 mL/min); gradient program: eluent C: 0%, 5 CV; 0 → 100%, 30 CV; 100%, 2 CV. The fractions containing SAHCHC (10) and iso-SAHCHC (iso-10) were identified by MS, pooled together, and the volume was reduced to 20 mL by freeze-drying. The solution was loaded with an Isolera flash chromatography system (Biotage) onto a Telos Flash NH₂ column (20 g, Kinesis). As mobile phase, a linear gradient of 30 mM NH₄CHO (pH 3.0) and acetonitrile was applied; gradient program: acetonitrile: 95%, 3 CV; 95% → 40%, 30 CV; 40%, 5 CV. The fractions containing SAHCHC (10) and iso-SAHCHC (iso-10) were pooled together, acetonitrile was removed under reduced pressure, and the water phase was freeze-dried and dissolved in 600 μL H₂O. The
sample was further purified using an Agilent 1200 HPLC system with an XBridge Amide column (5 μm, 150 × 10 mm, Waters). For elution, a linear gradient of 10 mM NH₄CHO₂ (pH 6.8) and acetonitrile was used (flow rate: 3.4 mL/min); gradient program: acetonitrile: 95%, 0–6 min; 95% → 40%, 6–36 min; 40%, 36–52.5 min. The fractions containing SAHCHC (10) and iso-SAHCHC (iso-10) were pooled together, acetonitrile was removed under reduced pressure and the remainder was freeze-dried (yield: 6 mg per 100 mL culture).
VI. High-performance liquid chromatography with mass spectrometry detection (HPLC-MS)

Sample preparation. Samples for RP-HPLC were acidified with 0.1–1% formic acid, centrifuged (1 min, 19600 × g), filtered (0.2 μm cellulose acetate filter, CS-Chromatographie) and diluted 1:40 with H2O. Samples for HILIC-HPLC were centrifuged (1 min, 19600 × g), filtered (Vivaspin 500, 10000 Da, Sartorius) and diluted 1:3 with H2O.

RP-HPLC-MS analysis of 2,3-trans-CHD (4), SDHCHC (8), iso-SDHCHC (iso-8) and SEPHCHC (7). RP-HPLC-MS analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies) coupled to an API 2000 or API Qtrap 4500 triple quadrupole mass spectrometer (AB SCIEX) with a Multospher RP C₁₈, AQ-3 μm EC column (60 × 2 mm, CS-Chromatographie). As mobile phase, a gradient of 0.1% vol/vol formic acid and acetonitrile was applied (flow rate: 0.3 mL/min, column temperature: 20 °C, injection volume: 2–5 μL); gradient program: acetonitrile: 0%, 0–1.8 min; 0% → 45%, 1.8–3.9 min; 45% → 80%, 3.9–9.0 min; 80% → 0%, 9.0–9.5 min; 0%, 9.5–18.0 min. The compounds were detected by mass spectrometry in negative ion mode using the parameters (API Qtrap 4500) listed in Table S2. For quantification, MRM scans were performed using the parameters (API Qtrap 4500) and transition ions listed in Tables S3 and S4, respectively. As standard, the purified iso-SDHCHC (iso-8) was used; the regioisomers SDHCHC (8) and iso-SDHCHC (iso-8) show the same relative intensity of the transition ions. For SEPHCHC (7), a purified regioisomer was used as standard (to be published elsewhere).

HILIC-HPLC-MS analysis of 2,3-trans-CHA (5), SAHCHC (10) and iso-SAHCHC (iso-10). HILIC-HPLC-MS analysis was performed with an Agilent 1100 HPLC system (Agilent Technologies) coupled to an API 2000 or API Qtrap 4500 triple quadrupole mass spectrometer (AB SCIEX) with an XBridge Amide column (5 μm, 100 × 2.1 mm, Waters). As mobile phase, a gradient of 10 mM NH₄CHO₂ in H₂O (pH 6.8) and acetonitrile was applied (flow rate: 0.3 mL/min, column temperature: 30 °C, injection volume: 2–5 μL); gradient program: acetonitrile: 90%, 0–2 min; 90% → 40%, 2–12 min; 40%, 12–15 min. SAHCHC (10) and iso-SAHCHC (iso-10) were detected by mass spectrometry in negative ion mode using the parameters (API Qtrap 4500) listed in Table S2. The substrate 2,3-trans-CHA (5) was detected and quantified by UV-visible absorption at 278 nm. For quantification, MRM scans were performed using the parameters (API Qtrap 4500) and transition ions listed in Tables S3 and S4, respectively. As a standard, the purified iso-SAHCHC (iso-10) was used. For SAHCHC (10) and iso-SAHCHC (iso-10), the regioisomers show the same relative intensity of the transition ions. The standard deviation was ±10.9% (determined by six repeated injections).
Table S2. Q1 scan parameters.

| Parameter                        | RP-HPLC-MS | HILIC-HPLC-MS |
|----------------------------------|------------|---------------|
| polarity                         | negative   | negative      |
| curtain gas                      | 30         | 30            |
| temperature                      | 400 °C     | 400 °C        |
| gas 1                            | 40         | 20            |
| gas 2                            | 60         | 30            |
| declustering potential (DP)      | –11 V      | –11 V         |
| focusing potential (FP)          | –260 V     | –260 V        |
| entrance potential (EP)          | –10 V      | –10 V         |
| voltage                          | –3000 V    | –3000 V       |

Table S3. MRM scan parameters.

| Parameter                        | SDHCHC  | SEPHCHC | 2,3-trans-CHD | SAHCHC |
|----------------------------------|---------|---------|---------------|--------|
| polarity                         | negative| negative| negative      | negative|
| curtain gas                      | 43      | 43      | 43            | 30     |
| collision gas                    | 2       | 2       | 2             | 2      |
| temperature                      | 400 °C  | 400 °C  | 400 °C        | 300 °C |
| gas 1                            | 60      | 60      | 60            | 20     |
| gas 2                            | 50      | 50      | 50            | 20     |
| declustering potential (DP)      | –90 V   | –90 V   | –90 V         | –21 V  |
| entrance potential (EP)          | –8 V    | –8 V    | –8 V          | –4 V   |
| collision cell entrance potential (CEP) | –18 V | –30 V   | –14 V         | –20 V  |
| collision energy (CE)            | –20 V   | –18 V   | –16 V         | –22 V  |
| collision cell exit potential (CXP) | –4 V   | –8 V    | –4 V          | –4 V   |
| voltage                          | –4000 V | –4000 V | –4000 V       | –3000 V|
Table S4. Isolated ion MRM scans.

| Compound                  | Q1 (m/z) | Q3 (m/z) | Specific parameter |
|---------------------------|----------|----------|--------------------|
| SDHCHC (8)/ iso-SDHCHC (iso-8) (258.1 Da) | 256.852  | 213.100  | CE: –10 V CE: –6 V |
|                           | 256.852  | 151.200  | CE: –18 V          |
|                           | 256.852  | 95.000   | CE: –22 V          |
| SEPHCHC (7) (328.1 Da)    | 327.176  | 281.400  | CE: –16 V CE: –6 V |
|                           | 327.176  | 133.200  | CE: –30 V CE: –4 V |
| 2,3-trans-CHD (4) (156.1 Da) | 154.938  | 83.000   | CE: –22 V          |
| SAHCHC (10)/ iso-SAHCHC (iso-10) (257.1 Da) | 255.928  | 211.700  | CE: –10 V CE: –6 V |
MS/MS spectra of the purified regioisomers iso-SDHCHC (iso-8) and iso-SAHTHC (iso-10), and the postulated fragmentations with the experimental (and theoretical) m/z ratios.

Figure S4. MS/MS spectrum of purified iso-SDHCHC (iso-8).

Figure S5. MS/MS spectrum of purified iso-SAHTHC (iso-10).
VII. NMR spectroscopy

NMR spectra were recorded at 24 °C on a Bruker DRX 400 spectrometer operating at 400 MHz for $^1$H NMR and at 100.6 MHz for $^{13}$C NMR acquisitions. Chemical shifts of the spectra are reported in ppm with the solvent (D2O) resonance as internal standard (4.69 ppm).

Table S5. Chemical shifts and correlations of isolated iso-SDHCHC (iso-8) in D$_2$O.

| Experiment Position | $^1$H in ppm ($J$ in Hz) | $^{13}$C δ in ppm | HMBC Coupling Partner | H,H-COSY Coupling Partner |
|---------------------|--------------------------|-------------------|-----------------------|---------------------------|
| 1                   | 3.14–3.19, m, 1H         | 51.7              |                       | H3, H6                    |
| 2                   |                          | 136.4             |                       |                           |
| 3                   | 6.98–7.03, m, 1H         | 140.6             |                       | H1, H4a/b                 |
| 4a/b                | a; 2.28, ddt, 1H (19.7, 7.8, 3.0) b; 2.66, ddt, 1H | 32.2              | C2                    | H3, H5                    |
| 5                   | 3.69, dt, 1H (5.4, 8.4)  | 68.6              | C3, C6                | H4a/b, H6                 |
| 6                   | 3.75, dd, 1H (8.4, 7.6)  | 73.5              | C3, C5                | H5, H1                    |
| 7                   |                           | 170.8             |                       |                           |
| 1’                  |                          | 202.4             |                       |                           |
| 2’a/b               | a; 2.80, dt, 1H (18.0, 6.4) b; 3.07, dt, 1H (18.0, 6.4) | 32.9              | C1’, C3’, C4’         | H3’                       |
| 3’a/b               | 2.39, m                  | 30.3              | C1’, C4’              | H2’a/b                    |
| 4’                  |                          | 180.2             |                       |                           |
Figure S6. $^1$H NMR spectrum (400 MHz) of iso-SDHCHC (iso-8) in D$_2$O.
Figure S7. $^1$H NMR spectrum (400 MHz) of iso-SDHCHC (iso-8) in D$_2$O (6–8 ppm).

Figure S8. $^1$H NMR spectrum (400 MHz) of iso-SDHCHC (iso-8) in D$_2$O (2–4 ppm).
Figure S9. HSQC spectrum of iso-SDHCHC (iso-8) in D$_2$O.
Figure S10. HMBC spectrum of iso-SDHHC (iso-8) in D$_2$O.
Figure S11. H,H-COSY spectrum of iso-SDHCHC (iso-8) in D$_2$O.
Figure S12. $^{13}$C NMR spectrum (100.6 MHz) of iso-SDHCHC (iso-8) in D$_2$O.
Table S6. Chemical shifts and correlations of isolated iso-SAHCHC (iso-10) in D$_2$O.

![Chemical structure of iso-SAHCHC](image)

| Experiment Position | $^1$H $\delta_H$ in ppm ($J$ in Hz) | $^{13}$C $\delta_C$ in ppm | HMBC Coupling partner | H,H-COSY Coupling partner |
|---------------------|------------------------------------|---------------------------|----------------------|--------------------------|
| 1                   | 3.30–3.40, m, 1H                   | 48.3                      | C6, C7               | H3, H4a/b, H6            |
| 2                   |                                    | 135.8                     |                      |                          |
| 3                   | 6.90–7.00, m, 1H                   | 138.4                     | C1, C5, C1’          | H1, H4a/b                |
| 4a/b                | a; 2.30–2.40, m, 1H                 | 32.4                      | C2 (a), C3, C5, C6   | H1, H3, H5              |
|                     | b; 2.65–2.70, m, 1H                 |                           |                      |                          |
| 5                   | 3.81, dddd, 1H (9.7, 5.6)          | 65.8                      | C6                   | H4a/b, H6               |
| 6                   | 3.30, dd, 1H (9.7)                 | 55.6                      | C1, C5, C7           | H1, H5                  |
| 7                   |                                    | 177.7                     |                      |                          |
| 1’                  |                                    | 202.2                     |                      |                          |
| 2’a/b               | a; 2.67, dt, 1H (18.1, 6.2)        | 33.0                      | C1’, C3’, C4’        | H3’                      |
|                     | b; 3.12, ddd, 1H (18.1, 7.8, 6.6)  |                           |                      |                          |
| 3’                  | 2.35–2.50, m, 2H                   | 30.0                      | C1’, C2’, C4’        | H2’a/b                  |
| 4’                  |                                    | 179.9                     |                      |                          |
Figure S13. $^1$H NMR spectrum (400 MHz) of iso-SAHCCHC (iso-10) in D$_2$O.
Figure S14. $^1$H NMR spectrum (400 MHz) of iso-SAHCCHC (iso-10) in D$_2$O.
Figure S15. $^1$H NMR spectrum (400 MHz) of iso-SAHCCH (iso-10) in D$_2$O.
Figure S16. H,H-COSY spectrum of iso-SAHCHC (iso-10) in D$_2$O.

Figure S17. HMBC spectrum of iso-SAHCHC (iso-10) in D$_2$O.
Figure S18. HSQC spectrum of iso-SAHCHC (iso-10) in D$_2$O.
Figure S19. $^{13}$C NMR spectrum (100.6 MHz) of iso-SAHCCHC (iso-10) in D$_2$O.
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