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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Extended substrate range of thiamine diphosphate-dependent MenD enzyme by coupling of two C–C-bonding reactions

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Abstract

Carboligations catalyzed by aldolases or thiamine diphosphate (ThDP)-dependent enzymes are well-known in biocatalysis to deliver enantioselective chain elongation reactions. A pyruvate-dependent aldolase (2-oxo-3-deoxy-6-phosphogluconate aldolase [EDA]) introduces a chiral center when reacting with the electrophile, glyoxylic acid, delivering the (*S*)-enantiomer of (4*S*)-4-hydroxy-2-oxoglutarate [(*S*)-HOG]. The ThDP-dependent enzyme MenD (2-succinyl-5-enol-pyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (SEPHCHC synthase)) enables access to highly functionalized substances by forming intermolecular C–C bonds with Michael acceptor compounds by a Stetter-like 1,4- or a benzoin-condensation 1,2-addition of activated succinyl semialdehyde (ThDP adduct formed by decarboxylation of 2-oxoglutarate). MenD-catalyzed reactions are characterized by high chemo- and regioselectivity. Here, we report (*S*)-HOG, in situ formed by EDA, to serve as new donor substrate for MenD in 1,4-addition reactions with 2,3-*trans*-CHD (2,3-*trans*-dihydroxy-cyclohexadiene carboxylate) and acrylic acid. Likewise, (*S*)-HOG serves as donor in 1,2-additions with aromatic (benzaldehyde) and aliphatic (hexanal) aldehydes. This enzyme cascade of two subsequent C–C bond formations (EDA aldolase and a ThDP-dependent carboligase, MenD) generates two new stereocenters.

Keywords Aldolase EDA · ThDP-dependent enzyme · MenD · 4-hydroxy-2-oxoglutarate · Carboligations · Stetter-like 1,4-additions · 1,2-additions

Introduction

MenD [2-**s**uccinyl-5-**e**nol-**p**yruvyl-6-**h**ydroxy-3-**c**yclo**h**exene-1-**c**arboxylate synthase (SEPHCHC synthase)] is a thiamine diphosphate (ThDP)-dependent enzyme in menaquinone biosynthesis of bacteria (Emmons et al. 1985; Jiang et al. 2007). Its physiological role is the formation of SEPHCHC (**3**) from isochorismate (**2**; acceptor) and 2-oxoglutarate (**1**; donor) in a so-called Stetter-like 1,4-addition reaction. This involves the non-oxidative decarboxylation of **1**, and the subsequent 1,4-addition with **2** to yield **3** (see Fig. 1; Jiang et al. 2007). Apart from its physiological reaction, MenD catalyzes synthetically useful 1,4-additions with other α,β-unsaturated carbonyl or carboxyl functionalities such as 2,3-*trans*-CHD (**7**; Kurutsch et al. 2009) or acrylic acid (**8**; Beigi et al. 2014).

Other ThDP-dependent Stetter-like enzymes, the so-called Stetterases, as PigD (Dresen et al. 2010), SeAAS, and HapD (Kasparyan et al. 2014), also use 2-oxoacids as donor and various α,β-unsaturated acids, ketones, and nitriles as acceptors (Beigi et al. 2014). The enzymatic mechanism of carboligation catalyzed by MenD is similar to other ThDP-dependent enzymes: 2-oxoacids as donor substrates form adducts with ThDP and are decarboxylated toward a ThDP-bound aldehyde ("active aldehyde"). The bound ThDP cofactor reverses the polarity ("umpolung") of the carbonyl enabling the addition of the donor aldehyde to an electrophilic acceptor forming a new C–C bond (Jordan 2003). Many ThDP-dependent enzymes show high enantioselectivity and can be used—in addition to their physiological functions in cell metabolism—in vitro for enantioselective C–C ligation reactions (Pohl et al. 2004; Müller et al. 2013; Schmidt et al. 2016, Giovannini et al. 2016).

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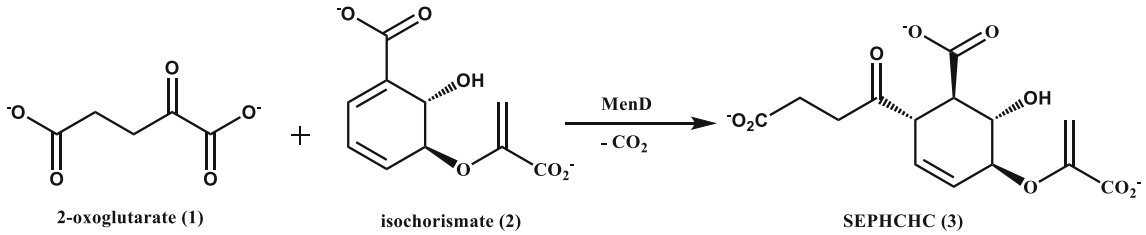

Fig. 1 1,4-Addition of 2-oxoglutarate (**1**) and isochorismate (**2**) catalyzed by MenD to yield SEPHCHC (**3**)

Interestingly, MenD catalyzes in vitro also 1,2-addition reactions on aromatic acceptors such as benzaldehyde (**9**) or on aliphatic aldehydes such as hexanal (**10**) with **1** as donor compound leading to 2-hydroxy ketones (Kurutsch et al. 2009; Beigi et al. 2013a, b; Westphal et al. 2013a, b; Pertusi et al. 2017). These products are interesting building blocks for pharmaceuticals or can be used as chiral precursors of 1,2-diols (Kulig et al. 2012). Whereas MenD displays a relatively high specificity for 1,4-reaction acceptors (Kurutsch et al. 2009; Beigi et al. 2014), its specificity for 1,2-reaction acceptors is more relaxed (Kurutsch et al. 2009; Beigi et al. 2013a, b; Westphal et al. 2013a, b; Pertusi et al. 2017). Besides its physiological donor compound, **1**, **4** and oxaloacetate have been reported as further, though much less active, donor substrates for MenD (Kurutsch et al. 2009). Further, and especially higher functionalized, donor compounds have not been reported so far for MenD.

Aldolases and ThDP-dependent carboligases are well-known enzymes in biocatalytic enantioselective chain elongations. Whereas a plethora of aldolase- *or* ThDP-dependent enzyme-catalyzed asymmetric C–C bond formations has been described over the last decades in chemo-enzymatic syntheses (for recent reviews, see Clapés et al. 2010; Clapés and Garrabou 2011; Brovetto et al. 2011; Fesko and Gruber-Khadjawi 2013; Müller et al. 2013; Windle et al. 2014; Prier and Arnold 2015; Schmidt et al. 2016; Giovannini et al. 2016; Busto 2016; López-Iglesias et al. 2016), cascade reactions wherein an aldolase forms a donor compound for a subsequent ThDP-carboligase reaction or vice versa have rarely been reported in the realm of biocatalysis (Zimmermann et al. 1999; Schürmann and Sprenger 2001; Climent et al. 2010; Hernandez et al. 2015; Guérard-Hélaine et al., 2017a). Nature, however, displays several such (reversible) cascade reactions, e.g., in the pentose phosphate pathway, the ThDP-dependent transketolase delivers a donor compound [sedoheptulose 7-phosphate] which can be used by transaldolase (a transferase) as substrate and vice versa (Sprenger 1995); another example is the combination of 2-oxo-3-deoxy-6-phosphogluconate (2-keto-3-deoxy-6-phosphogluconate, KDPGlc) aldolase (the Entner-Doudoroff aldolase (EDA); Kovachevich and Wood 1955) which cleaves KDPGlc into 4 and glyceraldehyde 3-phosphate; both products can then be taken by the ThDP-dependent 1-deoxyxylulose 5-phosphate synthase (DXS) (Sprenger et al. 1997) to form 1-deoxyxylulose 5-phosphate by decarboxylation of **4**.

One reason why the coupling of an aldolase with a ThDP-dependent carboligase is rare in biocatalysis lies in the typically specific substrate scope of these enzymes. Well-known donor compounds of ThDP-dependent enzymes are 2-oxoacids such as **4** (e.g., for pyruvate decarboxylase (PDC), acetohydroxyacid synthase (AHAS), DXS, YerE, PigD, and others), benzoylformate (benzoylformate decarboxylase (BFD)), hydroxypyruvate (transketolase (TK), pyruvate:ferredoxine oxidoreductase (PFOR)), or 2-oxoglutarate (MenD, SucA, Kgd; Müller et al. 2013; Beigi et al. 2013b). Such 2-oxoacids, however, are rarely reported as products of aldolase carboligations (Samland and Sprenger 2006; Ogawa et al. 2007; Smirnov et al. 2007; Walters and Toone 2007; Walters et al. 2008; Clapés et al. 2010; Brovetto et al. 2011; Busto 2016). Here, we present the aldolase product (*S*)-4-hydroxy-2-oxoglutarate (HOG, **6**) (see Fig. 2) as a novel donor compound for ThDP-dependent MenD.

We noticed that a pyruvate-dependent class I aldolase, KDPGlcaldolase (=EDA) from *Escherichia coli* which in nature catalyzes the reversible cleavage of KDPGlc, has been reported to perform aldol reactions with **4** as donor and the aldehyde acceptor **5**, to produce (*S*)-4-hydroxy-2-oxoglutarate [(*S*)-HOG (**6**); Floyd et al., 1992) (see Fig. 3, module 1)]. **6** is utilized as donor compound by MenD to perform 1,4- or 1,2-addition reactions on various acceptor compounds. This two-step tandem (cascade) reaction allows for two carbon chain elongation reactions and results in the addition of functionalized side chains of isochorismate (**2**) and 2,3-*trans*-CHD (2,3-*trans*-dihydroxy-cyclohexadiene carboxylate, **7**). We show that **6** serves also as donor for 1,4-addition on acrylic acid (**8**), and for 1,2-addition on the aliphatic acceptor, hexanal (**10**), as well as aromatic benzaldehyde (**9**). We report a new biocatalytic cascade strategy based on a one-pot reaction combining KDPGlc aldolase (EDA) and a ThDP-dependent carboligase, e.g., MenD. We introduce **6** as chiral donor and thereby tap the

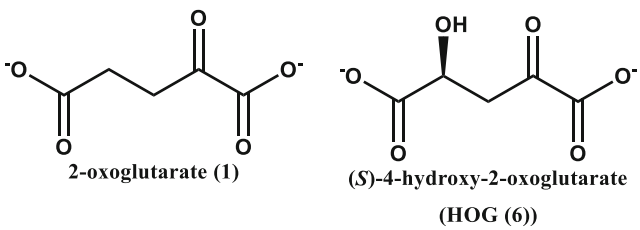

Fig. 2 4-Hydroxy-2-oxoglutarate (HOG, **6**), an analogue of the physiological donor substrate of MenD, 2-oxoglutarate (**1**)

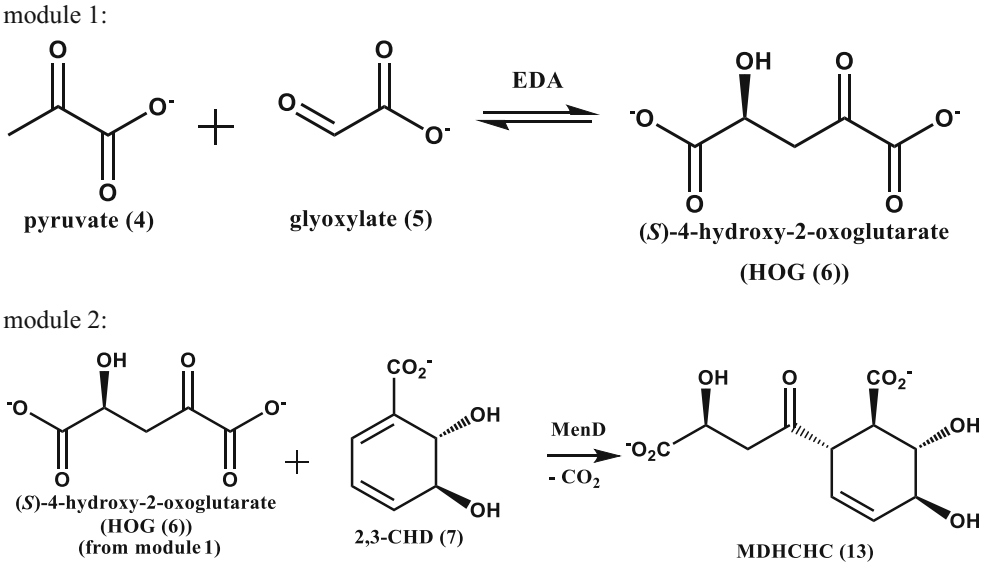

Fig. 3 Reaction scheme for a two-step reaction of aldol condensation of pyruvate (**4**) and glyoxylate (**5**) catalyzed by EDA (module 1) and subsequent 1,4-addition by MenD of its product, HOG (**6**) on 2,3-*trans*-CHD (**7**) (module 2)

potential of the three important C–C bond-forming reactions, namely the aldol, Stetter, and acyloin reaction.

Materials and methods

Chemicals and enzymes

All chemical reagents, solvents, buffers, salts, 2-oxoacids, and aldehydes were obtained in the highest available purity from Sigma-Aldrich (Steinheim, Germany) or from Fluka (Darmstadt, Germany) companies and were used without further purification, unless otherwise indicated. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Roth (Karlsruhe, Germany). Racemic (*RS*)-4-hydroxy-2-oxoglutarate (*rac*-HOG, *rac*- **6**) was obtained from Sigma-Aldrich; *rac*-HOG (**6**) was also synthesized via a base-catalyzed aldol condensation between oxaloacetate and glyoxylate (**5**) according to Riedel et al. (2011). 2,3-*trans*-CHD (**7**) was obtained enantiopure by fermentation from recombinant *E. coli* strains as described previously (Franke et al. 2001). Pyruvate (**4**) and [2,3-^{13}C]-pyruvate ([2,3-^{13}C]-**4a**) were used as their sodium salts. [2,3-^{13}C]-**4a** was purchased from Euriso-Top GmbH (Saarbrücken, Germany). Restriction enzymes (*Nde*I, *Nco*I, *Hin*dIII), 10 mM dNTPs mixture, T4 DNA Ligase, and Phusion High-Fidelity DNA polymerase with appropriate buffers, were purchased from New England Biolabs (NEB, Frankfurt am Main, Germany). The expression vector pET28a(+) was obtained from Novagen (Schwalbach, Germany). The *E. coli* strain BL21(DE3) pLysS was obtained from Invitrogen (Karlsruhe, Germany). Primers were obtained from Biomers (Ulm, Germany).

Cloning, gene expression, and purification of enzymes

The cloning of the *menD* gene (bps 2382259 through 2383929 of NCBI accession number AP009048.1) from chromosomal DNA of *E. coli* K-12 LJ110 (W3110, CGSC 4474) has been described elsewhere (Kurutsch et al. 2009), and the DNA sequence of the *menD* gene construct is given in the Supplementary Information. Expression and purification of MenD with an N-terminal 10× histidine tag (plasmid pET19b_*menD*) was carried out according to Kurutsch et al. (2009) with the following modifications: *E. coli* BL21(DE3) pLysS (Invitrogen) cells (Studier 1991) were transformed with the expression vector pET19b_*menD* and selected for ampicillin and chloramphenicol resistance. Single colonies were inoculated for overnight cultures in LB medium with ampicillin (final concentration 100 mg/L) and chloramphenicol (final concentration 25 mg/L). The main culture was performed in shake flasks at 180 rpm and 37 °C. The optical densities (OD) of bacterial cultures were determined at a wavelength of 600 nm (OD_{600}) on a UV/vis spectrophotometer, Cary 50 (Agilent Technologies, Waldbronn, Germany). At an OD_{600} of 0.5, expression was induced by addition of IPTG (1 mM final concentration). The culture was shaken for 16 h at 180 rpm and 20 °C. Cells were harvested by centrifugation at 8000×*g*, 4 °C, and 20 min using a Multifuge Avanti JXN26 (rotor JLA-8.1) centrifuge (Beckman Coulter, Krefeld, Germany). Purification of MenD as histidine-tagged protein was performed by immobilized metal affinity chromatography (IMAC) by gravity flow (Porath et al. 1975, Hochuli et al. 1987). Purification was done according to Kurutsch et al. (2009) with an additional washing step (100 mM imidazole). Cell pellets were resuspended in lysis buffer (50 mM KPi

buffer, pH 8.0, containing 150 mM NaCl, 1 mM $MgCl_2$ 1 mM ThDP, 20 mM imidazole), thawed at 30 °C and 90 rpm for 30 min, and then incubated for 30 min on ice for cell disruption by lysozyme (encoded by pLysS). Cells were additionally disrupted by sonication on ice (8 × 30 s, Sonoplus HD 200 with ultrasonic converter UW 200, Bandelin, Berlin, Germany). The debris was harvested by centrifugation at 40,000×*g*, 4 °C, 1 h (Multifuge Avanti JXN26, rotor JA-25.50 centrifuge (Beckman Coulter). The cleared lysate was loaded on a gravity flow column containing Ni-NTA Superflow resin (Qiagen, Hilden, Germany). The column was pre-equilibrated with 50 mM KP_i buffer containing 20 mM imidazole, 1 mM $MgCl_2$, 150 mM NaCl, and 1 mM ThDP (pH 8.0). After a washing step with 50 mM KP_i buffer containing 100 mM imidazole, 1 mM $MgCl_2$, 150 mM NaCl, and 1 mM ThDP (pH 8.0), the purified protein was eluted with 50 mM KP_i buffer containing 300 mM imidazole, 1 mM $MgCl_2$, 150 mM NaCl, and 1 mM ThDP (pH 8.0).

MenD-containing fractions were pooled, imidazole was removed, and MenD was concentrated by centrifugal filters (Amicon Ultra, 10 kDa, Merck Millipore, Darmstadt, Germany). Protein purity was checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), and protein concentrations were determined according to Bradford (1976) with BSA as standard protein for calibration. After this purification protocol, the protein concentrations were in the range of 10 to 15 mg mL^{-1}, consistent with a total amount of 170 mg MenD per 1 L culture (see Supplementary Fig. S1 for SDS-PAGE). Expression und purification of MenD_I474A F475G variant was done likewise.

Cloning of *eda* gene

The gene *eda* (bps 1933829 through 1934470 of NCBI accession number AP009048.1) encoding KDPGlc aldolase (EDA) was amplified via PCR from chromosomal DNA of *E. coli* K-12 wild-type strain LJ110 (W3110, CGSC 4474) using primers which introduced a *Nde*I restriction site at the 5′-end and a *Hin*dIII restriction site at the 3′-end. The PCR oligodeoxynucleotides used were as follows: 5′Nde1-_fw: TTT TCA TAT GAA AAA CTG GAA AAC AAG TGC AGA ATC 3′ and 5′Hind3-_rev: TTT TAA GCT TTT ACA GCT TAG CGC CTT CTA CAG 3′. After restriction digestion of the PCR product with *Nde*I and *Hin*dIII, the PCR amplificate was inserted into the expression vector pET28a(+) (Novagen) which had been cleaved likewise, creating a N-terminal 6× histidine tag. The resulting construct was full-length sequenced by GATC Biotech (Konstanz, Germany). Details of PCR amplification and the DNA sequence of *eda* are given in the Supplementary Information.

Expression and purification of EDA was done as described for MenD with the following modifications: recombinant strain BL21(DE3) pLysS/ pET28a(+)_*eda* were grown in LB medium with kanamycin (50 mg/L^{-1} final concentration) and chloramphenicol (25 mg/L final concentration). After the addition of IPTG (1 mM final concentration), the cells were shaken for 4 h at 180 rpm and 30 °C and then harvested by centrifugation as described above. The cell pellets were resuspended in lysis solution (5% of the culture volume, lysis solution: 50 mM $NaH_2PO_4 \cdot 2H_2O$, pH 8.0, 10 mM imidazole, 300 mM NaCl). The protein was purified on a Ni-NTA column as described above except that it was eluted with lysis solution containing 250 mM imidazole. The protein was desalted and taken up in 50 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2 \cdot 6H_2O$. After this purification protocol, the protein concentration was about 10 to 15 mg mL^{-1}, consistent with a total amount of 203 mg EDA per 1 L culture (see Supplementary Fig. S2 for SDS-PAGE).

General procedure for enzymatic reactions

Purified enzymes (purity > 90% according to SDS-PAGE, see Supplementary Figs. S1 and S2) were used for all preparations. After purification, enzymes were stored in reaction buffer. Verification of the enzymes catalyzing the C–C bond formation was done by performing control experiments without enzyme. Bradford assays for protein determination were measured at 595 nm on a microplate reader EON (Biotek, Bad Friedrichshall, Germany).

EDA-catalyzed reactions The reactions of 50 mM pyruvate (**4**) and 100 mM glyoxylate (**5**) were performed in Tris-HCl buffer (2 mL; 50 mM Tris-HCl buffer, 10 mM $MgCl_2 \cdot 6H_2O$, pH 8.0) at 30 °C and 750 rpm for 24 h using a thermomixer (TSC ThermoShaker, Analytik Jena, Jena, Germany). The final concentration of EDA was set to 0.2 mg mL^{-1}.

LC-MS analysis (LC-MS method 1, M_r 162.0, m/z = 161.0, t_R = 0.6 min) and ^{1}H-NMR verified formation of HOG (**6**) from pyruvate (**4**) and glyoxylate (**5**) for EDA.

MenD-catalyzed reactions The reactions were performed in 50 mM KP_i buffer pH 8.0 containing 0.1 mM ThDP, 2 mM $MgCl_2 \cdot 6 H_2O$, total volume of 2.0 mL) at 30 °C and 750 rpm for 24 h using a thermomixer (after 24 h no further consumption of acceptor substrate was detected). The final concentration of MenD was set to 0.7 mg mL^{-1}, 20-mM acceptor substrate (e.g., a total of 40 μmol in 2 mL volume), and 50-mM donor substrates **1** were used; if the product from the EDA reaction was used as donor, the concentration was estimated to be 50 mM (HOG, **6**) based upon the complete disappearance of **4**. For reactions with benzaldehyde (**9**) and hexanal (**10**), 5 vol% methyl *tert*-butyl ether (MTBE, final concentration) were added.

For in situ reactions with EDA, the reaction solutions (including (**4** as EDA donor at 50 mM and **5** at 100 mM) were

first incubated for 2 h at 30 °C, 750 rpm with 0.2 mg mL^{-1} EDA in 50 mM KP_i buffer pH 8.0 containing 0.1 mM ThDP and 2 mM $MgCl_2 \cdot 6\ H_2O$. MenD acceptor substrates were set at 20 mM (e.g., a total of 40 μmol in the assay) initial concentration. Thereafter, MenD_WT or MenD_I474A F475G was added (0.7 mg mL^{-1}) and reactions were further incubated for 24 h at 30 °C and 750 rpm. Enzymatic reactions were done in triplicate in closed Eppendorf tubes and were measured also in triplicate. In control experiments without enzyme addition, no significant decrease of acceptor substrate and no formation of product after 24 h incubation were observed.

For nuclear magnetic resonance (NMR) analysis, the reaction mixtures with benzaldehyde (**9**) as acceptor substrate were extracted three times with 300 μL $CDCl_3$. In the case of 2,3-*trans*-CHD (**7**) as acceptor substrate, 20% (*v*/*v*) D_2O was added to 600 μL of reaction mixture for NMR analysis.

LC-MS/MS studies with in situ production of [^{13}C-labeled] MDHCHC (13)

For this coupled enzymatic reaction, 50 mM of [2,3-^{13}C]-pyruvic acid (**4a**), 100 mM **5**, and 20 mM **7** (produced according to Franke et al. 2001) were incubated in 1 mL reaction buffer (50 mM KP_i buffer, pH 8.0, 2 mM MgCl2· 6H2O, 1 mM ThDP) at 30 °C, 750 rpm for 24 h. Final enzyme concentrations were 0.2 mg mL^{-1} EDA and 0.7 mg mL^{-1} MenD.

Analytical procedures

HPLC method 1

Decrease of acceptor substrate 2,3-*trans*-CHD (**7**) was measured by HPLC-DAD on a Dionex Ultimate 3000 system (Thermo Scientific) at 275 nm. After acidification of the samples with 1% formic acid and centrifugation (14,000 rpm, 5 min, and room temperature), substances were separated by RP chromatography on a Multospher RP C18 AQ-3-μm EC column (60 × 2 mm, CS-Chromatographie, Langerwehe, Germany) at 20 °C. Eluent A was 0.1% formic acid in H_2O and eluent B was acetonitrile. Gradient elution started with 100% (*v*/*v*) eluent A at $t = 0$ min. At $t = 1.8$ min, a linear gradient for 1.9 min was started which reached 45% (*v*/*v*) eluent B and 55% (*v*/*v*) eluent A and then to 20% eluent A and 80% eluent B in 5.1 min. The flow rate was 0.3 mL/min, the column temperature 20 °C, and the injection volume 5 μL.

HPLC method 2

For reactions with 2,3-*trans*-CHD (**7**) as acceptor and HOG (**6**) as donor substrates, HILIC technology was used. After acidifying the samples with 1% formic acid and centrifugation (14,000 rpm, 4 min, room temperature) the supernatant was diluted in acetonitrile and the substances were separated after centrifugation (14,000 rpm, 15 min, at room temperature) on an Agilent 1100 system at 275 nm on an amide column (column: XBridge Amide 5 μm (100 × 2.1 mm, Waters), gradient elution: (A) 10 mM NH_4CHO_2 in H_2O (pH 6.8) and (B) acetonitrile; gradient elution started with 10% (*v*/*v*) eluent A at $t =$ 0 min. At $t = 2.0$ min, a linear gradient for 10 min was started which reached 60% (*v*/*v*) eluent A and 40% (*v*/*v*) eluent B and then 3 min isocratic elution (flow rate: 0.3 mL/min) at room temperature; the injection volume was 1–10 μL.

HPLC method 3

For quantification of decrease of acceptor substrates, acrylic acid (**8**) and hexanal (**10**) method 2 was used with the following modifications:

After acidifying the samples with 1% formic acid and centrifugation (14,000 rpm, 4 min, and room temperature), the supernatant was diluted in acetonitrile and the substances were separated after centrifugation (14,000 rpm, 15 min, and room temperature) on an Agilent 1100 system at 275 nm on an amide column (column: XBridge Amide 5 μm (100 × 2.1 mm, Waters), gradient elution: (A) 10 mM NH_4CHO_2 in H_2O (pH 6.8) and (B) acetonitrile; gradient elution started with 5% (*v*/*v*) eluent A at $t = 0$ min. At $t = 0$ min, a linear gradient for 2.5 min was started which reached 60% (*v*/*v*) eluent A and 40% (*v*/*v*) eluent B. After 1.1 min under the same conditions, a linear gradient for 0.6 min was started which reached 5% eluent A and 95% eluent B and then 1.8 min isocratic elution (flow rate 1.2 mL/min) at 40 °C; the injection volume was 1–10 μL.

Product purification of MDHCHC (13)

The product MDHCHC (**13**) was purified by semi-preparative HPLC on a HILIC column (XBridge prep Amide 5 μm 150 mm × 10 mm, waters, Eschborn, Germany) using the following conditions on a FPLC (Biorad, Munich, Germany): (A) H_2O + 10 mM ammonium formate (NH_4CHO_2) in H_2O (pH 6.8) and (B) acetonitrile; A/B = 27:73, 10.0 mL min^{-1} for 9.6 min and 8 °C. The MDHCHC (**13**) containing fractions were lyophilized and NMR analyses were performed in D_2O. The lyophilized product was purified by preparative RP-HPLC column (Luna C18(2), 5 μm, 250 mm × 21.2 mm, Phenomenex, Aschaffenburg, Germany) to separate MDHCHC (**13**) and the substrate HOG (**6**) using following conditions on a Äkta Purifier (Amersham Bioscience/GE Healthcare, München) at room temperature: (A) 0.1% formic acid in H_2O and (B) methanol; gradient elution started with 90% (*v*/*v*) eluent A and 10% (v/v) eluent B at $t = 0$ min. At $t =$ 0 min, a linear gradient for 10 min was started which reached 70% (*v*/*v*) eluent B and 30% (*v*/*v*) eluent A. After 5 min under the same conditions, a linear gradient for 5 min was started

which reached 90% eluent A and 10% eluent B. The flow rate was 6.35 mL/min, and the column temperature 20 °C.

LC-MS method 1

Product identification was performed by liquid chromatography-mass spectrometry (Agilent 1260 HPLC system, Agilent, Santa Clara, USA) coupled to a quadrupole mass spectrometer Agilent 6130 (Agilent, Santa Clara, USA) using electrospray ionization in the negative ion mode. After separation by RP chromatography on a Multospher RP C18 AQ-3-μm EC column (60 × 2 mm, CS-Chromatographie, Langerwehe, Germany) at 20 °C, the substances were analyzed by mass spectrometry. Eluent A was 0.1% formic acid in H_2O and eluent B was acetonitrile. Gradient elution started with 100% (*v*/*v*) eluent A at $t = 0$ min. At $t = 0.9$ min, a linear gradient for 1.0 min was started which reached 45% (*v*/*v*) eluent B and 55% (*v*/*v*) eluent A and then to 20% eluent A and 80% eluent B in 2.5 min. The flow rate was 0.6 mL/min, the column temperature 20 °C, and the injection volume 1–10 μL.

LC-MS method 2

Product identification was performed by liquid chromatography-mass spectrometry (Agilent 1100 HPLC system, Agilent, Santa Clara, USA) coupled to a triple quadrupole mass spectrometer QTRAP 4500 (AB Sciex, Darmstadt, Germany) using electrospray ionization in the negative ion mode. After separation by RP chromatography on a Multospher RP C18 AQ-3-μm EC column (60 × 2 mm, CS-Chromatographie, Langerwehe, Germany) at room temperature the substances were analyzed by mass spectrometry. Eluent A was 0.1% formic acid in H_2O and eluent B was acetonitrile. Gradient elution started with 100% (*v*/*v*) eluent A at $t = 0$ min. At $t = 1.8$ min, a linear gradient for 1.9 min was started which reached 45% (*v*/*v*) eluent B and 55% (*v*/*v*) eluent A and then to 20% eluent A and 80% eluent B in 5.1 min. The flow rate was 0.3 mL/min and the injection volume 1–10 μL.

LC-MS method 3

Product identification of MDHCHC (**13**) with LC-MS/MS was performed by liquid chromatography-mass spectrometry (Agilent 1100 HPLC system, Agilent Technologies, Waldbronn, Germany) coupled to a triple quadrupole mass spectrometer QTRAP 4500 (AB Sciex, Darmstadt, Germany) using electrospray ionization in the negative ion mode. After separation by HILIC (column: XBridge Amide 5 μm (100 × 2.1 mm, Waters) at room temperature, the substances were analyzed by mass spectrometry. A gradient elution was used: 10 mM NH_4CHO_2 in H_2O (pH 6.8)/acetonitrile = 10:90 for 2 min, in 10 min to 60:40, for 3 min 60:40 flow rate: 0.3 mL/min. The LC-MS chromatogram usually showed three product peaks.

GC

GC analysis was performed on a Agilent 7890A Series GC system (Agilent Technologies, Waldbronn, Germany) equipped with a HP-5 column (Agilent, 30 m × 0.32 mm, 0.25-μm thick stationary phase), using the following conditions: injector temp 250 °C, detector temperature 300 °C, flow rate 25 mL min^{-1}, temperature program 60 °C for 3 min, then 20 °C min^{-1} to 280 °C for 4 min. Decrease of benzaldehyde (**9**) was measured. One hundred microliters of the reaction mixture was acidified with 1 μL formic acid and extracted with 200 μL of ethyl acetate (containing 0.1 μL/mL decane as internal standard).

NMR

Nuclear magnetic resonance (NMR) spectra were recorded on a DRX 400 instrument (Bruker, Billerica, USA) operating at 400 and 100 MHz for ^1H and ^{13}C acquisitions, respectively. Chemical shifts (δ) of ^1H and ^{13}C NMR spectra are reported in ppm with a solvent resonance as an internal standard (^1H NMR: $CHCl_3$ 7.24 ppm, CHD_2OD 3.30 ppm; ^{13}C NMR: $CDCl_3$ 77.0 ppm, CD_3OD 49.0 ppm). Coupling constants (*J*) are reported in hertz (Hz). Splitting patterns are indicated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet. NMR data were evaluated with the software ACD/NMR Processor Academic Edition (ACD/Labs, Toronto, Canada. Besides ^1H NMR and ^{13}C NMR analyses, the structure of HOG (**6**) was determined by HSQC and HMBC. The product of reaction of 20 mM 2,3-CHD (**7**), 50 mM pyruvate (**4**), and 100 mM glyoxylate (**5**) catalyzed by 0.7 mg/mL MenD was analyzed by ^1H NMR and TOCSY experiments.

Circular dichroism (CD) spectra were measured using a Jasco J-810 spectrometer (Jasco International Co., Tokyo, Japan).

Analytical data of the products

Aldol condensation (see Supplementary Fig. S3) (*S*)-4-Hydroxy-4-oxoglutarate = HOG (**6**), LC-MS method 1. $C_5H_6O_6$, M_r 162.0, $m/z = 161.0$, $t_R = 0.6$ min; CAS: 1187-99-1.^1H-NMR (D_2O): $\delta = 2.95–3.2$ (2H, CH_2), 4.30–4.35 (H, C*HOH*CO_2H).

^{13}C-NMR (D_2O): $\delta = 203$ (C2), 181 (C5), 169 (C1), 68 (C4), 44 (C3). These ^1H NMR and ^{13}C NMR data confirmed the results of Guérard-Hélaine et al. (2017b).

1,4-Addition reaction products (1*R*,5*S*,6*S*)-2-(3-Carboxylato-3-hydroxypropanoyl)-5,6-dihydroxycyclohex-3-ene-1-carboxylate = MDHCHC (**13**), product (see Supplementary Fig.

S5) of 1,4-addition reaction of HOG (**6**) and 2,3-*trans*-CHD (**7**), LC-MS method 3. $C_{11}H_{14}O_8$, M_r 274.2, m/z = 273.0: t_R = 11.9 min (MDHCHC **13**), t_R = 12.3 min (iso-MDHCHC **14**) and t_R = 13.2 min (structure not determined), MDHCHC (**13**), and iso-MDHCHC (**14**): new CAS substances. ^{1}H-NMR: δ = 5.6 ppm (2H, CH_{olef}).

(1*R*,5*S*,6*S*)-2-(3-Carboxylatopropanoyl)-5,6-dihydroxycyclohex-3-ene-1-carboxylate = SDHCHC (**11**), product (see Supplementary Fig. S4) of 1,4-addition reaction of 2-oxoglutarate (**1**) and 2,3-*trans*-CHD (**7**), LC-MS method 2. $C_{11}H_{14}O_7$, M_r 258.2, m/z = 257.0, t_R = 3.9 min (SDHCHC (**11**), t_R = 2.9 min (iso-SDHCHC **12**), and t_R = 5.0 min (structure not determined), CAS: 1333341-60-8 (SDHCHC **11**), CAS: 1333341–61-9 (iso-SDHCHC **12**).

4-Oxoheptanedioate (**15**), product (see Supplementary Fig. S6) of 1,4-addition reaction of 2-oxoglutarate (**1**) and acrylic acid (**8**), LC-MS method 1. $C_7H_{10}O_5$, M_r 174.1, m/z = 173.0; t_R = 1.0 min, CAS: 502-50-1.

(*S*)-2-hydroxy-4-oxoheptanedioate (**16**), product (see Supplementary Fig. S7) of 1,4-addition reaction of HOG (**6**) and acrylic acid (**8**), LC-MS method 1. $C_7H_{10}O_6$, M_r 190.1, m/z = 189.0, t_R = 2.2 min, CAS: 1093176-29-4.

1,2-Addition reaction products (*R*)-5-Hydroxy-4-oxo-5-phenylpentanoate (**17**), product (see Supplementary Fig. S8) of 1,2-addition reaction of 2-oxoglutarate (**1**) and benzaldehyde (**9**), LC-MS method 1. $C_{11}H_{12}O_4$, M_r 208.1, m/z = 207.0; t_R = 3.7 min, CAS: 1206795-80-3.

(2*S*,5*R*)-2,5-Dihydroxy-4-oxo-5-phenylpentanoate (**18**), product (see Supplementary Fig. S9) of 1,2-addition reaction of HOG (**6**) and benzaldehyde (**9**), LC-MS method 1. $C_{11}H_{12}O_5$, M_r 224.1, m/z = 223.0; t_R = 2.2 min. CAS: new substance. ^{1}H-NMR ($CDCl_3$): δ = 2.78–3.06 (2H,CH_2CO), 7.10–7.26 ppm (m, 5H, CH_{Ar}).

(2*S*,5*R*)-2,5-Dihydroxy-5-(2-fluorophenyl)-4-oxopentanoic acid.

CD: λ (nm) (mdeg) (KP_i buffer) = 284 (− 100). ^{1}H NMR (D_2O): δ = 2.53–2.90 (m, 2H, CH2), 4.45 (dd, J = 7.8, 3.7 Hz, 1H, CHOH), 5.38 (s, 1H, CHOH), 7.10–7.21 (m, 1H, CHar), 7.26 ppm (dd, J = 7.18, 7.4, 7.5 Hz, 1H, CHar), 7.306 (ddd, 7.9, 7.4, 1.8 Hz, 1H, CHar), 7.35–7.41 ppm (m, 1H, CHar).

(2*R*,5*R*)-2,5-Dihydroxy-5-(2-fluorophenyl)-4-oxopentanoic acid (2*R*,5*R*)). ^{1}H NMR (D_2O): δ = 5.40 (s, 1H, CHOH).

(*R*)-5-Hydroxy-4-oxodecanoic acid (**19**), product (see Supplementary Fig. S10) of 1,2-addition reaction of hexanal (**10**) and 2-oxoglutarate (**1**), LC-MS method 1. $C_{10}H_{18}O_4$, M_r 202.1, m/z = 201.1, t_R = 3.9 min, CAS: 1206729-77-2.

(2*S*,5*R*)-2,5-Dihydroxy-4-oxodecanoic acid (**20**), product (see Supplementary Fig. S11) of 1,2-addition reaction of hexanal (**10**) and HOG (**6**), LC-MS method 1. $C_{10}H_{18}O_5$, M_r 218.1, m/z = 217.1, t_R = 3.8 min, CAS: new substance.

Results

EDA-catalyzed synthesis of (*S*)-HOG (6)

HOG (**6**), an analogue of 2-oxoglutarate (**1**), has an additional hydroxyl group at the 4-position (see Fig. 2). Enzymatic formation of **6** from pyruvate (**4**) and glyoxylate (**5**) has been reported by the group of Turner (Floyd et al. 1992) using the *Escherichia coli* enzyme EDA (KDPGlc aldolase), a pyruvate-dependent class I aldolase (Kovachevich and Wood (1955), see Fig. 3, module 1). We cloned the *eda* gene from *E. coli* K-12 wild-type strain LJ110 and purified the recombinant His-tagged protein by IMAC (for details, see Materials and Methods section). EDA was very active on **4** and **5** hence (*S*)-(**6**) was formed in good yields (consumption of 50 mM pyruvate (**4**) after 24 h was > 99%, see Supplementary Fig. S3). ^{1}H and ^{13}C NMR data (see Supplementary Figs. S14–S18) showed the structure of **6** and were consistent with Guérard-Hélaine et al. (2017b). Additionally, HSQC and HMBC NMR confirmed the structure of **6**. Circular dichroism was used to analyze stereoselectivity of **6**. The CD spectrum of HOG (Eda) (**6**) shows a positive Cotton effect at 210 nm and 320 nm and a negative Cotton effect at 260 nm. This shows the enriched formation of one enantiomer of **6** compared to (*rac*)-HOG (*rac*-**6**) (see Supplementary Fig. S13); assuming full conversion, a total of 100 μmol (ca. 16 mg) of **6** (in a reaction volume of 2 mL) was obtained. Chromatographic separation of (*S*)-(**6**) from its educt **4**, however, proved to be difficult. Therefore, we used a one-pot, two-enzyme system for **6** formation and further reactions. First, purified EDA was added to a buffered system with **4** and **5** as substrates. After 2 h, when the aldolase reaction had come to equilibrium, MenD was added and the reaction was proceeded overnight (total time, 26 h).

(*S*)-HOG (6) as donor substrate for MenD-catalyzed 1,4-additions

MenD-catalyzed synthesis of 13 from 7 and in situ formed 6

The physiological 1,4 acceptor compound of MenD is isochorismate (**2**), a notoriously unstable product of chorismate (Hubrich et al. 2014). Proxy substrates are 2,3-*trans*-CHD (**7**, Kurutsch et al. 2009) or acrylic acid (**8**, Beigi et al. 2014) (see Fig. 4), leading to higher functionalized products. We assayed recombinant His-tagged MenD (Kurutsch et al. 2009) with the standard donor, **1**, and various acceptor compounds. As can be seen from Table 1, the data compared favorably with > 99% consumption of **8** with **1** as donor compound (Beigi et al. 2014, see Supplementary Fig. S4) and ca. 90% conversion with **7** in 24 h (Kurutsch et al. 2009).

Next, we assayed enzymatically prepared (*S*)-**6** (EDA-catalyzed product of **4** and **5**) for its propensity to act as a donor compound for MenD in one-pot cascade reactions.

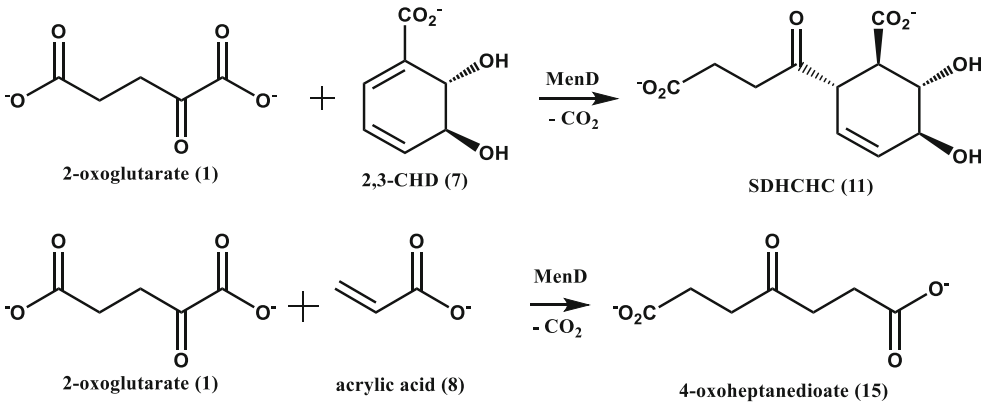

Fig. 4 1,4-Addition of 2-oxoglutarate (**1**) and 2,3-*trans*-CHD (**7**) or acrylic acid (**8**) catalyzed by MenD

6 was enzymatically synthesized in a one-pot system by EDA as described above. After 2 h at 30 °C, more than 90% of **4** was converted to **6** (data not shown). The subsequent MenD-catalyzed reaction with 20 mM **7** as acceptor resulted in 78% consumption of **7** as judged by HPLC-DAD (see Table 2). The control experiment with **1** as donor gave a consumption of **7** of 90% (see Table 1 and Fig. 4). These results ascertain **6** to be a suitable donor compound for MenD.

However, two novel products from the reaction of **6** and **7** were detected by LC-MS analysis (based on HILIC technology) indicating two substances with $m/z = 273.0$ (t_R = 11.9 min and $t_R = 12.3$ min) (see Supplementary Fig. S5). We assume this to be malyl-DHCHC [(1*R*,2*S*,5*S*,6*S*)-2-malyl-5,6-dihydroxycyclohex-3-enecarboxylate = MDHCHC (**13**)] (see Fig. 3, module 2). This was verified by LC-MS/MS and NMR studies (see Supplementary Fig. S5, 20–24).

In analogy to SDHCHC (**11**), which is formed from **1** and **7** and isomerizes to iso-SDHCHC (**12**, Kurutsch et al. 2009), MDHCHC (**13**, $t_R = 11.9$ min) should isomerize to the more stable iso-MDHCHC (**14**, $t_R = 12.3$ min; see Fig. 5). Analysis by LC-MS/MS gave retention times for MDHCHC (**13**) of 11.9 min and 12.3 min for iso-MDHCHC (**14**) (see Supplementary Fig. S5). For both compounds (**13** and **14**), parent anions of $m/z = 272.8$ and fragment ions of m/z = 255.0 $[M–H_2O–H]^-$, 228.8 $[M–CO_2–H]^-$, 210.9 $[M–CO_2–H_2O–H]^-$, 198.9 $[M–C_2H_2O_3–H]^-$, 192.9 $[M–CO_2–2H_2O–H]^-$, 174.8 $[M–CO–3H_2O\text{-}H]^-$, 166.8 $[M–2CO_2–H_2O–H]^-$, 155.0 $[M–C_2H_2O_3–CO_2–H]^-$, 148.9 $[M–2CO_2–2H_2O–H]^-$, 137.0 $[M–C_2H_2O_3–CO_2–H_2O–H]^-$, 119.1 $[M–C_2H_2O_3–CO_2–2H_2O–H]^-$, and 72.8 $[C_2HO_3]^-$ were observed (see Fig. 6). This fragmentation pattern is in accordance with the formation of **13** and **14**. A third, however weak, signal with $m/z = 273.0$ ($t_R = 13.2$ min) was also detected. We do not know at this time which structure can be attributed to this third form.

For a further study of the EDA-catalyzed formation of **6** and its subsequent fate in the product, **13**, labeling experiments were applied. [2,3–^{13}C]-Pyruvate (4a) was reacted with **5** by EDA and the MenD-catalyzed reaction was performed with **7**. LC-MS/MS data and fragmentation pattern (see Fig. 6) confirmed formation of [2,3-^{13}C]-labeled **6a** and in turn the formation of labeled MDHCHC (**13a**).

The reaction scheme in Fig. 3 (module 1) shows the aldol reaction of **4** and **5** by EDA (Floyd et al. 1992) and the subsequent 1,4-addition of **6** on **7** catalyzed by MenD (Fig. 3, module 2). We conclude that 1,4-addition of **6** on **7** leads to **13** and, probably due to a non-enzymatic spontaneous isomerization, also to iso-MDHCHC **14** (see Fig. 5).

Structure of the products as determined by ^{1}H NMR 1,4-Addition of **1** or **6** to the cyclohexadiene of **7** (double bond C5, C6) leads to products with a cyclohexene moiety. The ^{1}H NMR spectra showed the isomerization of **13** to **14** as

Table 1 MenD-catalyzed 1,2- and 1,4-addition reactions of activated succinyl semialdehyde

Donor substrate	Acceptor substrate	Decrease of acceptor [%] after 24 h	Conversion of acceptor (literature data) [%]
2-Oxoglutarate (**1**)	2,3-*trans*-CHD (**7**)	90	Not quantified (Kurutsch et al. 2009)
2-Oxoglutarate (**1**)	Acrylic acid (**8**)	> 99	> 99 (Beigi et al. 2014)
2-Oxoglutarate (**1**)	Benzaldehyde (**9**)	> 99	> 99 (Kurutsch et al. 2009)
2-Oxoglutarate (**1**)	Hexanal (**10**)	95	55 isolated yield (Kurutsch et al. 2009)

Conditions: 50 mM KP_i buffer pH 8.0, 2 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM ThDP, 20 mM acceptor substrate, 50 mM 2-oxoglutarate (**1**); 2.0 mL reaction volume. MenD: 0.7 mg mL^{-1}, 30 °C, 750 rpm; 24-h reaction time. Consumption is denoted as decrease of acceptor substrate measured by GC or HPLC-DAD (for detailed reaction conditions of GC or HPLC methods, see Materials and Methods section). Determinations were done in triplicates

Table 2 Comparison of MenD-catalyzed 1,2- and 1,4-addition reactions with 2-oxoglutarate (**1**) and (*S*)-HOG (**6**) as donor compounds

Donor substrate	Acceptor substrate	Decrease of acceptor [%] after 24 h; (method of detection)
2-Oxoglutarate (**1**)	2,3-*trans*-CHD (**7**)	90.4 ± 0.1 (HPLC-DAD)
(*S*)-HOG (**6**)	2,3-*trans*-CHD (**7**)	78.5 ± 0.1(HPLC-DAD)
2-Oxoglutarate (**1**)	Benzaldehyde (**9**)	> 99 (GC)
(*S*)-HOG (**6**)	Benzaldehyde (**9**)	> 99 (GC)
2-Oxoglutarate (**1**)	Acrylic acid (**8**)	> 99 (HPLC-DAD)
(*S*)-HOG (**6**)	Acrylic acid (**8**)	> 99 (HPLC-DAD)
2-Oxoglutarate (**1**)	Hexanal (**10**)	94.9 ± 4.0 (HPLC-DAD)
(*S*)-HOG (**6**)	Hexanal (**10**)	87.2 ± 3.4 (HPLC-DAD)

Conditions: 50 mM KP_i buffer pH 8.0, 2 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM ThDP, 20 mM acceptor substrate, 50 mM 2-oxoglutarate (**1**) or (*S*)-HOG (**6**) formed by EDA reaction; 2.0 mL reaction volume. MenD: 0.7 mg mL^{-1}, 30 °C, 750 rpm; 24 h reaction time. (*S*)-HOG (**6**) was formed by a 2-h pre-reaction at 30 °C of from 50 mM pyruvate (**4**) and 100 glyoxylate (**5**) ($v = 2$ mL, 50 mM KP_i buffer pH 8.0, 2 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM ThDP, EDA: 0.2 mg ml^{-1}. Consumptions are given as decrease of acceptor substrate measured by GC or HPLC-DAD (for detailed reaction conditions of GC or HPLC methods, see Materials and Methods section). Determinations in triplicates with standard deviations

described above during storage at 4 °C: the double bound in the cycle isomerizes from position 3,4 to position 2,3. The ^{1}H NMR signal for the olefinic proton(s) (shifts from 5.6 to 6.9 ppm, see Supplementary Figs. S20–S23). The formation of this cycle in the products of **7** with **1** and **6** was verified by TOCSY experiments (see Supplementary Fig. S24). These results are consistent with data from LC-MS experiments.

To further characterize the product of MenD-catalyzed 1,4-addition of **6** with **7**, we performed the transformations on a preparative scale and purified the reaction product by preparative HILIC to remove remaining **7** from the mixture. Then, by preparative RP-HPLC, **6** was separated from the mixture. During purification of **13**, isomerization to **14** occurred. LC-MS data verified separation of **4**, **5**, and **7**. The stereochemistry of the product, however, could not be determined. We assume that the products SDHCHC, iso-SDHCHC, MDHCHC, and iso-MDHCHC have the same configuration as reported for SEPHCHC (1*R*,2*S*,5*S*,6*S*, Jiang et al. 2007).

1,4-Addition reactions with acrylic acid (148)

Further substrate studies were performed with acrylic acid (**8**) as aliphatic Michael acceptor. With **1** as donor, this leads to the formation of an aliphatic yet non-chiral diacid with an oxo group, 4-oxoheptanedioate (**15**, Beigi et al. 2014) (see Fig. 4, Supplementary Fig. S6). With **6** as donor substrate, the MenD-catalyzed reaction should give an aliphatic chiral diacid, 2-hydroxy-4-oxoheptanedioate (**16**) (see Fig. 7). In the MenD-catalyzed reaction of **8** with **6**, more than 99% of the acceptor was converted (see Table 2). Product formation was detected by LC-MS ($m/z = 189.0$ $[M–H]^-$), consumption of the acceptor **8** with **1** was also almost quantitative (Table 1, see Supplementary Fig. 7). Determination of product structure by ^{1}H NMR was not successful (see Supplementary Fig. S25).Still, **8** appears to be a favorite acceptor for MenD in 1,4-addition reactions. In a very recent paper (Sudar et al. 2018), MenD was successfully used to add 1 on acrylonitrile in a gram scale format.

(*S*)-6 as donor for MenD-catalyzed 1,2-additions

MenD-catalyzed synthesis of 18 from benzaldehyde (9) and in situ formed (*S*)-6

Apart from its physiological function, MenD is able to perform 1,2-addition reactions with various aromatic and aliphatic aldehydes (Kurutsch et al. 2009; Beigi et al. 2013a; Westphal et al. 2013a; Pertusi et al. 2017) leading to

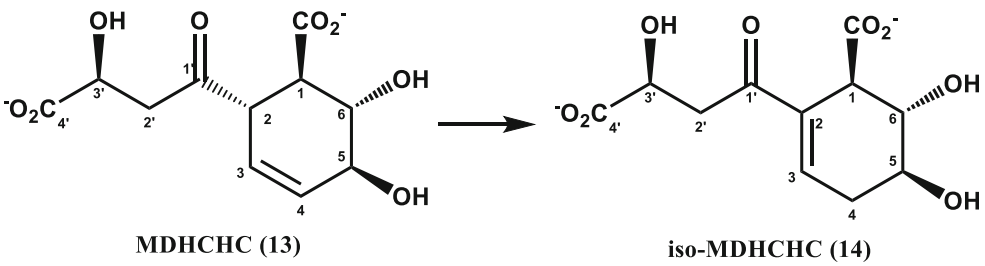

Fig. 5 Predicted isomerization of MDHCHC (**13**) to iso-MDHCHC (**14**)

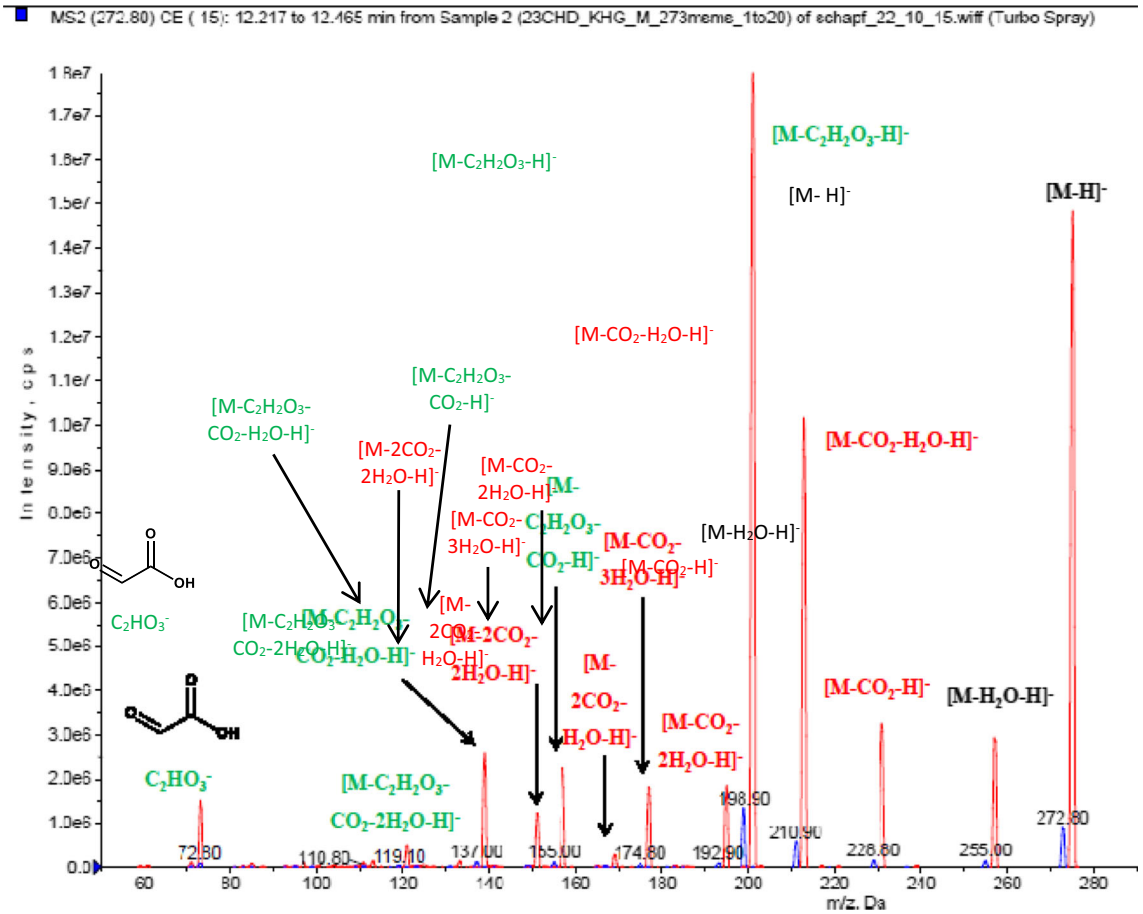

Fig. 6 Fragmentation pattern of the putative product MDHCHC (**13**). The postulated fragmentation mechanism is explained in Fig. S12 (SI) (blue: fragmentation of MDHCHC; red: fragmentation of ^{13}C-labeled MDHCHC). Incubation of 50 mM pyruvate (4) or [2,3-^{13}C]-pyruvate (4a) and 100 mM glyoxylate (5), and 20 mM 2,3-trans-CHD (7) with 0.2 mg mL^{-1} EDA and 0.7 mg mL^{-1} MenD for 24 h at 30 °C, 750 rpm (after 2 h addition of MenD) (HILIC-LC-MS/MS, malyl-DHCHC: M_r = 274 g/mol, m/z 273.0; [2,3-^{13}C]-malyl-DHCHC: M_r = 276 g/mol, m/z = 275.0; two ways of fragmentation: path 1: red, path 2: green)

functionalized 2-hydroxy ketones (see Fig. 8). These are valuable chiral building blocks and potent precursors of γ- and δ-lactones which are present in the structure of several natural products and are important intermediates in organic synthesis (Sukumaran and Hanefeld 2005; Kulig et al. 2012; Sehl et al. 2012). Therefore, we assayed whether **6** serves as donor compound for MenD-catalyzed 1,2-additions. First, purified MenD was incubated with benzaldehyde (**9**) and **6** in analytical scale. GC analysis was used to estimate the extent of product formation. Two derivatized product peaks appeared according to GC-MS analysis. These most likely stem from conditions during GC runs. The first peak corresponds to the decarboxylated 2-hydroxy ketone oxidized to the 1,2-diketone; the second peak shows in addition lactone formation of the 5-hydroxy-4-oxopentanoyl moiety. This behavior is typical for 2-hydroxy ketones derived from MenD catalysis, i.e., with **1** as donor substrate (Kurutsch et al. 2009). Determination of product structure by NMR was not successful (see Supplementary Fig. S26). Using LC-MS, one single product peak was detected [(2*S*,5*R*)-2,5-dihydroxy-4-oxo-5-phenylpentanoate (**18**)] with **1** (see Supplementary Fig. S8) and **6** as (see Supplementary Fig. S9) donor substrates,

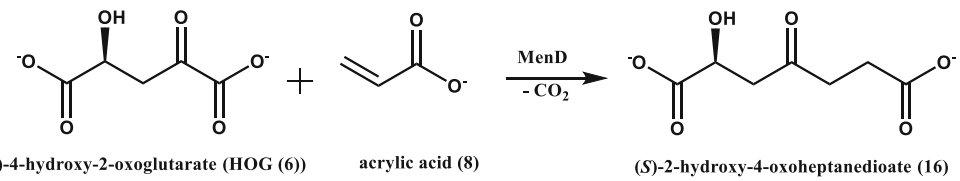

Fig. 7 Putative 1,4-addition of HOG (**6**) and acrylic acid (**8**) catalyzed by MenD

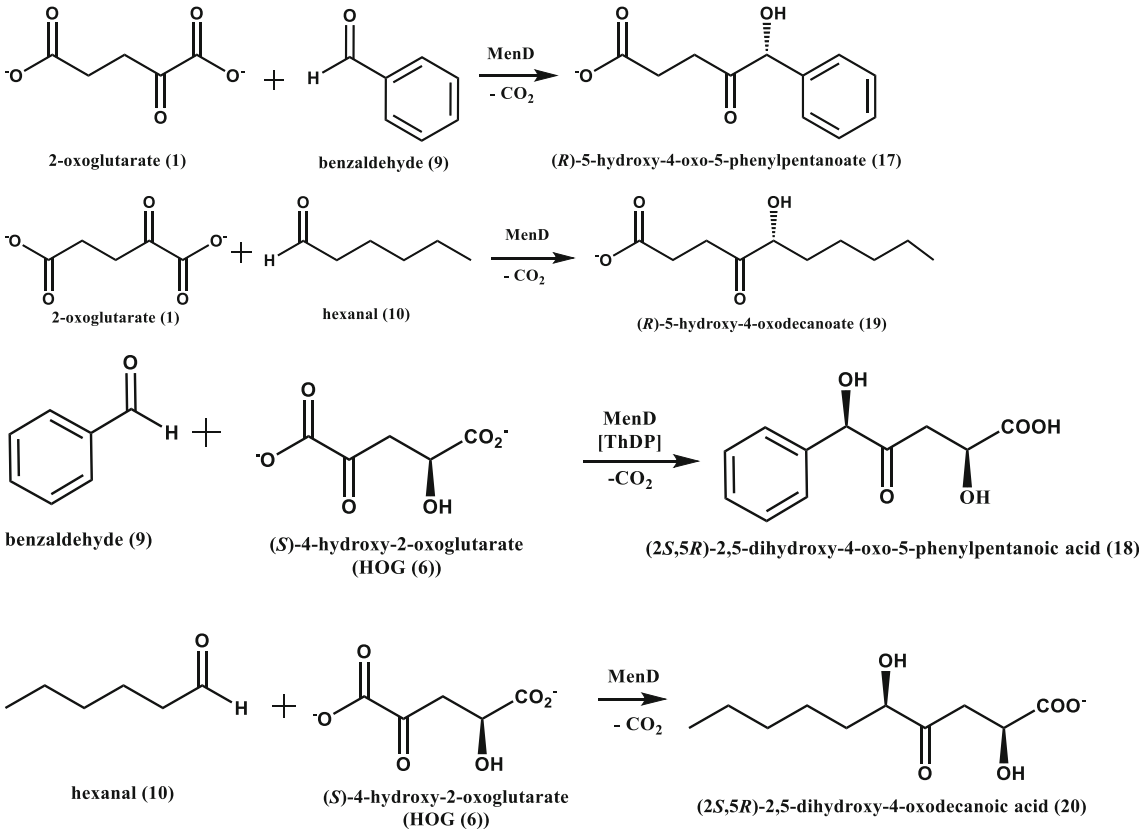

Fig. 8 1,2-Addition of 2-oxoglutarate (**1**) or HOG (**6**) and aliphatic/aromatic aldehydes

respectively. In contrast to **7** as acceptor, the consumption of **9** with **6** as donor was complete (see Table 2).

In order to determine the enantio- and diastereoselectivity of MenD-catalyzed 1,2-additions, we analyzed the products formed by the addition of (*R*,*S*)-HOG (*rac*-**6**, commercially available) and, moreover, synthesized according to Riedel et al. (2011)) and (*S*)-HOG (**6**), respectively, to 2-fluorobenzaldehyde (for NMR spectra of the products of MenD-catalyzed addition a. The isolated products (see Supplementary Figs. S27 and S28) showed a good separation of the signal of the carbinol-CH. The product of the enzymatically prepared (*S*)-HOG (**6**) showed one singlet at 5.38 ppm, whereas (*R*,*S*)-HOG (*rac*-**6**) resulted in two signals at 5.38 ppm (main product, 2*S*,5*R* configuration, see below) and 5.40 ppm (minor product, putatively 2*R*,5*R* configuration). Moreover, the Eda-MenD product (2*S*,5*R*)-5-(2-fluorophenyl)-2,5-dihydroxy-4-oxopentanoate showed a negative Cotton effect at 280 nm, clearly indicating (*R*)-configuration of the newly formed stereocenter (see Supplementary Fig. S14). This is in line with the stereoconfiguration of previous products of 1,2-addition of 1 to several benzaldehyde derivatives (Kurutsch et al. 2009; Hailes et al. 2013). From this, it can be clearly stated that the MenD-catalyzed 1,2-addition proceeds as a highly enantioselective reaction and, moreover, that both enantiomers of **6** are accepted for 1,2-additions.

MenD-catalyzed synthesis of 20 from hexanal (10) an in situ formed (*S*)-6

Hexanal (**10**) is an aliphatic acceptor for MenD-catalyzed 1,2-addition reaction (Kurutsch et al. 2009). The product with **1** is a C10 carboxylate with an internal 2-hydroxy ketone function (**19**) (Fig. 8). In our hands, reaction of **1** with **10** gave a consumption of the acceptor of 95% (Table 1). For **10** and **6**, product formation **20** was detected by LC-MS ($m/z = 217.1$ $[M–H]^-$, $t_R = 3.8$ min); a consumption of the acceptor of 87% (±3%) was determined. Determination of product structure by NMR was not successful (see Supplementary Fig. S29).

In summary, MenD accepts **6** with aromatic and aliphatic acceptor substrates in 1,2- and in 1,4-addition reactions.

1,2- and 1,4-addition reactions by a MenD variant (MenD_I474A F475G)

Wild-type MenD catalyzes the highly enantioselective formation of (*R*)-2-hydroxy ketones, often with ee > 99% (Kurutsch et al. 2009). This selectivity has recently been almost reversed to ~ 75% *ee* (*S*) by a two-amino acid exchange (I474A F475G, Westphal et al. 2013a, b, 2014). In our hands, the standard reaction of **1** and **9** catalyzed by this MenD variant led to a

consumption of **9** of 86%. With **6** as donor compound and **9** as acceptor, the MenD variant gave almost complete consumption (94%). LC-MS analysis confirmed product formation. The 1,2-addition of the donor **1** on **10** had not been reported before for this MenD variant. We therefore analyzed this reaction and found a consumption of 91% (± 4%) of **10**. With **6** as donor, a consumption of 63% (± 4) of **10** was found (data not shown).

A 1,4-addition reaction had not been reported for the MenD_I474A F475G variant so far. We tested **1** as donor with **8** which resulted in a quantitative consumption of **8**. **6** as donor substrate gave a 76% consumption of **8**. To our astonishment, no consumption of **1** or **6** on **7** as 1,4-acceptor could be detected (no product formation was detected by LC-MS nor a decrease of **7** over the reaction time).

Discussion

The aldolase product (*S*)-4-hydroxy-2-oxoglutarate (HOG,**6**) was identified as a new donor substrate in thiamine diphosphate (ThDP)-dependent enzyme-catalyzed 1,2- and 1,4-additions. The MenD-catalyzed 1,4-addition of **6** with **7** was characterized by LC-MS/MS and NMR spectroscopy. The product **13** of the enzymatic reaction isomerizes by rearrangement of the double bond to the more stable α,β-unsaturated isomer iso-MDHCHC (**14**). The MenD-catalyzed 1,4-addition of **6** with acrylic acid (**8**) gives a chiral diacid with a central 2-hydroxy ketone motif. This product is a possible precursor for new polymers or polyesters based on the two carboxylic acid groups. Crosslinking might be also possible through the hydroxyl or oxo functionality.

The 1,2-addition of **6** was studied with benzaldehyde (**9**) and hexanal (**10**) as acceptor substrates which were quantitatively converted into the corresponding 2-hydroxy ketones **(18)** and **(20)**, respectively. Thus, **6** is a new donor for MenD both for 1,2- and 1,4-addition reactions.

As already observed for *Ec*MenD (Kurutsch et al. 2009, Beigi et al. 2014), these reactions are characterized by excellent chemoselectivity. Under tested conditions, only the desired products were detected in significant amounts. Neither self-ligation of **6** or **9**, nor products with changed chemo- or regioselectivity of acceptor and donor substrate were detected. Reaction of **6** with **8** or **10** opens the field for new kinds of monohydroxy-4-oxo diacids respectively dihydroxy-4-oxo acids. Such functionalized acids contain a polar part with a 2-hydroxy carboxylic group, a 2-hydroxy ketone function, and in addition an alkyl (e.g., propyl) side chain which could allow a plethora of subsequent enzymatic or chemical follow-up reactions.

To our astonishment, the (*S*)-selective MenD_I474A F475G variant (Westphal et al. 2013b), while performing 1,4-addition on **8**, did not utilize **7** as acceptor. Neither with the cognate donor, **1**, nor with the different **6** did we find product formation or a decrease of **7**. It remains to be elucidated whether the (*S*)-selectivity is incompatible with the 1,4-addition reaction when bulky substrates such as **7** are applied.

In summary, the aldolase product (*S*)-(**6**) is established as a new donor substrate of ThDP-dependent MenD-catalyzed carboligations. As all starting materials can be generated by the central metabolism in vivo from established carbon sources, the new transformations enable strategies toward metabolic engineering and sustainable synthetic biology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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