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MenD as a versatile catalyst for asymmetric synthesis

Anja Kurutsch[a], Michael Richter[a], Volker Brecht[a], Georg A. Sprenger[b], Michael Müller[a,*]

[a] Institute of Pharmaceutical Sciences, Albert-Ludwigs-Universität, Albertstraße 25, 79104 Freiburg, Germany
[b] Institute of Microbiology, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

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Dedicated to Prof. Eckhard Leistner, who pioneered the diversity of isochorismate chemistry.

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ABSTRACT

The thiamine diphosphate (ThDP)-dependent enzyme 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (MenD) from *Escherichia coli* K12, formerly known as SHCHC-synthase, catalyses the decarboxylation of α-ketoglutarate and the subsequent addition of the resulting succinyl-THDP to isochorismate. Here, the enzyme is tested for unphysiologial C–C bond-forming reactions.

Condensation of α-ketoglutarate after decarboxylation to a broad range of aldehydes gave α-hydroxyketones with isolated yields from 26 to 87% and 94 to 98% *ee* for addition to aromatic aldehydes. MenD accepts a wide range of aldehydes as acceptor substrates to produce chiral α-hydroxyketones with conserved regioselectivity where the activated succinylsemialdehyde serves selectively as the donor. Regioselectivity is inverted only for condensation of α-ketoglutarate with pyruvate (activated acetaldehyde) as donor. Besides α-ketoglutarate, pyruvate and oxalacetate are accepted as donors in combination with benzaldehyde and 2-fluorobenzaldehyde as acceptors, however with decreased activity of C–C bond formation.

The physiological 1,4-addition of α-ketoglutarate to isochorismate was investigated for acceptor substrate variability. (2*S*,3*S*)-2,3-Dihydroxy-2,3-dihydrobenzoate (2,3-CHD), which lacks the pyruvyl found in isochorismate, is converted to (5*S*,6*S*)-2-succinyl-5,6-dihydroxycyclohex-2-enecarboxylate. In contrast to the addition to carbonyls, the active site of MenD does appear to impose specific constraints on the acceptor substrate for 1,4-addition with α,β-unsaturated carboxylic acids.

1. Introduction

One of the key features of many successful bioactives and pharmaceuticals is chirality. In this context the synthesis of single enantiomers has become more and more important as often only one enantiomer is bioactive while the other can be inactive or even toxic. For this purpose enzyme catalysis offers many advantages compared to non-enzymatic synthesis: extraordinary regio- and stereoselectivity are common properties of enzymes. High yields of the desired enantiomer combined with minimal formation of by-products and environmental compatible reaction conditions independent from petroleum-derived educts are obvious benefits of enzymatic synthesis [1]. Thiamine diphosphate (ThDP)-dependent enzymes are already used in large scale organic synthesis catalysing the formation of chiral α-hydroxyketones [2,3], itself versatile building blocks for stereoselective synthesis. Several ThDP-dependent enzymes are well characterised for their catalytic potential relating to substrate diversity and stereoselectivity [4,5].

The ThDP-dependent enzyme MenD from *Escherichia coli* (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase) is particular with respect to its physiological reaction [6,7]. Its high resolution crystal structure was recently determined by Dawson et al. [8]. It catalyses the second step of the biosynthesis of menaquinones (Vitamin K_2 derivatives) (**1**) (Scheme 1), which play an important role as electron shuttle. The absence of the menaquinone biosynthetic machinery in humans and animals makes it an interesting target for development of bioactives like antibiotics [9].

Regarding the enzymatic mechanism MenD is in principal similar to other ThDP-dependent enzymes: α-keto acids form adducts with ThDP and are decarboxylated toward a ThDP-bound aldehyde ("active aldehyde"). The bound ThDP reverses the polarity of the carbonyl enabling the addition of the donor aldehyde to an electrophilic acceptor, thereby forming a 2-hydroxyketone [10].

However, beholding the natural donor and acceptor substrates, MenD is extraordinary in two ways: it accepts α-ketoglutarate (**3**) as natural donor and, more importantly, it is the only enzyme known so far to catalyse physiologically a Stetter-like 1,4-addition to an α,β-unsaturated carboxylic acid [7,11,12]. The ThDP adduct with succinylsemialdehyde is added to the β-carbon of the unsaturated carboxylic acid isochorismate (**2**) [13]. Forming a new C–C bond

* Corresponding author. Tel.: +49 761 6320; fax: +49 761 6351.
E-mail address: michael.mueller@pharmazie.uni-freiburg.de (M. Müller).

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Scheme 1. MenD-catalysed reaction. The enzyme catalyses the second step of the biosynthesis of menaquinones starting from chorismate.

stereoselectively the product 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC, **4**) is observed. A first hint for the occurrence of an intermediate between **2** and **6** was recognised by Simantiras and Leistner in 1991 [12]. Guo et al. proved in 2007 the existence of **4** by NMR and characterized MenH, which catalysed the elimination of pyruvate (**5**) from **4** resulting in 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC, **6**) (Scheme 1) [6,14]. This unrivalled transformation led us to investigate this interesting natural reaction for biocatalytic purpose broadening acceptor and donor substrate spectrum. The main focus of this investigation is therefore the biocatalytic potential of MenD and its utility in asymmetric synthesis regarding a side reaction of many ThDP-dependent enzymes, the addition of α-keto acids to different aldehydes, and furthermore the physiological 1,4-addition applying several unsaturated carboxylic acids.

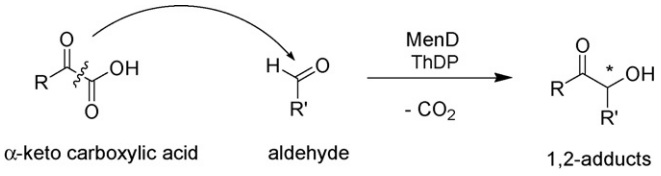
2. Results and discussion

2.1. 1,2-Addition products

The closest sequence homology of MenD is reported for the AHAS I with 14% [7,15]. Referring to the fact that many of the ThDP-dependent enzymes including AHAS I [16] catalyse the formation of chiral hydroxyketons accepting a broad substrate range, also the capacity of MenD for this unphysiological 1,2-addition of α-ketoglutarate to aldehydes was investigated.

2.1.1. Acceptor substrate variation

Purified MenD was incubated with α-ketoglutarate and different aromatic (**7**), aliphatic (**9**) and α,β-unsaturated aldehydes (**11**) as potential acceptor substrates in analytical scale (Scheme 2). For each type of aldehydes one reaction was run on preparative scale. GC–MS analysis was used to judge the extent of product formation. In each case reaction took place, two derivatised product peaks appeared. One peak corresponds to the decarboxylated α-hydroxyketone oxidised to the diketone, the second peak shows in addition lactone formation of the 4-oxopentanoyl moiety. Product verification by NMR analysis proves that these derivatisation processes are a result of GC–MS analysis conditions. For non-volatile products, LC–MS analysis was successfully applied as qualitative hint for product formation, detecting product peaks. In this case ^{1}H NMR was used to quantify product formation by calculation of the proton ratio of substrate and product. Therefore, 20% D_2O were added for in situ measurements to avoid false ratios through extraction.

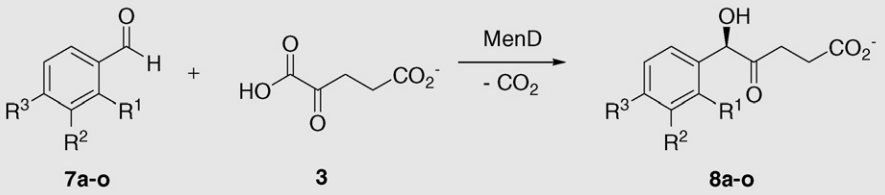


Scheme 2. Basic reaction scheme of the MenD-catalysed reaction with aldehydes as acceptors. Purified MenD was incubated with α-ketoglutarate as donor and different aromatic, aliphatic and α,β-unsaturated aldehydes as potential acceptor substrates.

The MenD-catalysed condensation products of **3** with different types of aldehydes gave high isolated yields of up to 87%. Good asymmetric induction (94–98% *ee*) was observed for a broad range of α-hydroxyketones (Table 1).

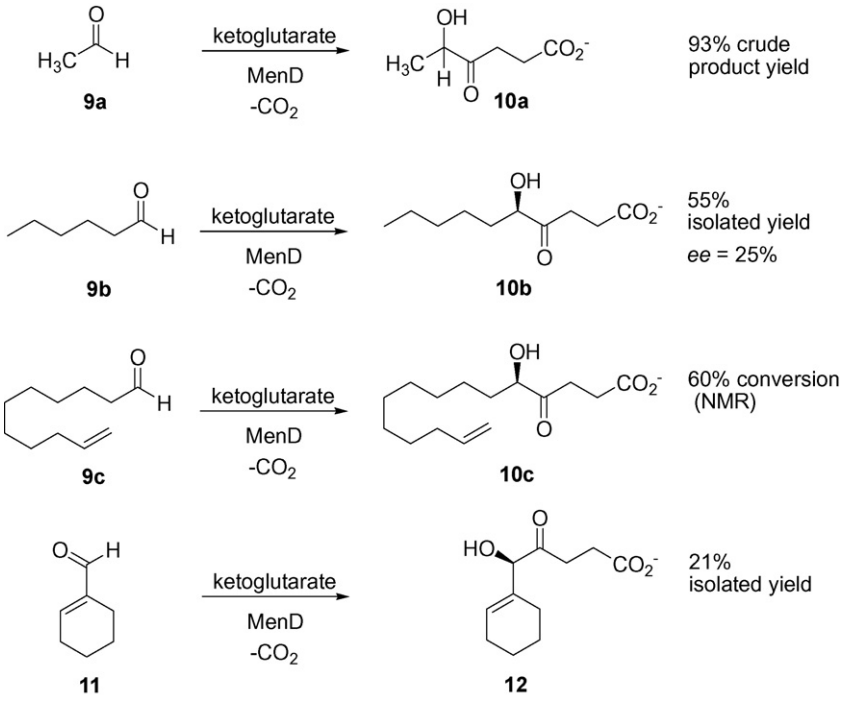
For the reaction of benzaldehyde with α-ketoglutarate the carboligase activity was determined as 0.2–0.25 $U_{MenD1,2Lig}$ mg^{-1}

Table 1
Conversion rate, isolated yield and *ee*-values of the MenD-catalysed 1,2-addition reactions of α-ketoglutarate to aldehydes (benzaldehyde derivatives as acceptors) determined by GC–MS or NMR.



Product	R^1	R^2	R^3	Conversion rate [%] GC–MS/NMR	Isolated yield [%]	*ee* [%]
8a	H	H	H	>99	65	94
8b	I	H	H	90	n.d.	n.d.
8c	Cl	H	H	80	n.d.	n.d.
8d	Br	H	H	65	n.d.	n.d.
8e	CH3	H	H	12	n.d.	n.d.
8f	F	H	H	>99	87	95
8g	H	F	H	88	n.d.	n.d.
8h	H	OCH3	H	50	n.d.	n.d.
8i	H	I	H	95	79	98[a]
8k	H	H	F	>99	n.d.	n.d.
8l	H	H	Cl	>99	n.d.	n.d.
8m	H	H	OH	66 (NMR)	n.d.	n.d.
8n	OH	OH	H	45	n.d.	n.d.
8o	H	OH	OH	53 (NMR)	n.d.	n.d.

[a] *ee* determined from methyl ester **18a**.



Scheme 3. Isolated yields of α-hydroxyketones determined for the MenD-catalysed reactions corresponding to the aliphatic (**9a**–**c**) and α,β-unsaturated aldehydes (**11**).

protein. The conversion rate, the isolated yield and the *ee*-values for substituted benzaldehydes (**7a**–**o**) as acceptors are shown in Table 1. High product formation was obtained for aromatic halide-substituted aldehydes as acceptor, irrespective of the substitution position. Methoxy- (**7h**) and methyl-substituted benzaldehydes (**7e**) in contrast show significant lower conversion. The regioselectivity drops for heteroaromatic aldehydes like thiophenal, as fast isomerisation from 5-hydroxy-4-oxo-products to 4-hydroxy-5-oxo-products was observed. No reaction could be detected for amino- and nitro-substituted benzaldehydes.

For aliphatic (**9a**–**c**) and α,β-unsaturated aldehydes (**11**) [17] the isolated product yields achieved are moderate (21–55%). Nevertheless, there is no hint for pronounced steric restriction with respect to aliphatic aldehyde substrates. Even for C_{11} aldehydes MenD showed no considerable decrease in activity compared to C_5 aldehydes (Scheme 3).

Chiral phase HPLC, chiral phase GC, chiral phase LC–MS, optical rotation and circular dichroism (CD) were used to analyse stereoselectivity. The absolute configuration was deduced by comparison of the CD spectra of **8a**, **8f**, **8i**, **10b**, **10c** and **12** to that of *R*-phenylacetylcarbinol (PAC, **14a**) (Fig. 1) [18]. Each compound shows a negative Cotton effect at 270–280 nm and a positive Cotton effect of much weaker amplitude at 315 nm consistently with **14a**. Hence, we conclude that each of the α-hydroxyketones formed by MenD has *R* absolute configuration. The highest *ee* values were determined for different benzaldehyde derivatives (Table 1).

2.1.2. Donor substrate variation

Benzaldehyde (**7a**) and 2-fluorobenzaldeyde (**7f**) were chosen as acceptors for all donor variation experiments (Scheme 4). The physiological donor **3** was substituted by oxaloacetate (**13a**), which is in comparison to **3** one methylene moiety shorter, pyruvate (**5**) and 2-oxobutyrate. No conversion at all was detected for 2-oxobutyrate by GC–MS, whereas both **13a** and **5** incubated with the acceptor aldehydes react to **14a** and 2-fluoro-PAC (**14b**), however with low conversion rates of about 2–24% for donor **13a** and 1–5% for **5**. 4-Hydroxy-3-oxo-4-phenylbutanoate and 4-(2-fluorophenyl)-4-hydroxy-3-oxobutanoate formation is not detected by GC–MS, hence, **13a** must either be decarboxylated before condensation or afterwards. Higher conversion rates for incubation with **13a** argues for 3-oxo-propanoate addition first and decarboxylation afterwards. *R* configuration was determined for both products (**14a** and **b**) by chiral phase HPLC. No conversion could be observed applying 3-bromo-2-oxopropanoate, 2-oxo-3-phenylpropanoate or 2-(furan-2-yl)-2-oxoacetate as donors.

2.2. Regioselectivity by substrate variation

An interesting induction of regioselectivity by applying different substrate combinations was observed when different α-keto acids were tested for MenD-catalysed carboligation (Scheme 5). Carboligation of solely pyruvate shows traces of racemic acetoin detected using chiral GC. Incubation of α-ketoglutarate only with MenD resulted not in verifiable α-hydroxyketone formation. Incubation of pyruvate (**5**) with α-ketoglutarate **(3**), however, shows enzyme-catalysed product formation. The triphenyltetrazolium (TTC)-test for activity was used to get a first hint for formation

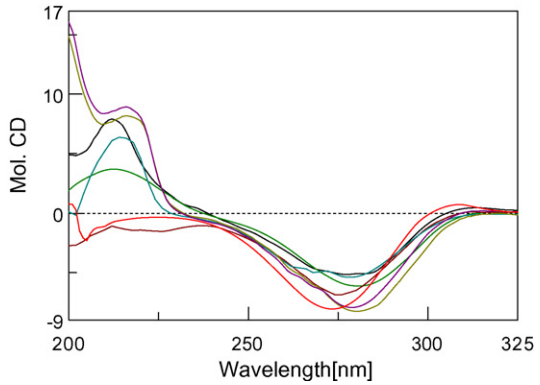
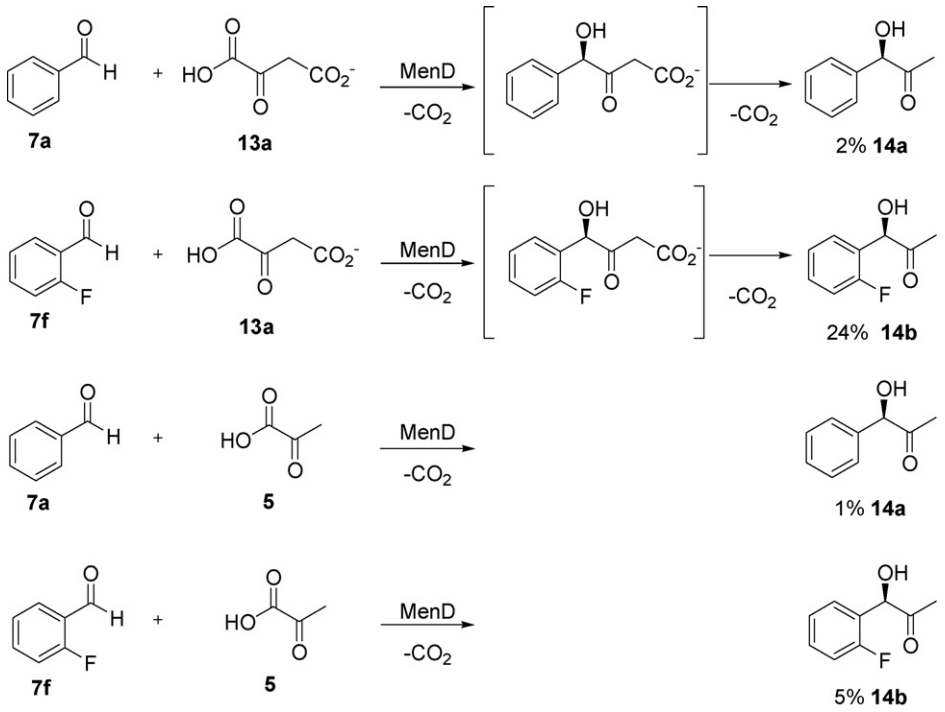


Fig. 1. CD spectra of **8a**, **8f**, **8i**, **10b**, **10c** and **12**. They were used to deduce the absolute configuration by comparison to that of *R*-phenylacetylcarbinol (PAC, **14a**).



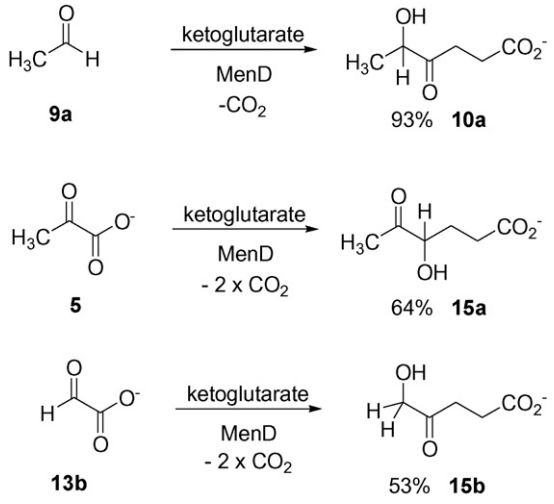
Scheme 4. Reactions regarding the donor substrate variation. Benzaldehyde (**7a**) and 2-fluorobenzaldeyde (**7f**) were chosen as acceptors. Conversion rates are denoted.

of α-hydroxyketones [19]. Surprisingly, the regioselectivity was inverted. The α-keto acid **3** changed its normal donor role and functions as acceptor substrate for acetaldehyde (decarboxylated **5**) resulting in 4-hydroxy-5-oxohexanoate (**15a**).

Dependence of regioselectivity on the substrates in enzymatic transformations was previously observed by Gocke et al. [20]. However, with acetaldehyde (**9a**) as the substrate, 5-hydroxy-4-oxohexanoate (**10a**) is isolated as product (see above). Applying glyoxylate (**13b**) the usual regioselectivity is observed as proven by ^{1}H NMR of achiral 5-hydroxy-4-oxopentanoate (**15b**). Determination of stereoselectivity by chiral phase GC, optical rotation and CD shows all condensation products (pyruvate or acetaldehyde as substrates) to be racemic. Optical rotation and CD measurements were performed with the free acid and the methyl carboxylate form of the products while for chiral GC the methylated forms were used only. Racemisation through methylation or GC analysis could thus be excluded. According to NMR high regioselectivity was observed (Fig. 2). The selective formation of regioisomers is assumed to be due to regioselective yet not stereoselective enzyme catalysis rather than by isomerisation of the α-hydroxyketone.

Mechanistically this effect is not understood. Allosteric binding of **5** might cause changes in enzyme conformation. In competition experiments containing **5**, **3** and **7a** only the condensation of **7a** and **3** was detected by GC–MS (results not shown).

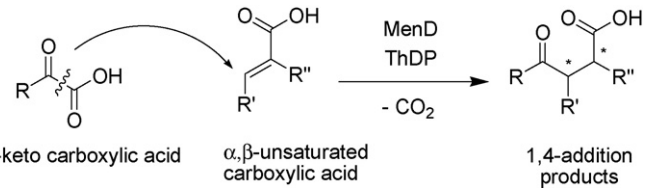
3-Bromo-2-oxopropanoate and 2-oxobutyrate were also tested for donor activity in combination with ketoglutarate as acceptor, however α-hydroxyketone formation was not observed according to NMR analysis.



Scheme 5. Induction of regioselectivity by applying different substrate combinations mediated by MenD catalysis.

2.3. 1,4-Addition products

We elucidated also the MenD-catalysed 1,4-addition of succinylsemialdehyde (decarboxylated **3**), to α,β-unsaturated carbonyl or carboxyl functionalities. Through this reaction chiral products with two potential new stereocenters can be formed (Scheme 6). Therefore, different α,β-unsaturated carboxylic acids were tested as substrates for carboligation activity with MenD (Scheme 7). Substances **16a**–**c** are commercially available, **16d** [21] and **16e** were synthesised, and **16f**–**h** were accessible through fermentation processes using *E. coli* strains developed in previous works [22,23].



Scheme 6. MenD-catalysed 1,4-addition of succinylsemialdehyde (decarboxylated **3**), to α,β-unsaturated carboxylic acid resulting in two potential new stereocenters (asterisks).

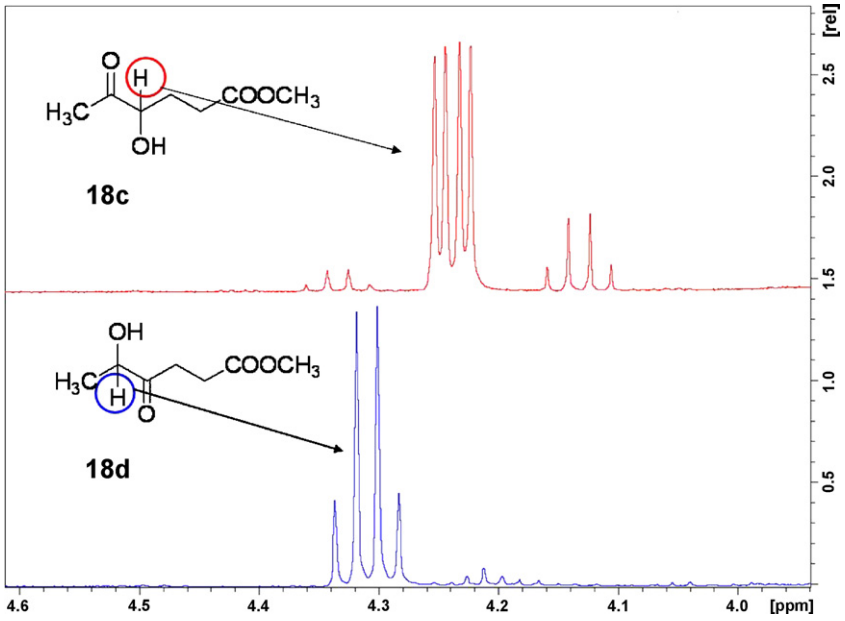
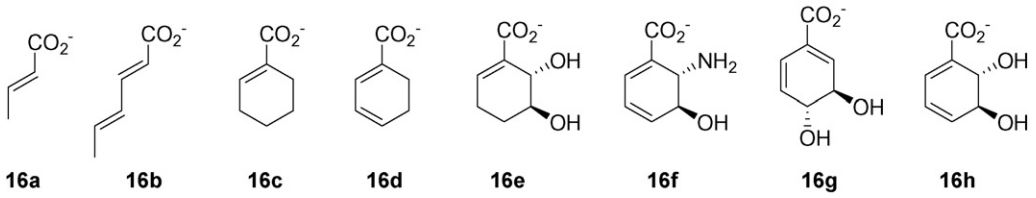


Fig. 2. Proton signals according to ^{1}H NMR revealed high regioselectivity of the methylated reaction products **18c** (1) and **18d** (2). The proton and the corresponding signal is coloured in red or blue, respectively. Applying pyruvate (**5**) and α-ketoglutarate (**3**), the latter takes the acceptor role and acetaldehyde (decarboxylated **5**) acts as donor, inverting the accustomed regioselectivity forming **15a**. **15a** is methylated by TMS-diazomethane to **18c** (1). Condensation of acetaldehyde (**9a**) and **3** led to the accustomed regioisomer **10a**. Methylation of **10a** led to formation of **18d** (2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)



Scheme 7. α,β-Unsaturated carboxylic acids tested as substrates for MenD-catalysed 1,4-addition of α-ketoglutarate (**3**).

The MenD-catalysed 1,4-transformations emerged to be very limited with respect to other substrates. Substances **16a–g** were not accepted at all within detection limits of NMR. Probably two double bonds in conjugation to the carboxylic acid are required for activity. Displacement like in **16g**, or absence of the second double bond (**16e**) interrupts conjugation to the hydroxyl moiety in position 3. Furthermore the replacement of the hydroxyl moiety in position 2 by an amino group (**16f**) led to no product formation. The only substrate accepted by MenD apart from the physiological substrate isochorismate was (2*S*,3*S*)-2,3-dihydroxy-2,3-dihydrobenzoate (2,3-CHD, **16h**) which lacks the pyruvyl function in position 3. This reaction was chosen as positive control for the other tested substrates. 66–83 $mU_{MenD1,4Lig}\,mg^{-1}$ protein was the calculated activity for the 1,4-addition applying 2,3-CHD. The acceptance of 2,3-CHD and the subsequent addition of succinylsemialdehyde supports the results of Guo et al. [6] that elimination of pyruvate is not essentially catalysed by MenD. Our results show in addition that the pyruvyl residue is not even required for catalytic activity. Unfortunately, chemical synthesis of (*R*)-5-hydroxycyclohexa-1,3-dienecarboxylate was not successful in our hands.

Reaction conditions for the condensation of α-ketoglutarate to 2,3-CHD (**16h**) were investigated regarding pH and buffer concentration. Highest conversion rates were obtained between pH 7 and pH 11 with a maximum at pH 8.5. The addition of NaCl in concentration varying from 50 to 200 mM had no influence on activity at pH 8. Also buffer concentration could be varied from 25 to 500 mM potassium phosphate without a significant effect on the conversion rate at pH 8. Performing this enzymatic synthesis using N-terminal or using C-terminal poly-histidine tagged MenD makes no difference on the rate of product formation. The first product formed through enzyme-catalysed addition of formal succinylsemialdehyde is (5*S*,6*S*)-2-succinyl-5,6-dihydroxycyclohex-3-enecarboxylate (**17a**). After 3 d isomerisation took place and the thermodynamically more stable product (5*S*,6*S*)-2-succinyl-5,6-dihydroxycyclohex-2-enecarboxylate (**17b**) is formed (Fig. 3). The products were obtained in almost pure form without further purification. The isomeric substances **17a** and **17b** are soluble in H_2O, however, neither DMSO nor methanol solves the compounds within the detection limit of NMR. To enable the use of the highly functionalised compounds **17a** or **17b** as building blocks a suitable method for derivatisation should be developed.

Applying MenD to **3** in presence of unsaturated ketones like (*E*)-non-3-en-2-one, (*E*)-4-phenylbut-3-en-2-one, 1-cyclohexenylethanone and unsaturated esters like methyl cyclohex-1-enecarboxylate showed no carboligation activity at all.

3. Experimental

3.1. Materials

All chemical reagents, solvents, buffer, salts, α-keto acids and aldehydes were obtained from Sigma Aldrich, Fluka or Acros and used without further purification, if not indicated.

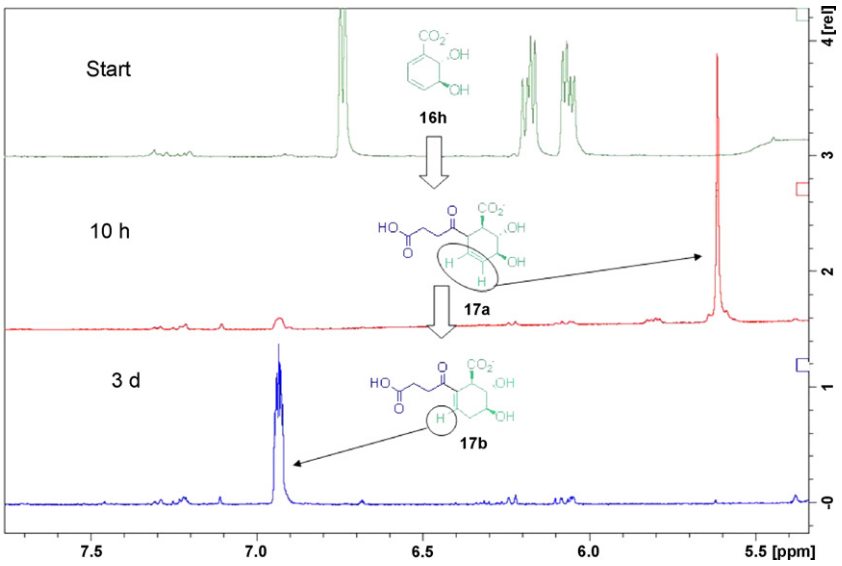


Fig. 3. NMR signals for monitoring the 1,4-addition of succinylsemialdehyde (decarboxylated **3**) to 2,3-CHD (**16h**). The first product formed through enzyme-catalysed addition is **17a** (signal shown in red). Within 3 d an isomerisation took place and led to the thermodynamically more stable product **17b** (signal coloured in blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

DNA-manipulating agents like restriction enzymes (XhoI, NdeI), T4 DNA Ligase, Phusion™ DNA Polymerase and High-Fidelity DNA Polymerase (HF) were purchased from New England Biolabs (NEB), the PfuTurbo® DNA Polymerase was obtained from Stratagene. For cell lysis Lysozyme from Fluka was used.

3.2. Analyses

High-performance liquid chromatography (HPLC-DAD) was carried out on a HP 1100 chromatography system (Agilent). Combination of HPLC-DAD with MS/MS (LC–MS) was performed using API2000 with Photospray™ or TurbolonSpray™ (Applied Biosystems). For determination of the enantiomeric excess the columns Chiralcel OD-H (Daicel Inc., 250 mm × 4 mm), Chiral OB (CS-Chromatographie-Service, 250 mm × 4 mm) and Chiral OM (CS-Chromatographie-Service, 250 mm × 4 mm) were used. MS and GC–MS analysis was carried out on the HP 6890N Series GC-system (EI, 70 eV) and the HP 5973 Network Mass Selective Detector (Agilent) using the column FS-Supreme-5 (CS-Chromatographie-Service), L = 30 m, diameter = 0.25 mm, film = 0.25 μm. As temperature gradient was used $T_{0\,min}$ = 60 °C, $T_{3\,min}$ = 60 °C, $T_{14\,min}$ = 280 °C, $T_{19\,min}$ = 280 °C. GC on chiral phase was performed on GC-2010 (FID) at 70 °C equipped with the injector AOC-20 (Shimadzu) and a FS-Lipodex D-column. Nuclear magnetic resonance (NMR) spectra were recorded at 24 °C on a DRX 400 (Bruker) operating at 400 and 100 MHz for ^{1}H and ^{13}C acquisitions, respectively. Chemical shifts (δ) of ^{1}H NMR and ^{13}C NMR spectra are reported in ppm with a solvent resonance as an internal standard. Circular dichroism (CD) was measured using a spectral polarimeter J-810 (Jasco International). Bradford was measured at 595 nm and optical density (OD) of bacterial cultivation was determined at 600 nm on the spectrophotometer UV mini – 1240 (Shimadzu). Optical rotation measurements were done on the polarimeter Modell 341 (PerkinElmer). ATR-IR-Spectra were taken on the infrarot-spectrometer Perkin-Elmer 1605 FT (P1065) (PerkinElmer).

3.3. Methods

3.3.1. Molecular cloning and gene expression

Standard molecular biology techniques were followed [24]. The *menD* gene from *E. coli* K12 was PCR-amplified using primers which introduced a NdeI restriction side at the 5′ end and a XhoI restriction side at the 3′ end. The oligodesoxynucleotides used in the gene amplification were *GGCATTCAT[ATGTCAGTAAGCGCATTTAAC] (menD forward), GGCATTCTCGAG[TAAATGGCTTACCTG CGCCAG] (menD reverse for pET-22b(+)) and GGCATTCTCGAG[TCATAAATGGCTTACCTG CGC] (menD reverse for pET-19b)*. After restriction digestion of *menD* containing amplified DNA and of the corresponding vectors, *menD* was inserted into a pET-22b(+) vector (Novagen) adding a C-terminal 6× histidine tag, and in addition into pET-19b (Novagen) tagging MenD at the N-terminus with an 10× histidine tag. The resulting constructs were full length sequenced by 4baselab to confirm in-frame cloning. The obtained plasmids were transformed in *E. coli* BL21(DE3) cells and grown in LB-medium with ampicillin (final c = 100 μg/mL). At an OD_{600} = 0.6 MenD expression was induced by adding IPTG (0.4 mM final concentration). After 12 h shaking at 120 rpm at 20 °C the cells were harvested and pelleted by centrifugation at 2000 rpm using 5804 R, rotor F-34-6-38 (Eppendorf).

3.3.2. Protein purification

The resuspended cell pellet containing lysozyme (1 mg/mL) was lysed by sonification on ice in a buffer volume of 5% of the culture volume. The lysis buffer (pH 8) consisted of 50 mM phosphate, 20 mM imidazole, 150 mM NaCl, 1 mM ThDP, 1 mM $MgCl_2 \cdot 6H_2O$. The debris was harvested through centrifugation at 4000 rpm. Purification of MenD as histidine-tagged protein was performed by IMAC (Immobilized metal affinity chromatography) [25].

25 mL supernatant was purified on Poly-prep chromatography columns (Biorad) packed with 2 mL Ni-NTA by gravity flow. The column was equilibrated with lysis buffer, the supernatant was added and the column was washed with washing buffer (pH 8) containing 50 mM phosphate, 50 mM imidazole, 150 mM NaCl, 1 mM ThDP, 1 mM $MgCl_2 \cdot 6H_2O$. The protein was subsequently eluted with elution buffer containing 300 mM imidazole. The single fractions were qualitatively screened in 96 well plates for protein by Bradford assay. Protein containing fractions were pooled and desalted by gel permeation chromatography using PD-10 Columns filled with Sephadex™ G-25M (GE Healthcare Amersham Biosciences). MenD could be stored at 4 °C for 4 weeks without significant loss of activity in desalting buffer (50 mM phosphate, 1 mM ThDP, 2 mM $MgCl_2 \cdot 6H_2O$, pH 8). The protein concentrations were determined

according to Bradford [26]. For calibration, bovine serum albumin was used. Following this purification protocol protein concentration between 2 and 4 mg/mL was obtained, consistent to a total amount of 35–70 mg MenD per 5 g cells.

3.4. Enzymatic synthesis

3.4.1. Reaction buffer

The reaction medium consisted of 50 mM phosphate, 2 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM ThDP, adjusted to pH 8. For in situ ^1H NMR experiments the reaction buffer contained 20% (v/v) D_2O. Depending on substrate solubility 20% (v/v) DMSO or 5% (v/v) MTBE were added as indicated.

3.4.2. MenD as catalyst

For all preparations purified C-terminal his-tagged MenD was used unless otherwise indicated. MenD was stored in desalting buffer. Protein concentration was adjusted by dilution of the protein solution with reaction buffer. Verification of MenD catalysing the C–C-bond forming was done by performing negative control experiments with the supernatants of pET-vectors expression without insert *menD*, crude and purified by IMAC.

3.4.3. TTC (2,3,5-triphenyltetrazolium)-assay for enzymatic activity of MenD

Activity was determined by detecting α-hydroxyketone formation [19]. Therefore 1.5 mL reaction buffer containing 20 mM acceptor substrate, 50 mM α-keto acid, 300 μL DMSO and 200 μg MenD at pH 8.0 was incubated for 12 h. 100 μL of the 1.5 mL preparation was filled in a micro-plate (0.5 mL, Greiner Bio-one) and the indicator reaction was started by adding 10 μL 2,3,5-triphenyltetrazoliumchloride (0.4% (v/v) in ethanol) and 30 μL 1 M NaOH. Control reactions were performed with 0.5 mM und 1 mM acetoin. After 2 h a significant red colour indicated α-hydroxyketone formation by MenD.

3.4.4. 1,2-Carboligase activity

The 1,2-carboligase activity was determined by the conversion rate of 20 mM benzaldehyde (**7a**) and 50 mM α-ketoglutarate (**3**) forming (*R*)-5-hydroxy-4-oxo-5-phenylpentanoate (**8a**). The substrates were dissolved in 1.5 mL reaction buffer containing 200 μg MenD, pH 8.0. After incubation at 30 °C and 300 rpm for 20 h the solution was acidified by 1% (v/v) formic acid and extracted with ethyl acetate. The calculation of the conversion rate of **7a** to (*R*)-5-hydroxy-4-oxo-5-phenylpentanoate (**8a**) was done using GC–MS analysis. 1 $U_{MenD1,2Lig}$ was thereby defined as 1 μmol **7a** which was converted by the enzyme at 30 °C in 1 min. The specific 1,2-carboligase-activity of MenD was determined to be 200–250 $mU_{MenD1,2Lig}\ mg^{-1}$ protein.

3.4.5. 1,4-Carboligase activity

The 1,4-carboligation was performed at 20 mM 2,3-CHD (**16h**) and 50 mM α-ketoglutarate (**3**) in reaction buffer. The appearance of the product 2-(3-carboxypropanoyl)-5,6-dihydroxycyclohex-3-enecarboxylate (**17a**) was monitored by ^1H NMR. This 1,4-addition reaction was carried out at 30 °C with 400 μg MenD in reaction buffer (20% (v/v) D_2O) for 12 h. For judging the extent of product formation the change of the integral ratio of 2,3-CHD to **17a** was determined by ^1H NMR. 1 $U_{MenD1,4Lig}$ was defined as 1 μmol **16h** which was converted by the enzyme at 30 °C in 1 min. The specific 1,4-carboligase-activity of MenD was determined to be 66–83 $mU_{MenD1,4Lig}\ mg^{-1}$ protein.

3.4.6. Enzymatic synthesis in analytical scale

MenD (70–120 μg/mL final) in 1.5 mL reaction buffer (20% (v/v) DMSO or 5% (v/v) MTBE as indicated) was incubated with 20 mM acceptor aldehydes as substrates and 50 mM donor substrate (**3**) at 30 °C and 300 rpm using a thermomixer (Eppendorf). After 16, 24 and 44 h 100 μL of the reaction mixture was extracted with 200 μL ethyl acetate (1% (v/v) formic acid) for GS–MS analysis. The GC chromatogram usually showed two product peaks. One of the peaks appeared as a lactone formed of the 4-oxopentanoyl moiety by dehydration. In most of the cases after 24 h no further conversion was detectable. For NMR analysis the complete preparation was extracted with 15 μL formic acid and 600 μL $CDCl_3$. The following compounds (conversion rate [%], GC–MS or NMR) were thus obtained.

*3.4.6.1. (R)-5-Hydroxy-5-(2-iodophenyl)-4-oxopentanoic acid (**8b**) (90%, GC–MS).* $C_{11}H_{11}IO_4$, M_r 333.97; GC–MS (EI): R_t = 8.64 min, *m/z* (%) 288 (1) $[M-CO_2, 2H]^+$, 231 (100) $[C_7H_4IO]^+$, 203 (17) $[C_6H_4I]^+$, 76 (36) $[C_6H_4]^+$, 51 (19) $[C_4H_3]^+$; $R_{t(lactone)}$ = 11.0 min, *m/z* (%) 231 (1) $[C_7H_4IO]^+$, 176 (38), 104 (100) $[C_7H_4O]^+$, 85 (12) $[C_4H_6O_2]^+$, 77 (28) $[C_6H_5]^+$, 51 (12) $[C_4H_3]^+$.

*3.4.6.2. (R)-5-Hydroxy-5-(2-chlorophenyl)-4-oxopentanoic acid (**8c**) (80%, GC–MS).* $C_{11}H_{11}ClO_4$, M_r 242.03 (100.0%), 244.03 (32.0%); GC–MS (EI): R_t = 9.1 min, *m/z* (%) 196 (1) $[M-CO_2, 2H]^+$, 141 (33) $[C_7H_4ClO]^+$, 139 (100) $[C_7H_4ClO]^+$, 113 (6) $[C_6H_4Cl]^+$, 111 (25) $[C_6H_4Cl]^+$, 75 (15) $[C_3H_7O_2]^+$, 57 (10) $[C_3H_5O]^+$, 50 (4) $[C_4H_2]^+$; $R_{t(lactone)}$ = 11.9 min, *m/z* (%) 224 (1) $[M]^+$, 141 (34) $[C_7H_4ClO]^+$, 139 (100) $[C_7H_4ClO]^+$, 113 (5) $[C_7H_4ClO]^+$, 111 (20) $[C_6H_4Cl]^+$, 85 (23) $[C_4H_6O_2]^+$, 75 (12) $[C_3H_7O_2]^+$, 50 (2) $[C_4H_2]^+$.

*3.4.6.3. (R)-5-Hydroxy-5-(2-bromophenyl)-4-oxopentanoic acid (**8d**) (65%, GC–MS).* $C_{11}H_{11}BrO_4$, M_r 285.98 (100.0%), 287.98 (97.3%); GC–MS (EI): R_t = 9.7 min, *m/z* (%) 240 (1) $[M-CO_2, 2H]^+$, 185 (100) $[C_7H_4BrO]^+$, 183 (100) $[C_7H_4BrO]^+$, 157 (27) $[C_6H_4Br]^+$, 155 (25) $[C_6H_4Br]^+$, 76 (20) $[C_6H_4]^+$, 57 (18) $[C_3H_5O]^+$, 50 (9) $[C_4H_2]^+$; $R_{t(lactone)}$ = 11.9 min, *m/z* (%) 185 (100) $[C_7H_4BrO]^+$, 183 (80) $[C_7H_4BrO]^+$, 157 (12) $[C_6H_4Br]^+$, 155 (16) $[C_6H_4Br]^+$, 85 (52) $[C_4H_6O_2]^+$, 77 (36) $[C_6H_5]^+$, 56 (12) $[C_4H_8]^+$.

*3.4.6.4. (R)-5-Hydroxy-5-(2-methylphenyl)-4-oxopentanoic acid (**8e**) (12%, GC–MS).* $C_{12}H_{14}O_4$, M_r 222.09; GC–MS (EI): R_t = 8.7 min, *m/z* (%) 176 (1) $[M-CO_2, 2H]^+$, 119 (100) $[C_8H_7O]^+$, 91 (45) $[C_7H_7]^+$, 65 (11) $[C_5H_5]^+$, 57 (2) $[C_3H_5O]^+$; $R_{t(lactone)}$ = 11.6 min, *m/z* (%) 119 (100) $[C_8H_7O]^+$, 91 (36) $[C_7H_7]^+$, 85 (7) $[C_4H_6O_2]^+$, 65 (9) $[C_5H_5]^+$.

*3.4.6.5. (R)-5-Hydroxy-5-(3-fluorophenyl)-4-oxopentanoic acid (**8g**) (88%, GC–MS).* $C_{11}H_{11}FO_4$, M_r 226.06; GC–MS (EI): R_t = 7.8 min, *m/z* (%) 180 (4) $[M-CO_2, 2H]^+$, 123 (100) $[C_7H_4FO]^+$, 95 (42) $[C_6H_4F]^+$, 75 (13) $[C_3H_7O]^+$, 57 (16) $[C_3H_5O]^+$; $R_{t(lactone)}$ = 11.0 min, *m/z* (%) 208 (3) $[M]^+$, 123 (100) $[C_7H_4FO]^+$, 95 (31) $[C_6H_4F]^+$, 85 (38) $[C_4H_4O_2]^+$, 75 (9) $[C_3H_7O]^+$, 57 (3) $[C_3H_5O]^+$.

*3.4.6.6. (R)-5-Hydroxy-5-(3-methoxyphenyl)-4-oxopentanoic acid (**8h**) (50%, GC–MS).* $C_{12}H_{14}O_5$, M_r 238.08; GC–MS (EI): R_t = 9.7 min, *m/z* (%) 192 (7) $[M-CO_2, 2H]^+$, 135 (100) $[C_8H_7O_2]^+$, 107 (25) $[C_7H_7O]^+$, 92 (14) $[C_6H_4O]^+$, 77 (18) $[C_6H_5]^+$, 57 (2) $[C_3H_5O]^+$; $R_{t(lactone)}$ = 12.4 min, *m/z* (%) 220 (7) $[M]^+$, 135 (100) $[C_8H_7O_2]^+$, 107 (16) $[C_7H_7O]^+$, 92 (6) $[C_6H_4O]^+$, 85 (5) $[C_4H_4O_2]^+$, 77 (9) $[C_6H_5]^+$, 57 (1) $[C_3H_5O]^+$.

*3.4.6.7. (R)-5-Hydroxy-5-(4-fluorophenyl)-4-oxopentanoic acid (**8k**) (>99%, GC–MS).* $C_{11}H_{11}FO_4$, M_r 226.06; GC–MS (EI): R_t = 8.0 min, *m/z* (%) 180 (5) $[M-CO_2, 2H]^+$, 123 (100) $[C_7H_4FO]^+$, 95 (38) $[C_6H_4F]^+$, 75 (16) $[C_3H_7O]^+$, 57 (16) $[C_3H_5O]^+$; $R_{t(lactone)}$ = 11.0 min, *m/z* (%) 208 (1) $[M]^+$, 123 (100) $[C_7H_4FO]^+$, 95 (13) $[C_6H_4F]^+$, 85 (20) $[C_4H_4O_2]^+$, 75 (5) $[C_3H_7O]^+$, 57 (2) $[C_3H_5O]^+$.

*3.4.6.8. (R)-5-Hydroxy-5-(4-chlorophenyl)-4-oxopentanoic acid (**8l**) (>99%, GC–MS).* $C_{11}H_{11}ClO_4$, M_r 242.03 (100.0%), 244.03 (32.0%); GC–MS (EI): R_t = 9.2 min, *m/z* (%) 196 (2) $[M-CO_2, 2H]^+$, 141 (32) $[C_7H_4ClO]^+$, 139 (100) $[C_7H_4ClO]^+$, 113 (10) $[C_6H_4Cl]^+$, 111 (30) $[C_6H_4Cl]^+$, 75 (15) $[C_3H_7O_2]^+$, 57 (10) $[C_3H_5O]^+$, 50 (3) $[C_4H_2]^+$; $R_{t(lactone)}$ = 12.5 min, *m/z* (%) 224 (1) $[M]^+$, 141 (30) $[C_7H_4ClO]^+$, 139 (100) $[C_7H_4ClO]^+$, 113 (8) $[C_6H_4Cl]^+$, 111 (30) $[C_6H_4Cl]^+$, 85 (32) $[C_4H_4O_2]^+$, 75 (15) $[C_3H_7O_2]^+$, 57 (2) $[C_3H_5O]^+$, 50 (7) $[C_4H_2]^+$; ^{1}H NMR ($CDCl_3$): δ = 2.56–2.62 (m, 2H, CH_xH$_y$CO, CH_xH$_y$CO$_2$H), 2.71–2.75 (m, 2H, CH$_x$H$_y$CO, CH$_x$H$_y$CO$_2$H), 4.21 (br s, 1H, CHO*H*), 5.16 (s, 1H, C*H*OH), 7.30 (d, *J* = 8.5 Hz, 2H, CH$_{m\text{-Ar}}$), 7.39 ppm (d, *J* = 8.5 Hz, 2H, CH$_{o\text{-Ar}}$). ^{13}C NMR ($CDCl_3$): δ = 27.4 (CO_2HCH_2), 32.3 ($COCH_2$), 79.0 (CHOH), 128.7 (2× C$_{Ar}$), 129.2 (2× C$_{Ar}$), 134.8 (CCl), 136.2 (C$_q$), 176.7 (CO_2H), 207.2 ppm (CO).

*3.4.6.9. (R)-5-Hydroxy-5-(4-hydroxyphenyl)-4-oxopentanoic acid (**8m**) (66%, NMR).* $C_{11}H_{12}O_5$, M_r 224.07; MS (-Q1 scan, TurbolonSpray^{TM} source, ESI): *m/z* (%) 223 (32) $[M-H^+]^-$, 205 (24) $[M-H_2O, H^+]^-$, 161 (100) $[M-CO_2, H_2O, H^+]^-$, 133 (53) $[M-CO_2, H_2O, C_2H_2, H^+]^-$, 121 (18), 93 (62) $[C_6H_5O]^-$, 65 (10); ^{1}H NMR (MeOH-d_4): δ = 1.88–1.98 (m, 1H, CH_xH$_y$CO$_2$H), 2.08–2.26 (m, 1H, CH$_x$H$_y$CO$_2$H), 2.08–2.26 (m, 1H, CH_xH$_y$CO), 2.73–2.82 (m, 1H, CH$_x$H$_y$CO), 4.82 (s, 1H, C*H*COH), 6.71 (d, *J* = 8.4 Hz, 2H, CH$_{m\text{-Ar}}$), 7.15 ppm (d, *J* = 8.4 Hz, 2H, CH$_{o\text{-Ar}}$).

*3.4.6.10. (R)-5-(2,3-Dihydroxyphenyl)-5-hydroxy-4-oxopentanoic acid (**8n**) (45%, GC–MS).* $C_{11}H_{12}O_6$, M_r 240.06; MS (-Q1 scan, TurbolonSpray^{TM} source, ESI): *m/z* 239 $[M-H^+]^-$, 221 $[M-H_2O, H^+]^-$, 177 $[M-CO_2, -H_2O, H^+]^-$, 137 $[C_7H_5O_3]^-$, 91 $[C_7H_7]^-$.

*3.4.6.11. (R)-5-(3,4-Dihydroxyphenyl)-5-hydroxy-4-oxopentanoic acid (**8o**) (53%, NMR).* $C_{11}H_{12}O_6$, M_r 240.06; MS (-Q1 scan, TurbolonSpray^{TM} source, ESI): *m/z* 239 $[M-H^+]^-$, 221 $[M-H_2O, H^+]^-$, 177 $[M-CO_2, -H_2O, H^+]^-$, 137 $[C_7H_5O_3]^-$, 91 $[C_7H_7]^-$; ^{1}H NMR (DMSO-d_6): δ = 2.31 (dt, *J* = 6.7, 2.1 Hz, 2H, CH_2CO$_2$H), 2.66 (t, *J* = 6.7 Hz, 2H, CH$_2$CO), 4.88 (d, *J* = 4.3 Hz, 1H, C*H*OH), 5.71 (d, *J* = 4.3 Hz, 1H, CHO*H*), 6.62 (dd, *J* = 8.0, 1.8 Hz, 1H, CH$_{Ar}$), 6.69 (d, *J* = 8.0 Hz, 1H, CH$_{Ar}$), 6.73 ppm (d, *J* = 1.2 Hz, 1H, CH$_{Ar}$).

3.4.7. Enzymatic synthesis in semi-preparative scale

MenD (60 μg/mL final) was incubated in 10–50 mL of reaction buffer with 10–20 mM acceptor substrates (aldehydes or α,β-unsaturated aldehydes) and 40–50 mM donor substrate (ketoglutarate) at 30 °C in a batch reactor, equipped with a magnetic stirrer. The vessel was sealed by a septum and put under nitrogen atmosphere. After 16, 24 and 42 h 100 μL of the reaction mixture was extracted with 200 μL ethyl acetate (1% (v/v) formic acid) for GS–MS analysis. After 42 h the mixture was extracted with ethyl acetate (3× batch volume). The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. Purification by flash chromatography was performed if indicated. The following products were thus obtained (yield, *ee* if determined).

3.4.8. Aromatic aldehydes as acceptors

*3.4.8.1. (R)-5-Hydroxy-4-oxo-5-phenylpentanoic acid (**8a**) (65% isolated yield, yellow oil, ee = 94%).* $C_{11}H_{12}O_4$, M_r 208.07; GC–MS (EI): R_t = 8.1 min, *m/z* (%) 162 (2) $[M-CO_2, 2H]^+$, 105 (100) $[C_7H_5O]^+$, 77 (48) $[C_6H_5]^+$, 51 (12) $[C_4H_3]^+$; $R_{t(lactone)}$ = 11.3 min, *m/z* (%) 190 (1) $[M]^+$, 105 (100) $[C_7H_5O]^+$, 85 (12) $[C_4H_6O_2]^+$, 77 (28) $[C_6H_5]^+$, 51 (7) $[C_4H_3]^+$; MS (-Q1 scan, TurbolonSpray^{TM} source, ESI): *m/z* (%) 207 $[M-H^+]^-$, 189 $[M-H_2O, H^+]^-$, 161 $[C_{10}H_9O_2]^-$, 145 $[C_{10}H_{10}O]^-$; HPLC-DAD (Chiralcel OM, 25 °C, 0.75 mL min^{-1}, isohexane/2-propanol = 90:10), R_t = 29.8 min (*S*-enantiomer), R_t = 33.3 min (*R*-enantiomer, main product); IR: $\tilde{\nu}$ = 3436 (O–H), 3153 (COO–H), 2905 (C–H), 1710 (C=O), 1345, 1216 (C–O), 1163, 1015 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ = 2.47–2.58 (m, 2H, CH_xH$_y$CO, CH_xH$_y$CO$_2$H)), 2.65–2.78 (m, 2H, CH$_x$H$_y$CO, CH$_x$H$_y$CO$_2$H), 5.17 (s, 1H, C*H*OH), 7.32–7.42 ppm (m, 5H, CH$_{Ar}$); ^{1}H NMR (DMSO): δ = 2.36 (t, *J* = 6.6 Hz, 2H, CH_2CO$_2$H), 2.74 (t, *J* = 6.6 Hz, 2H, CH$_2$CO), 5.08 (s, 1H, C*H*OH), 5.90 (br s, 1H, CHO*H*), 7.27–7.39 ppm (m, 5H, CH$_{Ar}$); ^{13}C NMR ($CDCl_3$): δ = 27.7 (*C*H$_2$CO$_2$H), 32.4 (*C*H$_2$CO), 79.7 (*C*HOH), 127.4 (2× C$_{m\text{-Ar}}$), 128.8 (C$_{Ar}$), 129.0 (2× C$_{o\text{-Ar}}$), 137.6 (Cq), 177.8 (CO_2H), 207.8 ppm (CO); optical rotation: $[\alpha]_D^{22} = -112$ (*c* = 0.86 g/100 mL in methanol); CD: λ (nm) (Δε) (CH$_3$CN) = 216 (48.2), 279 (−22.3), 315 (1.3).

*3.4.8.2. (R)-5-Hydroxy-5-(2-fluorophenyl)-4-oxopentanoic acid (**8f**) (87% isolated yield, yellow oil, ee = 95%).* $C_{11}H_{11}FO_4$, M_r 226.06; GC–MS (EI): R_t = 8.0 min, *m/z* (%) 180 (7) $[M-CO_2, 2H]^+$, 123 (100) $[C_7H_4FO]^+$, 95 (50) $[C_6H_4F]^+$, 75 (22) $[C_3H_7O]^+$, 57 (23) $[C_3H_5O]^+$; $R_{t(lactone)}$ = 11.1 min, *m/z* (%) 208 (1) $[M]^+$, 123 (100) $[C_7H_4FO]^+$, 95 (18) $[C_6H_4F]^+$, 85 (27) $[C_4H_4O_2]^+$, 75 (8); HPLC-DAD (Chiralcel OM, 25 °C, 0.75 mL min^{-1}, isohexane/2-propanol = 90:10) R_t = 23.4 min (*S*-enantiomer), R_t = 24.9 min (*R*-enantiomer, main product); ^{1}H NMR ($CDCl_3$): δ = 2.48–2.63 (m, 2H, CH_xH$_y$CO, CH_xH$_y$CO$_2$H), 2.65–2.80 (m, 2H, CH$_x$H$_y$CO CH$_x$H$_y$CO$_2$H), 5.45 (s, 1H, C*H*OH), 7.07–7.17 (m, 2H, CH$_{Ar}$), 7.27–7.37 ppm (m, 2H, CH$_{Ar}$); ^{13}C NMR ($CDCl_3$): δ = 27.6 (*C*H$_2$CO$_2$H), 32.1 (d, $J_{C\text{-}F}$ = 2.6 Hz, *C*H$_2$CO), 73.4 (d, $J_{C\text{-}F}$ = 3.6 Hz, CHOH), 115.9 (d, $^2J_{C\text{-}F}$ = 21 Hz, C$_{m\text{-Ar}}$), 124.8 (d, $^4J_{C\text{-}F}$ = 3.8 Hz, C$_{m\text{-Ar}}$), 125.0 (d, $^2J_{C\text{-}F}$ = 13.5 Hz, C$_q$), 129.0 ($^3J_{C\text{-}F}$ = 3.2 Hz, C$_{p\text{-Ar}}$), 130.5 ($^3J_{C\text{-}F}$ = 8.3 Hz, C$_{o\text{-Ar}}$), 160.4 ($^1J_{C\text{-}F}$ = 249 Hz, CF), 177.9 (CO_2H), 206.9 ppm (CO); optical rotation: $[\alpha]_D^{22} = -98.2$ (*c* = 0.85 g/100 mL in methanol); CD: λ (nm) (Δε) (CH$_3$CN) = 208$_{min}$ (15.9), 214$_{max}$ (19.7), 278 (−21.9).

*3.4.8.3. (R)-5-Hydroxy-5-(3-iodophenyl)-4-oxopentanoic acid (**8i**) (79% isolated yield, yellow oil).* $C_{11}H_{11}IO_4$, M_r 333.96; GC–MS (EI): R_t = 10.6 min, *m/z* (%) 288 (9) $[M-CO_2, 2H]^+$, 231 (100) $[C_7H_4IO]^+$, 203 (20) $[C_6H_4I]^+$, 76 (21) $[C_6H_4]^+$, 50 (6) $[C_4H_2]^+$; $R_{t(lactone)}$ = 13.4 min, *m/z* (%) 288 (1) $[M-CO]^+$, 231 (100) $[C_7H_4IO]^+$, 203 (26) $[C_6H_4I]^+$, 85 (26) $[C_4H_6O_2]^+$, 76 (17) $[C_6H_4]^+$, 50 (6) $[C_4H_2]^+$; IR: $\tilde{\nu}$ = 3392 (O–H), 3054 (COO–H), 2921 (C–H), 1707 (C=O), 1566, 1188 (C–O), 1063 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ = 2.48–2.62 (m, 2H, CH_xH$_y$CO, CH_xH$_y$CO$_2$H), 2.66–2.76 (m, 2H, CH$_x$H$_y$CO, CH$_x$H$_y$CO$_2$H), 5.11 (s, 1H, C*H*OH), 7.13 (t, *J* = 7.8 Hz, 1H, CH$_{m\text{-Ar}}$), 7.31 (d, *J* = 7.8 Hz, 1H, CH$_{o\text{-Ar}}$), 7.70 (d, *J* = 7.8 Hz, 1H, CH$_{p\text{-Ar}}$), 7.71 ppm (s, 1H, CH$_{o\text{-Ar}}$); ^{13}C NMR ($CDCl_3$): δ = 27.7 (*C*H$_2$CO$_2$H), 32.4 (*C*H$_2$CO), 78.8 (*C*HOH), 94.8 (CI), 126.7 (C$_{Ar}$), 130.8 (C$_{Ar}$), 136.4 (C$_{Ar}$), 138.0 (C$_{Ar}$), 139.9 (C$_q$), 177.7 (CO_2H), 207.2 ppm (CO); optical rotation: $[\alpha]_D^{22} = -76.7$ (*c* = 0.72 g/100 mL in methanol); CD: λ (nm) (Δε) (CH$_3$CN) = 212 (52.3), 277 (−31.35), 314 (2.3).

*3.4.9. Aliphatic aldehydes as acceptors (acetaldehyde (**9a**), hexanal (**9b**), undec-10-enal (**9c**))*

*3.4.9.1. rac-5-Hydroxy-4-oxohexanoic acid (**10a**) (93% crude product yield, pale yellow oil).* $C_6H_{10}O_4$, M_r 146.06; ^{1}H NMR (MeOH-d_4): δ = 1.35 (d, *J* = 7.0 Hz, 3H, CH$_3$), 2.59 (t, 2H, *J* = 6.4 Hz, CH_2CO$_2$H), 2.87 (t, 2H, *J* = 6.4 Hz, COCH$_2$), 4.25 ppm (q, *J* = 7.0 Hz, 1H, C*H*OH). ^{1}H NMR (DMSO-d_6): δ = 1.18 (d, 3H, *J* = 7 Hz), 2.38–2.44 (m, 2H, CH_2CO$_2$H), 2.75–2.81 (m, 2H, CH$_2$CO) 4.05 ppm (q, 1H, *J* = 7 Hz); Optical rotation and CD indicate racemic product formation.

*3.4.9.2. (R)-5-Hydroxy-4-oxodecanoic acid (**10b**) (55% isolated yield, colourless oil, ee n.d.).* $C_{10}H_{18}O_4$, M_r 202.12; ^{1}H NMR (MeOH-d_4): δ = 0.94 (t, *J* = 6.9 Hz, 3H, CH$_3$), 1.34–1.38 (m, 6H, CH$_3$CH_2CH_2CH_2), 1.58–1.65 (m, 1H, CH_xH$_y$CHOH), 1.72–1.81 (m, 1H, CH$_x$H$_y$CHOH), 2.56–2.60 (m, 2H, CH_2CO$_2$H), 2.84–2.88 (m, 2H, CH$_2$CO), 4.12 ppm (dd, *J* = 4.3, 8.2 Hz, 1H, C*H*OH); ^{13}C NMR (MeOH-d_4): δ = 12.9 (CH$_3$), 22.1 (CH$_3$*C*H$_2$), 24.4 (*C*H$_2$CH$_2$CHOH), 26.9 (*C*H$_2$CO$_2$H), 31.4 (CH$_3$CH$_2$*C*H$_2$), 32.4 (*C*H$_2$CO), 33.1(*C*H$_2$CHOH), 76.6 (CH$_2$*C*HOH), 174.9 (CO_2H), 212.3 ppm (CO); optical rotation: $[\alpha]_D^{22} = -21$

(c = 0.50 g/100 mL in methanol); CD: λ (nm) ($\Delta\varepsilon$) (CH_3CN) = 274 (−2.95).

*3.4.9.3. (R)-5-Hydroxy-4-oxopentadec-14-enoic acid (**10c**) (60% conversion (NMR), yellow oil, ee n.d.).* $C_{15}H_{26}O_4$, M_r 270.18; GC–MS (EI): R_t = 10.0 min, m/z (%) 167 (16) $[C_{11}H_{19}O]^+$, 149 (71) $[C_{11}H_{17}]^+$, 121 (5) $[C_9H_{13}]^+$, 107 (22) $[C_8H_{11}]^+$, 93 (18) $[C_7H_9]^+$, 83 (49) $[C_6H_{11}]^+$, 55 (100) $[C_4H_7]^+$; $R_{t(lactone)}$ = 12.6 min, m/z (%) 167 (18) $[C_{11}H_{19}O]^+$, 149 (67) $[C_{11}H_{17}]^+$, 107 (16) $[C_8H_{11}]^+$, 93 (13) $[C_7H_9]^+$, 85 (100) $[C_4H_4O_2]^+$, 55 (71) $[C_4H_7]^+$; ^{1}H NMR ($CDCl_3$): δ = 1.19–1.50 (m, 8H, CH_2), 1.58–1.65 (m, 1H, CH_xH_yCHOH), 1.80–1.91 (m, 1H, CH_xH_yCHOH), 2.01–2.09 (q, 2H, J = 6.9 Hz, $CH_2CH_2CH_{olef}$), 2.70–2.86 (m, 4H, $CH_2CH_2CO_2H$), 4.26 (dd, 1H, J = 3.8 Hz, $CHOH$), 4.97–5.00 (m, 1H, $CH_xH_yCH_{olef}$), 5.02–5.04 (m, 1H, $CH_xH_yCH_{olef}$), 5.77–5.88 ppm (m, 1H, CH_2CH_{olef}); ^{13}C NMR ($CDCl_3$): δ = 24.7, 27.6 28.8, 29.0, 29.1, 29.2, 29.3, 32.3, 33.5, 33.7 (CH_2), 76.5 (CHOH), 114.1 (CH_{2olef}), 139.1 (CH_{2olef}), 177.7 (CO_2H), 203.2 ppm (CO); optical rotation: $[\alpha]_D^{22} = -50$ (c = 0.63 g/100 mL in methanol); CD: λ (nm) ($\Delta\varepsilon$) (CH_3CN) = 27_{min} (−21.8), 309_{max} (2.0).

*3.4.10. 1-Cyclohexene-1-carbaldehyde (**11**) as acceptor*

*3.4.10.1. (R)-5-Cyclohexenyl-5-hydroxy-4-oxopentanoic acid (**12**) (21% isolated yield, yellow oil).* $C_{11}H_{16}O_4$, M_r 212.1; GC–MS (EI): R_t = 7.6 min, m/z (%) 168 (2) $[M-CO_2]^+$, 111 (24) $[C_7H_{11}O]^+$, 83 (100) $[C_6H_{11}]^+$, 55 (44) $[C_3H_3O]^+$; $R_{t(lactone)}$ = 11.5 min, m/z (%) 109 (100) $[C_7H_9O]^+$, 81 (45) $[C_6H_9]^+$, 53 (7) $[C_4H_5]^+$; MS (-Q1 scan, TurbolonSpray™ source, ESI): m/z (%) 211 $[M-H^+]^-$, 167 $[M-CO_2, H^+]^-$; ^{1}H NMR ($CDCl_3$): δ = 1.51–1.67 (m, 5H, 2× CH_2, 1× $CH_xH_yC_q$), 2.07–2.15 (m, 3H, $CH_{olef}CH_2$, $CH_xH_yC_q$), 2.69–2.82 (m, 4H, $COCH_2CH_2CO_2H$), 4.57 (s, 1H, $CHOH$), 5.96–5.97 ppm (m, 1H, CH_{olef}); ^{13}C NMR ($CDCl_3$): δ = 22.1, 22.2 (CH_2), 22.8 ($CH_2C_{olef}H$), 25.3 (C_qCH_2), 27.7 (CH_2CO_2H), 32.1 (CH_2CO), 82.3 (CHOH), 129.9 (CH_{olef}), 135.2 (C_q), 177.7 (CO_2H), 207.2 ppm (CO); optical rotation: $[\alpha]_D^{22} = -111$ (c = 0.15 g/100 mL in methanol); CD: λ (nm) ($\Delta\varepsilon$) (CH_3CN) = 213 (5.3), 281 (−8.3), 319 (0.1).

*3.4.11. α-Hydroxyketones by condensation of pyruvate (**5**) and glyoxylate (**13b**), respectively, with α-ketoglutarate (**3**)*

*3.4.11.1. rac-4-Hydroxy-5-oxohexanoic acid (**15a**) (64% crude product yield, yellow oil).* $C_6H_{10}O_4$, M_r 146.06; ^{1}H NMR (DMSO-d_6): δ = 2.12 (s, 3H, $COCH_3$), 2.25–2.30 (m, 2H, CH_2), 2.47–2.52 (m, 2H, CH_2), 3.50 (br s, CHO*H*), 3.89 (q, J = 4.5 Hz, 1H, C*H*OH), 12.12 ppm (br s, 1H, CO_2H); Optical rotation and CD indicate racemic product formation.

*3.4.11.2. 5-Hydroxy-4-oxopentanoic acid (**15b**) (53% crude product yield, colourless oil).* $C_5H_8O_4$, M_r 132.04; ^{1}H NMR (DMSO-d_6): δ = 2.43 (t, J = 6.5 Hz, 2H, CH_2CO_2H), 2.63 (t, J = 6.5 Hz, 2H, $COCH_2$), 4.06 (s, 2H, CH_2OH, 5.15 ppm (br s, 1H, CH_2OH); ^{13}C NMR (DMSO-d_6): δ = 27.7 (CH_2CO_2H), 33.1 (CH_2CO), 67.8 (CH_2OH), 174.1 (CO_2H), 210.4 ppm (CO).

3.4.12. Phenyacetylcarbinol (PAC) derivatives

(R)-*Phenylacetylcarbinol* ((R)-*PAC*) (**14a**) (1% conversion (GC–MS) for incubation of benzaldehyde and pyruvate, 2% conversion (GC–MS) for incubation of benzaldehyde and oxaloacetate). $C_9H_{10}O_2$, M_r 150.07; GC–MS (EI): R_t = 7.7 min, m/z (%) 150 (2) $[M]^+$, 122 (100) $[M-CO]^+$, 107 (41) $[C_7H_7O]^+$, 77 (96) $[C_6H_5]^+$; HPLC-DAD (Chiralcel OM, 25 °C, 0.75 mL min^{-1}, isohexane/2-propanol = 90:10) R_t = 11.2 min (*S*-enantiomer), R_t = 13.3 min (*R*-enantiomer, main product), configuration determined by authenticated sample; *ee* n.d.

(R)-*2-Fluorophenylacetylcarbinol* ((R)-*2-Fluoro-PAC*) (**14b**) (5% conversion (GC–MS) for incubation of 2-fluorobenzaldehyde and pyruvate, 24% conversion (GC–MS) for incubation of benzaldehyde and oxaloacetate). $C_9H_9O_2F$, M_r 168.06; GC–MS (EI): R_t = 7.6 min, m/z (%) 168 (2) $[M]^+$, 140 (38) $[M-CO]^+$, 125 (100) $[C_7H_6FO]^+$, 97 (53) $[C_6H_6F]^+$, 77 (31) $[C_6H_5]^+$, 51 (11) $[C_4H_3]^+$; HPLC-DAD (Chiralcel OM, 25 °C, 0.75 mL min^{-1}, isohexane/2-propanol = 90:10) R_t = 10.4 min (*S*-enantiomer), R_t = 12.6 min (*R*-enantiomer, main product), configuration determined by authenticated sample; *ee* n.d.

3.4.13. 1,4-Addition product

*3.4.13.1. (5S,6S)-2-succinyl-5,6-dihydroxycyclohex-2-enecarboxylic acid (**17b**).* $C_{11}H_{14}O_7$, M_r 258.07; ^{1}H NMR (D_2O): δ = 2.36–2.48 (m, 3H, CH_2CO_2H, $CH_{olef}CH_xH_y$), 2.70–2.83 (m, 1H, $CH_{olef}CH_xH_y$), 2.88 (dt, J = 6.4, 17.9 Hz, $COCH_xH_y$), 3.14 (dt, J = 7.1, 17.9 Hz, $COCH_xH_y$), 3.18–3.23 (d, J = 7.3 Hz, 1H, $CHCO_2H$), 3.77–3.80 (m, 2H, $CHOH$), 7.09 ppm (t, J = 2.6 Hz, 1H, CH_{olef}); ^{13}C NMR (D_2O): δ = 34.2 (CH_2CO_2H), 35.2 (CH_2CH_{olef}), 36.2 (CH_2CO), 55.3 ($CHCO_2H$), 71.5 (CH_2CHOH), 76.6 ($CHCHOH$), 139.5 (C_q), 142.7 (CH_{olef}), 183.5 ($CHCO_2H$), 184.5 (CH_2CO_2H), 205.7 ppm (CO).

3.5. Synthesis of substrates for putative 1,4-addition

*3.5.1. Cyclohexa-1,3-dienecarboxylic acid (**16d**)*

Acrylic acid (7.6 mL, 8.0 g, 140 mmol) and 1-acetoxy-1,3-butadiene (10.4 mL, 10.0 g, 90 mmol) was dissolved in 55 mL toluene. 1,4-Dihydroquinone (0.31 g, 2.7 mmol) was added and the mixture was refluxed for 19 h. After addition of 55 mL ethyl acetate the organic layer was washed with brine and dried over $MgSO_4$. Purification by flash chromatography on silica gel using cyclohexane/ethyl acetate (4:1 (v/v)) with 1% (v/v) formic acid resulted in 2-acetoxycyclohex-3-enecarboxylic acid (4.38 g, 23 mmol) as a brown solid with a yield of 25% [21]. ^{1}H NMR ($CDCl_3$): δ = 1.77–1.98 (m, 1H, CH_2), 2.03 (s, 3H, CH_3), 2.04–2.08 (m, 1H, CH_2), 2.08–2.21 (m, 1H, CH_2), 2.22–2.33 (m, 1H, CH_2), 2.69 (dt, J = 3.5, 12.8 Hz, 1H, $CHCO_2H$), 5.56 (t, J = 4.3 Hz, 1H, CHOCO), 5.93 (m, 1H, CH), 6.03 (m, 1H, CH), 10.16 ppm (s, 1H, CO_2H).

2-Acetoxycyclohex-3-enecarboxylic acid (223 mg, 1.8 mmol) was dissolved 1 mL H_2O. 500 μL H_2SO_4 was added dropwise. After 1.5 h at 40 °C the mixture was extracted with ethyl acetate (3 × 5 mL). The combined organic phases were dried ($NaSO_4$) and the solvent was evaporated under reduced pressure. After purification by column chromatography on silica gel using cyclohexane/ethyl acetate (7:3 (v/v), 1% (v/v) formic acid) as eluent **16d** was obtained with a yield of 19.5% (43.5 mg, 0.35 mmol) as yellow oil. $C_7H_8O_4$, M_r 156.04; ^{1}H NMR (D_2O): δ = 2.28–2.35 (m, 2H, CH_2CH_{olef}), 2.44–2.51 (m, 2H, CH_2C_q), 6.08–6.14 (m, 1H, CH_2CH_{olef}), 6.20–6.25 (m, 1H, $CH_2CH_{olef}CH_{olef}$), 7.15 ppm (d, J = 5.0 Hz, 1H, $CH_{olef}C_q$). ^{13}C NMR (D_2O): δ = 20.2 (C_qCH_2), 22.8 ($C_{olef}CH_2$), 123.9 ($C_{olef}CH_2$), 126.4 (C_q), 134.7 ($CH_2CH_{olef}CH_{olef}$), 135.4 ($CH_{olef}C_q$), 172.8 ppm (CO_2H).

*3.5.2. (5S,6S)-5,6-dihydroxycyclohex-1-enecarboxylic acid (**16e**)*

(5*S*,6*S*)-5,6-Dihydroxycyclohexa-1,3-dienecarboxylic acid (**16h**) (2,3-CHD) (97.5 mg, 0.6 mmol) and 6 mg Pd/C (10%) were dissolved in 25 mL methanol. After 30 min at 0.5 bar and 20 °C the solvent was evaporated and the crude product **16e** was obtained (90% purity according to NMR) without need for further purification. $C_7H_{10}O_4$, M_r 158.06; MS (-Q1 scan, TurbolonSpray™ source, ESI): m/z (%) 157 (100) $[M-H^+]^-$, 139 (15) $[M-H_2O, H^+]^-$, 95 (43) $[M-CO_2, H_2O, H^+]^-$, 69 (17) $[M-CO_2, H_2O, C_2H_2, H^+]^-$; ^{1}H NMR ($D_2O$): δ = 1.68–1.76 (m, 1H, $CHOHCH_xH_y$), 1.81–1.88 (m, 1H, $CHOHCH_xH_y$), 2.24–2.28 (m, 2H, CH_2CH), 3.89–3.93 (m, 1H, CH_2CHOH, 4.35 (d, J = 4.5 Hz, 1H, C_qCHOH), 6.74 ppm (t, J = 3.6 Hz, 1H, CH_{olef}). ^{1}H NMR (MeOH-d_4): δ = 1.71–1.78 (m, 1H, $CHOHCH_xH_y$), 1.87–1.94 (m, 1H, $CHOHCH_xH_y$), 2.21–2.29 (m, 1H, $CHCH_xH_y$), 2.25–2.34 (m, 1H, $CHCH_xH_y$), 3.92–3.94 (m, 1H, CH_2CHOH, 4.35 (d, J = 3.7 Hz, 1H, C_qCHOH), 7.14 ppm (t, J = 3.9 Hz, 1H, CH_{olef}). ^{13}C NMR (MeOH-d_4): δ = 21.4 ($CHCH_2$), 23.1 ($COHCH_2$), 66.3 (C_qCOH), 69.2 (CH_2COH), 129.9 (C_q), 142.6 (CH_{olef}), 168.8 ppm (CO_2H).

3.6. Derivatisation by TMS-diazomethane

The carboxylic acid function of the products derived from enzymatic synthesis was methylated by TMS-diazomethane [27]. The reduced polarity of the products facilitated further purification or analysis. The products (0.1 mmol) were dissolved in 2 mL of toluene/methanol (3:2) at room temperature. 2 M solution of TMS-diazomethane in ether was added dropwise until the yellow colour of the solution remains. After 1 h excess of TMS-diazomethane was destroyed by adding 10 μL of acetic acid. Evaporation of the solvent under reduced pressure led to high yields of methylated products. If necessary further purification was performed by column chromatography on silica gel using cyclohexane/ethyl acetate (1% (v/v) formic acid) in product adapted ratio as eluent. The following methylated products were obtained without racemisation.

3.6.1. (R)-Methyl 5-hydroxy-5-(3-iodophenyl)-4-oxopentanoate (**18a**) (ee = 98%)

$C_{12}H_{13}IO_4$, M_r 347.99; HPLC-DAD (Chiralcel OD-H, 25 °C, 0.75 mL min^{-1}, isohexane/2-propanol = 90:10): R_t = 28.9 min (*R*-enantiomer, main product), 31.9 min (*S*-enantiomer); ^{1}H NMR ($CDCl_3$): δ = 2.52–2.59 (m, 2H, CH_2), 2.66–2.78 (m, 2H, CH_2), 3.68 (s, 3H, OCH_3), 5.12 (s, 1H, C*H*OH), 7.15 (t, J = 7.9 Hz, 1H, $H_{m\text{-}Ar}$), 7.34 (d, J = 7.9 Hz, 1H, $H_{o\text{-}Ar}$), 7.71 (d, J = 7.9 Hz, 1H, $H_{p\text{-}Ar}$), 7.73 ppm (s, 1H, $H_{o\text{-}Ar}$); ^{13}C NMR ($CDCl_3$): δ = 27.7, 32.6 (CH_2), 52.0 (OCH_3), 79.0 (CHOH), 94.8 (CI), 126.6 (CH_{Ar}), 130.6 (CH_{Ar}), 136.3 (CH_{Ar}), 137.9 (CH_{Ar}), 140.0 (C_q), 172.5 (CO_2CH_3), 207.3 ppm (CO).

3.6.2. (R)-Methyl 5-cyclohexenyl-5-hydroxy-4-oxopentanoate (**18b**) (26% isolated yield, colourless oil, ee n.d.)

$C_{12}H_{18}O_4$, M_r 226.12; GC–MS (EI): R_t = 11.07 min, m/z (%) 226 (1) $[M]^+$, 166 (28) $[M-CO_2, -2H]^+$, 111 (100) $[C_7H_{11}O]^+$, 81 (38) $[C_6H_9]^+$, 67 (86) $[C_5H_7]^+$, 55 (50) $[C_4H_7]^+$; ^{1}H NMR ($CDCl_3$): δ = 1.50–1.66 (m, 5H, CH_2), 2.08–2.15 (m, 3H, CH_2), 2.62–2.81 (m, 4H, CH_2), 3.70 (s, 3H, OCH_3), 4.55 (s, 1H, C*H*OH), 5.96–5.97 ppm (m, 1H, CH_{olef}).

3.6.3. rac-Methyl 4-hydroxy-5-oxohexanoate (**18c**) (17% isolated yield, pale yellow oil)

$C_7H_{12}O_4$, M_r 160.07; ^{1}H NMR (C_6D_6): δ = 1.45–1.54 (m, 1H, $CH_xH_yCO_2CH_3$), 1.64 (s, 3H, CH_3), 1.90–1.99 (m, 1H, $CH_xH_yCO_2CH_3$), 2.30–2.47 (m, 2H, CH_2CO), 3.40 (s, 3H, OCH_3), 3.86 ppm (dd, J = 3.8, 8.5 Hz, 1H, C*H*OH); ^{1}H NMR ($CDCl_3$): δ = 1.75–1.85 (m, 1H, $CH_xH_yCO_2CH_3$), 2.21–2.31 (m, 1H, $CH_xH_yCO_2CH_3$), 2.26 (s, 3H, CH_3CO), 2.43–2.63 (m, 2H, CH_2CHOH), 3.07 (s, 3H, $COOCH_3$), 4.24 ppm (dd, J = 8.8, 8.3 Hz); ^{13}C NMR ($CDCl_3$): δ = 25.2 (CH_3), 28.4 (CH_2CHOH), 29.2 ($CH_2CO_2CH_3$), 51.7(OCH_3), 75.7 ($CH_2CHOHCO$), 173.6 (CO_2CH_3), 209.3 ppm (CO). Optical rotation and CD indicate racemic product formation.

3.6.4. rac-Methyl 5-hydroxy-4-oxohexanoate (**18d**) (25% isolated yield, yellow oil)

$C_7H_{12}O_4$, M_r 160.07; ^{1}H NMR ($CDCl_3$): δ = 1.42 (d, J = 7.0 Hz, 3H, CH_3), 2.63–2.88 (m, 4H, CH_2CH_2), 3.69 (s, 3H, OCH_3), 4.31 ppm (q, J = 7.0 Hz, 1H, C*H*OH); ^{13}C NMR ($CDCl_3$): δ = 19.7 (CH_3), 27.5 ($CH_2CO_2CH_3$), 32.1 (CH_2CO), 51.9 (OCH_3), 72.7 (CHOH), 172.8 (CO_2CH_3), 210.9 ppm (CO).

3.6.5. (R)-Methyl 5-hydroxy-4-oxodecanoate (**18e**) (81% isolated yield, colourless oil, ee = 25%)

$C_{11}H_{20}O_4$, M_r 216.14; LC–MS (HPLC: Chiralcel OB, 25 °C, 0.75 mL min^{-1}, isohexane/2-propanol = 97:3; MS/MS: +MRM scan (mass 217/91), TurbolonSpray^{TM} source, ESI): R_t = 16.1 min (*R*-enantiomer, main product), R_t = 18.9 min (*S*-enantiomer); ^{1}H NMR ($CDCl_3$): δ = 0.90 (t, J = 6.9 Hz, 3H, CH_3), 1.31–1.55 (m, 6H, CH_2), 1.58–1.65 (m, 1H, CH_xH_yCHOH), 1.72–1.81 (m, 1H, CH_xH_yCHOH), 2.56–2.82 (m, 4H, $CH_2CH_2CO_2H$), 3.34 (br s, 1H, CHO*H*), 3.69 (s, 3H, OCH_3), 4.24 ppm (dd, J = 3.9, 7.5 Hz, 1H, C*H*OH); ^{13}C NMR ($CDCl_3$): δ = 13.9 (CH_3), 22.4, 24.4, 27.6 (CH_2), 31.6 (CH_2CO), 32.5 ($CH_2CO_2CH_3$), 33.6 (CH_2CHOH), 51.9 (OCH_3), 76.5 (CHOH), 172.8 (CO_2CH_3), 210.7 ppm (CO).

4. Conclusions

We have demonstrated here that MenD is a potent and versatile biocatalyst for asymmetric synthesis. MenD accepts a broad spectrum of acceptor and donor substrates. For the 1,2-addition [5] a wide variety of aliphatic, aromatic and unsaturated aldehydes is accepted and these are converted in an asymmetric condensation with ketoglutarate to the corresponding *R*-α-hydroxyketones. Electron withdrawing substituents at the aromatic ring increase the electrophilic character of the carbonyl and are hence increasing activity. Electron donating effects on the aldehyde function like methoxy substituents or electron rich aromatic systems like heteroaromatic systems result in a decreased conversion rate. Aliphatic and unsaturated aliphatic aldehydes are also accepted as acceptor substrates resulting in new chiral compounds. In principle, α-ketoglutarate as donor can be substituted by smaller substrates like oxaloacetate and pyruvate resulting, however, in a pronounced loss in activity.

After decarboxylation of α-ketoglutarate, succinylsemialdehyde functions as donor substrate. The only exception observed was the condensation of pyruvate with α-ketoglutarate. In this case regioselectivity was completely inverted forming racemic 4-hydroxy-5-oxohexanoic acid.

The interesting 1,4-addition of α-ketoglutarate to α,β-unsaturated carboxylic acids was restricted though. Besides isochorismate (data not shown), only 2,3-CHD (obtained through microbial fermentation of a recombinant *E. coli* strain [22,23]) was accepted to give the new unphysiologial products **17a** and **17b** containing 4 and 3 stereocentres, respectively.

Regarding the broad diversity in substrate spectrum and in mechanism, catalysing two different types of reaction, MenD offers high potential as biocatalyst in chemoenzymatic synthesis. New chiral building blocks are provided for further synthesis towards bioactive compounds.

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