Molecular Catalysis 467 (2019) 135–142

Contents lists available at ScienceDirect

Molecular Catalysis

journal homepage: www.elsevier.com/locate/mcat

Preparation of optically pure flurbiprofen *via* an integrated chemo-enzymatic synthesis pathway

Junichi Enoki[a,1], Max Linhorst[a,1], Florian Busch[a], Álvaro Gomez Baraibar[a], Kenji Miyamoto[b], Robert Kourist[c,*], Carolin Mügge[a,*]

[a] Junior Research Group for Microbial Biotechnology, Ruhr-University Bochum, 44780, Bochum, Germany
[b] Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Yokohama, 223-8522, Japan
[c] Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010, Graz, Austria

ARTICLE INFO

Keywords:
Biocatalysis
Asymmetric catalysis
Protecting groups
C-C coupling
Protein engineering

ABSTRACT

In the synthesis of chiral molecules, the incorporation of enantioselective enzymatic conversions within the synthetic route often presents a useful approach. For the substitution of a chemical step with an enzymatic reaction, however, the complete synthetic route leading to and from this reaction needs to be considered carefully. An integrated approach, taking the possibilities and challenges of both types of conversions into account, can give access to chemo-enzymatic processes with great potential for effective synthesis strategies. We here report on the synthesis of enantiopure flurbiprofen using arylmalonate decarboxylase (AMDase, EC 4.1.1.76) in a chemo-enzymatic approach. Interestingly, practical considerations required shifting the enzymatic step to an earlier position in the synthetic route than previously anticipated. Engineered enzyme variants made it possible to obtain both (*R*)- and (*S*)-enantiomers of the target compound in excellent optical purity (> 99%*ee*). The presented results underline that enzymes are most useful when they fit in a synthetic route, and that the optimization of biocatalytic steps and the planning of synthetic routes should be an integrated process.

Introduction

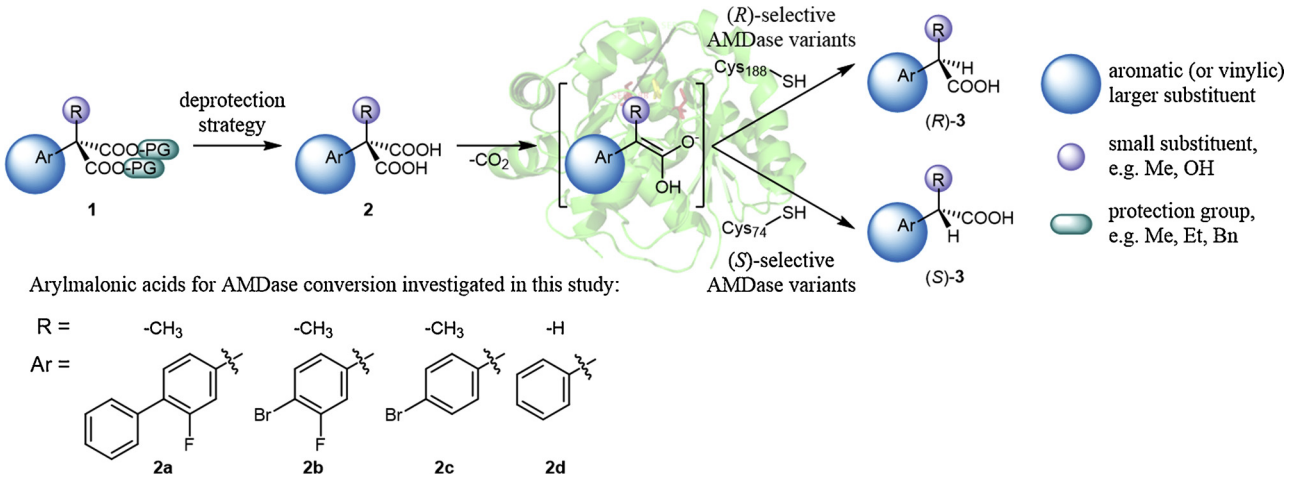
The production of drugs bearing a chiral center often relies on an appropriate natural – chiral – source or makes use of laborious methods of chiral synthesis that often include tedious steps of chiral resolution. Another approach to introduce the chiral center may be the direct asymmetric synthesis of intermediates by aid of highly effective enzymes [1]. Biocatalysis offers considerable advantages regarding selectivity and sustainability, but the development of cost-efficient and sustainable processes with enzymes requires a successful integration of biocatalytic steps into synthesis routes. Since enzymatic reactions often use a different substrate than the traditional chemical approach, the availability of the starting material is as important as the efficiency of the enzymatic reaction. An interesting case is presented by chiral α-substituted carboxylic acids. This class of compounds embraces many chiral building blocks and active pharmaceutical ingredients. While numerous enzymes have been suggested to produce such compounds, most suffer from narrow substrate spectra, insufficient enantioselectivity or other limitations [2]. In this context, arylmalonate decarboxylase from *Bordetella bronchiseptica* (AMDase, EC 4.1.1.76) has received considerable interest over the last years since it effectively promotes the stereospecific decarboxylation of α-disubstituted malonic acids, resulting in pure enantiomers of the respective monoacids [3–6]. It acts as stereospecific decarboxylase to malonic acids bearing an aryl or vinyl unit and a small substituent, such as methyl groups, at the quaternary α-carbon atom. So far, all known natural AMDases exclusively produce the (*R*)-enantiomer of the α-substituted monoacids due to a stereospecific protonation step, which is selectively conducted by a Cys-SH function (Scheme 1) [7,8].

Switching the position of this Cys from one side of the substrate binding site to the opposite by enzyme engineering gave access to variants with selectivity towards the (*S*)-enantiomers [9]. While the activity of the (*S*)-selective variants was significantly reduced, [12,13] focused directed evolution [14–16] and rational design [3] overcame this problem and even increased the activity of the (*R*)-selective variants. Compounds of the profen class, α-arylpropionic acids like (*R*)- and (*S*)-flurbiprofen or naproxen, could efficiently be produced by AMDase-driven decarboxylation of the respective malonic acid precursors in yields of > 98% and > 99%*ee* for the respective enantiomers [3,17].

One drawback of this synthesis is the intrinsic instability of the

* Corresponding authors.
E-mail addresses: kourist@tugraz.at (R. Kourist), carolin.muegge@rub.de (C. Mügge).
[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.mcat.2019.01.024
Received 1 November 2018; Received in revised form 17 January 2019; Accepted 21 January 2019



Scheme 1. Synthesis of chiral *α*-disubstituted carboxylic acids from *α*-disubstituted malonic acid precursors with (*R*)- and (*S*)-selective AMDase variants.

malonic acids **2**, resulting in partial spontaneous decarboxylation and therefore decreasing the *ee* of the final isolated product **3**. Furthermore, the classical synthesis of flurbiprofen **3a** proceeds via malonic acid dialkyl esters **1a** (Scheme 2). These are then cleaved by protolysis, resulting in ester hydrolysis and simultaneous decarboxylation of **2a**, therefore producing a racemic product *rac*-**3a** [18,11,10]. Efforts to produce (*R*)-flurbiprofen (*R*)-**3a** from the dimethyl ester $\mathbf{1a}^{\mathbf{Me}}$ resulted in a maximal 92%*ee* after biocatalysis with AMDase [19]. Slightly higher *ee* values (up to 96%*ee*) have been obtained with additional, laborious, steps of racemic resolution [20]. To make use of the high stereoselectivity of AMDase-based decarboxylation, the preparation of the *α*-disubstituted malonic acid **2** is a crucial issue: the aforementioned classical pathway will not suffice in producing a malonic acid **2** without the presence of at least some decarboxylated product *rac*-**3**. We have recently reported on the use of benzyl esters as alternative protecting groups since they can be cleaved by hydrogenolysis instead of protolysis [21]. Using this approach, we produced malonic acid precursors $\mathbf{1}^{\mathbf{Bn}}$ to flurbiprofen and naproxen without undesired spontaneous decarboxylation [3].

Even though this was an important milestone in the incorporation of the AMDase-based decarboxylation into a production process, the efficient synthesis of the malonic acid benzyl esters $\mathbf{1}^{\mathbf{Bn}}$ was challenging and required optimization. We therefore set out to develop an integrated chemo-enzymatic approach for the production of optically pure (*R*)- and (*S*)-flurbiprofen **3a** based on the enzymatic decarboxylation of *α*-disubstituted malonic acids **2** and their preparation from their dibenzyl ester precursors $\mathbf{1}^{\mathbf{Bn}}$, taking into account the whole synthesis pathway. In this context, it is of utmost importance to keep the balance between enzyme effectivity and an efficient overall synthetic route. Such an approach required to re-consider the position of the enzymatic asymmetrization within the route. This was made possible since enzyme engineering has provided variants with an improved activity for the synthesis of both enantiomers of different *α*-methyl arylpropionic acids **3** [3,14,15,19]. We report here on the full synthesis of enantiopure flurbiprofen **3a** by keeping in mind issues of catalyst compatibility, practicability and efficiency.

Results and discussion

Establishment of the synthetic route

Preparations of flurbiprofen have been reported by different methods [18,11,10,19]. They all include the formation of an aryl-aryl bond to create the biphenyl unit and the protolysis of a malonic dialkyl ester, ultimately leading to a racemic product. These steps are central processes in the overall pathway and require several catalysts; The use of metal catalysts, organic compounds and strong acids have been reported.

In our aim to work under the most economically and ecologically benign conditions possible, we found the use of Pd/C as a readily available and fairly stable hydrogenolysis catalyst a proper choice for the cleavage of the two benzyl ester functions that would replace the ethyl esters in our process. In addition, the same catalyst can be used in an efficient Suzuki coupling, where sodium tetraphenyl borate serves as fourfold phenyl group donor [11,22]. We therefore decided to design a synthesis pathway that would include

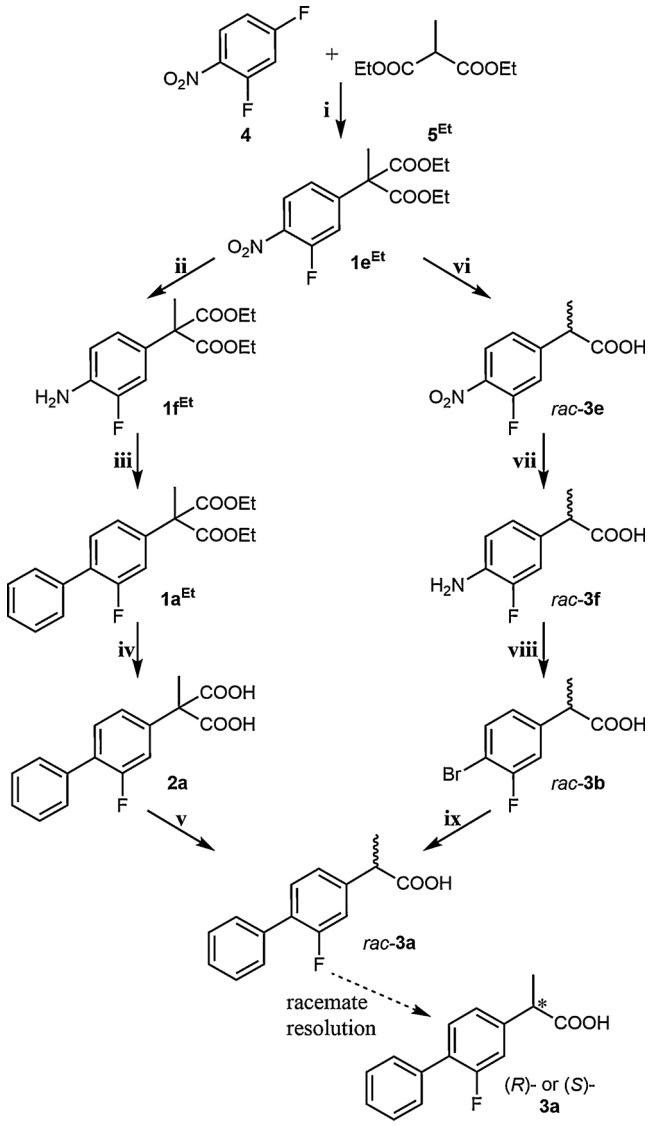
(a) the Suzuki coupling of a phenyl group to a suitable halogenated aromatic precursor,
(b) the hydrogenolysis of malonic acid dibenzyl esters, both catalyzed by the heterogenous Pd/C catalyst, and
(c) the stereoselective decarboxylation of a malonic acid by AMDase

at strategically relevant steps of the process.

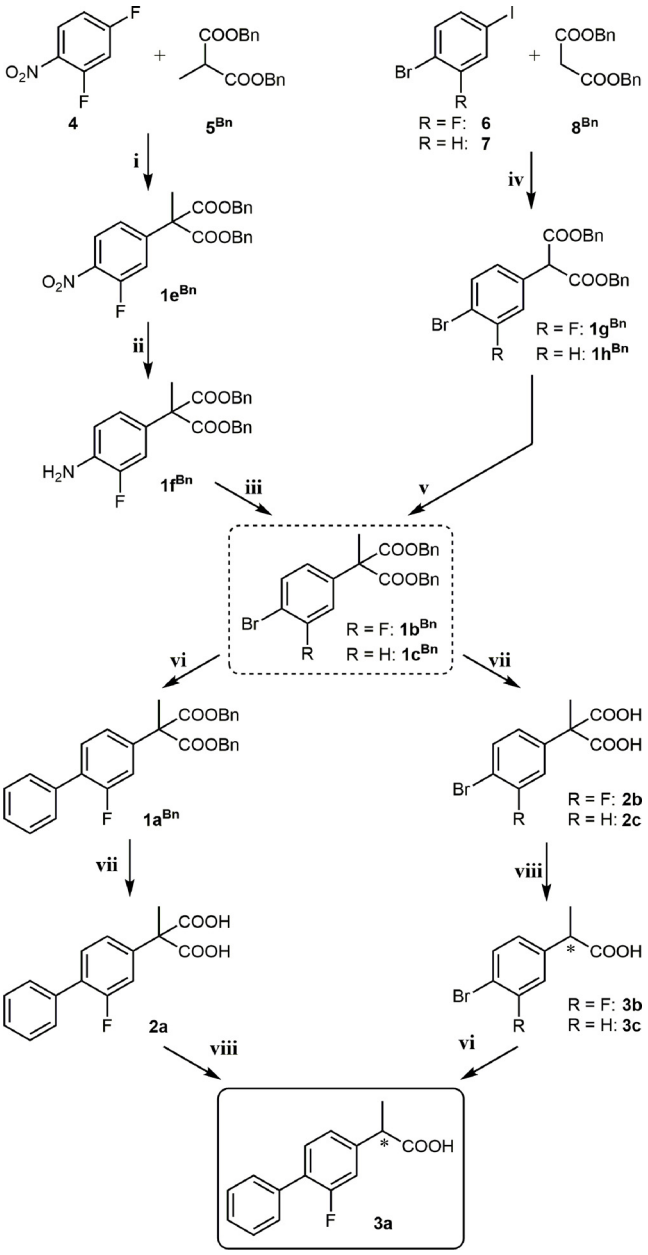
These three steps could be taken from one central intermediate of the process, namely dibenzyl *α*-(4-bromo-3-fluorophenyl)-*α*-methylmalonate $\mathbf{1b}^{\mathbf{Bn}}$ (Scheme 3). From there, two different reaction sequences are in principle thinkable. The first route in the synthetic order (a)-(b)-(c) (vi-vii-viii in Scheme 3) is closely related to the route reported by Hylton [10] and includes the decarboxylation of flurbiprofen malonic acid **2a** by AMDase as the final and already established step [3]. The second route in the synthetic order (b)-(c)-(a) (vii-viii-vi in Scheme 3) was also considered, even though in this case the reactivity of AMDase towards *α*-(4-bromo-3-fluorophenyl)-*α*-methylmalonic acid **2b** had not been investigated so far.

The synthesis of $\mathbf{1b}^{\mathbf{Bn}}$ was explored on different routes, with the left route in Scheme 3 being in close analogy with the industrial procedure [10]. All steps were re-drawn, using dibenzyl methylmalonate $\mathbf{5}^{\mathbf{Bn}}$ instead of its diethyl counterpart $\mathbf{5}^{\mathbf{Et}}$. The final Sandmeyer-type conversion of $\mathbf{1f}^{\mathbf{Bn}}$ was not successful in the isolation of $\mathbf{1b}^{\mathbf{Bn}}$ in our hands. The compound was therefore prepared *via* an alternative route from 1-bromo-2-fluoro-4-iodobenzene **6** and dibenzyl malonate $\mathbf{8}^{\mathbf{Bn}}$ in two steps involving Cu(I) catalyzed coupling [23] to give $\mathbf{1g}^{\mathbf{Bn}}$ and subsequent methylation of the *α*-carbon atom (Scheme 3, right route). The direct coupling of *α*-methyl dibenzyl malonate $\mathbf{5}^{\mathbf{Bn}}$ with **6** (or **7**) by this procedure did not succeed (data not shown), possibly due to hindered interaction of the *in situ* generated catalyst complex with the methyl substituted $\mathbf{5}^{\mathbf{Bn}}$. In parallel, also the homologous compound without the fluorine substituent $\mathbf{1c}^{\mathbf{Bn}}$ was synthesized *via* the same two-step pathway and used for comparison.

Next, we investigated the two possible routes to prepare (*R*)- and (*S*)-flurbiprofen **3a**. Following the pathway analogous to the one



Scheme 2. Reported procedures for the production of *rac*-flurbiprofen **3a** according to Hylton [10] (left) and Lu et al. (right) [11]. Reaction conditions: **i**, NaOH, DMF, r.t., 4.5 h; **ii**, H_2, Pd/C, EtOAc, r.t., 2.5 h; **iii**, [a] $NaNO_2$, H_2O, benzene, 70 °C, [b] HOAc, benzene, 70 °C, 8 h, [c] 85% H_2SO_4; **iv**, [a] NaOH, EtOH, r.t., 6 h, [b] HCl; **v**, HOAc, reflux, 17 h, **vi**, HOAc, H_2SO_4, H_2O, reflux, 24 h; **vii**, H_2, Pd/C, EtOH, r.t., 16 h; **viii**, [a] $NaNO_2$, 40% HBr, H_2O, 0 °C, [b] CuBr, 40% HBr, 60 °C; **ix**, Ph_4BNa, Na_2CO_3, Pd/C, reflux, 1 h.



Scheme 3. Explored synthesis routes towards optically pure flurbiprofen **3a**. Reaction conditions: **i**, NaOH, DMF, 0 °C, 12 h; **ii**, Fe/HCl, NH_4Cl, EtOH/H_2O, 65 °C, 12 h; **iii**, [a] $NaNO_2$, 48% HBr, H_2O, 0 °C, [b] CuBr, 48% HBr, reflux; **iv**, Cs_2CO_3, CuI/picolinic acid, dioxane, r.t. 24 h; **v**, NaH, CH_3I, DMF, 0 °C to r.t., 4 h; **vi**, Ph_4BNa, Na_2CO_3, Pd/C, H_2O or H_2O/dioxane, reflux, 1 h; **vii**, H_2, Pd/C, EtOAc, 5 h; **viii**, AMDase crude cell extract, Tris−HCl pH 8.5, 30 °C, 1 h.

reported by Hylton, [10] a phenyl group had to be coupled to the bromo substituent in **1b^{Bn}** by Suzuki coupling. Even though the reaction took place to some degree, it was not possible to achieve full conversion and an acceptable yield of **1a^{Bn}** under conditions suitable for this particular Suzuki coupling procedure (data not shown). A possible reason for this is the low solubility of **1b^{Bn}** in water at the one hand and the low solubility of the reactant sodium tetraphenylborate in less polar solvents on the other hand: It was not possible to dissolve both components in the same mixture to a sufficient degree, even when using organic solvents as additives. Therefore, even if the following steps have been properly described and performed before, [3] this route had to be considered impractical for the overall goal to achieve an efficient synthesis of flurbiprofen **3a**. Also, using the initially reported benzyl coupling method from **1f^{Bn}** to **1a^{Bn}** (Step iii in Scheme 2) [10] was out of the question for us due to the very unfavorable and ecologically risky conditions (amongst others, the use of benzene as solvent). We therefore turned our attention to the alternative route, demanding an efficient activity of AMDase towards substrates that have previously not been investigated.

The second possible route required the cleavage of the benzyl esters from **1b^{Bn}** to produce the malonic acid **2b**. Interestingly, the hydrogenolysis of **1b^{Bn}** occurred less easily as the one of **1a^{Bn}**: Cleavage of the Bn ester from **1a^{Bn}** had been achieved within 5 h at 0 °C, with higher reaction temperatures leading to partial decarboxylation of the malonic acid **2a** [3]. Contrary, **2b** was produced in 99% isolated yield upon hydrogenolysis for 5 h at 25 °C, with no detectable spontaneous decarboxylation to *rac*-**3b**. This presents a significant advantage over the first-described route since the stability of the malonic acid in absence of AMDase is a crucial factor for obtaining a high final *ee* value. The catalyst Pd/C was easily filtered off, making it available for re-use.

Next, the malonic acid was subjected to enzymatic decarboxylation

by different variants of AMDase. By converting 1 mmol (291 mg, in 50 mL reaction volume) of **2b** in presence of AMDase, both enantiomers of the arylpropionic acid were produced in > 99%*ee* and isolated yields of 99 and 95% for (*R*)-**3b** and (*S*)-**3b**, respectively. This was achieved within 1 h of reaction with crude cell extract containing AMDase in Tris-HCl buffer (pH 8.5), followed by extraction and crystallization of the pure products. In order to closer investigate the efficiency of this particular reaction, the decarboxylation of all structural analogs **2a-d** was conducted with the different AMDase variants (cf. below).

In the final step, Suzuki coupling with sodium tetraphenylborate in the presence of Pd/C afforded enantiopure **3a** with > 99%*ee* for both enantiomers under retention of the stereocenter. Isolated yields were 99% and 98% for (*R*)-**3a** resp. (*S*)-**3a**. The results prove the stability of the stereocenter under coupling conditions and show the high effectivity of the coupling method under aqueous conditions. Again, work-up of the final step was straightforward, since a simple precipitation-filtration sequence allowed us to recover the catalyst and isolate the pure product. It should be noted that the coupling of other aryl units should also be possible via this method. Since Flurbiprofen is the only pharmaceutically relevant drug in this class with a biphenyl-pharmacophore, we focused solely on the coupling of an unsubstituted phenyl group.

Development of the biocatalytic step

Key to the success of the overall route was the availability of optimized AMDase variants to efficiently produce both enantiomers of the *α*-arylpropioic acids **3**. The engineering of the hydrophobic pocket of the enzyme's active site had generated AMDase variants with improved activity for the synthesis of several arylpropionic acids, yet the effects were rather specific for different substrates. As it was difficult to anticipate which enzyme variants would be most suitable for the conversion of the intermediate **2b**, we screened several AMDase mutants available in our laboratory. It is known that electron-donating substituents (like isobutyl) at the aromatic system decrease the enzyme activity, [14,24] which is attributed to a lower stability of the intermediately formed enediolate [25–27]. Electron-withdrawing substituents increase the stability of the enediolate and accelerate the reaction. A typical example is flurbiprofen malonate **2a** that gradually undergoes spontaneous decarboxylation at neutral pH. Steric effects of substituents at the aromatic site are more difficult to rationalize. Nevertheless, AMDase and its variants have the capacity to produce arylpropionic acids with large aromatic substituents. We reasoned that the presence of a *p*-bromo-atom in **2b** would not be sterically challenging, and the overall weak electron-donating effect ([-I] ≈ [+M]) would be more than compensated by the electron-withdrawing effect ([-I] > [+M]) of the *m*-fluoro atom.

To understand the effect of the substituents on the enzymatic activity, we compared *α*-(*p*-bromo-*m*-fluorphenyl)-*α*-methylmalonate **2b** with *α*-(*p*-bromophenly)-*α*-methylmalonate **2c** and *α*-phenylmalonate **2d** as a reference. We investigated the specific activity of the four most promising AMDase variants available in our laboratory, namely the (*R*)-selective wildtype (AMDase wt) and improved variant IPLL [3] as well as the (*S*)-selective variants G74C/M159 L/C188 G (AMDase CLG) [15] and its improved variant G74C/M159 L/C188 G/V43I/A125 P/V156 L (AMDase CLG-IPL), [14] towards the crucial malonic acid **2b** as well as to structural homologs **2a**, **c** and **d**.

Fig. 1 shows the composition of the substrate binding pocket for the wildtype and the three engineered AMDase variants. It demonstrates how the (*R*)- and (*S*)-selective variants can perform a stereospecific re-protonation from a distinct side of the planar intermediate by swapping the position of the catalytic cysteine from the pro-(*R*)-side (C188) to the pro-(*S*)-side (C74) of the substrate binding site. All additional amino acid exchanges only alter the shape and size of the binding pocket, as there is no change in polarity or charge. By the introduction of the IPL (L) mutation set, the size of the hydrophobic pocket is altered. This may result in a more effective decarboxylation and as well change the binding of the substrate respective intermediate by limiting its flexibility within the active site.

Results of the biocatalytic activity investigations are summarized in Fig. 2 and Table S2. Phenyl malonate **2d** lacks the *α*-methyl group and is a fast-converted substrate for both (*R*)-selective variants. It is striking, however, that the (*S*)-selective variants are far less active, with the variant CLG-IPL only showing 8% relative activity (28 $U \cdot mg^{-1}$) of the wildtype (345 $U \cdot mg^{-1}$). In contrast, this variant is considerably faster than the wildtype in the conversion of **2a**. This demonstrates that the effect of amino acid substitutions in the hydrophobic pocket is very specific for individual substrates. While the steric effect of the missing *α*-methyl group in phenyl malonate **2d** affected the variants differently, the comparison of **2b** with **2c** clearly demonstrates that the presence of the *m*-fluoro atom in **2b** has an activating effect on the decarboxylation catalyzed by all variants. The activating effect of the *m*-fluoro substituent for all AMDase variants resulted in a fourfold (for CLG-IPL and IPLL) to eightfold (for CLG) improvement in the conversion of **2b** *vs.* **2c**.

In view of the desired synthesis of optically pure **3a**, we were pleased to find that (*R*)-selective AMDase IPLL showed activity in the conversion of **2b** (480 $U \cdot mg^{-1}$) comparable to **2a** (522 $U \cdot mg^{-1}$), for which the variant has initially been engineered [3]. This observation might be explained by a comparable size as well as roughly similar electronic effects ([+M] and [-I]) of the *para* phenyl (**2a**) and bromo (**2b**) substituents. The compounds thus seem to be similar substrates to variant AMDase IPLL, whilst their differences weigh more to the other investigated AMDase variants. (*S*)-selective AMDase CLG-IPL converted **2b** with a specific activity of 41 $U \cdot mg^{-1}$, which is satisfactory for synthetic applications. As this variant is faster than the wildtype in the conversion of **2a**, but has one third of the wildtype's relative activity towards **2b**, there is still potential for improvement by further engineering that is specific for the newly identified interesting substrate **2b**. The results show clearly that the availability of a reasonable variant library is crucial for the quick identification of enzyme mutants with high activity and the desired enantiopreference for the asymmetric decarboxylation of different *α*-arylmalonates, and thus significantly contributes to solving specific synthetic problems.

Conclusions

By shifting the enzymatic reaction to an earlier step of the route than previously anticipated, we developed an efficient preparation of a suitable *α*-arylmalonate as key intermediate for the chemo-enzymatic synthesis of optically pure flurbiprofen. The higher stability of **2b** compared to **2a** towards spontaneous decarboxylation facilitates the deprotection of its precursor considerably. By the identification of two AMDase variants with high activity and outstanding enantioselectivity in the decarboxylation of **2b**, we could establish a synthetic route of pure (*S*)- and (*R*)-flurbiprofen starting from simple and inexpensive starting materials. This underlined the importance of mutant libraries or enzyme collections for the rapid identification of suitable biocatalyst variants.

The results furthermore show that an integrated optimization of synthesis strategies and biocatalysis is an efficient approach for the development of synthetic routes leading to optically pure ingredients.

Experimental section

General experimental conditions

Syntheses were carried out under nitrogen atmosphere with anhydrous solvents using standard Schlenk techniques when required, otherwise regular air-exposed conditions were used. If not indicated otherwise, all chemicals used for synthesis and protein expression were obtained from standard commercial suppliers (Sigma Aldrich, Alfa Aesar, TCI, Fisher Scientific, abcr, VWR, Acros Organics) in the highest-

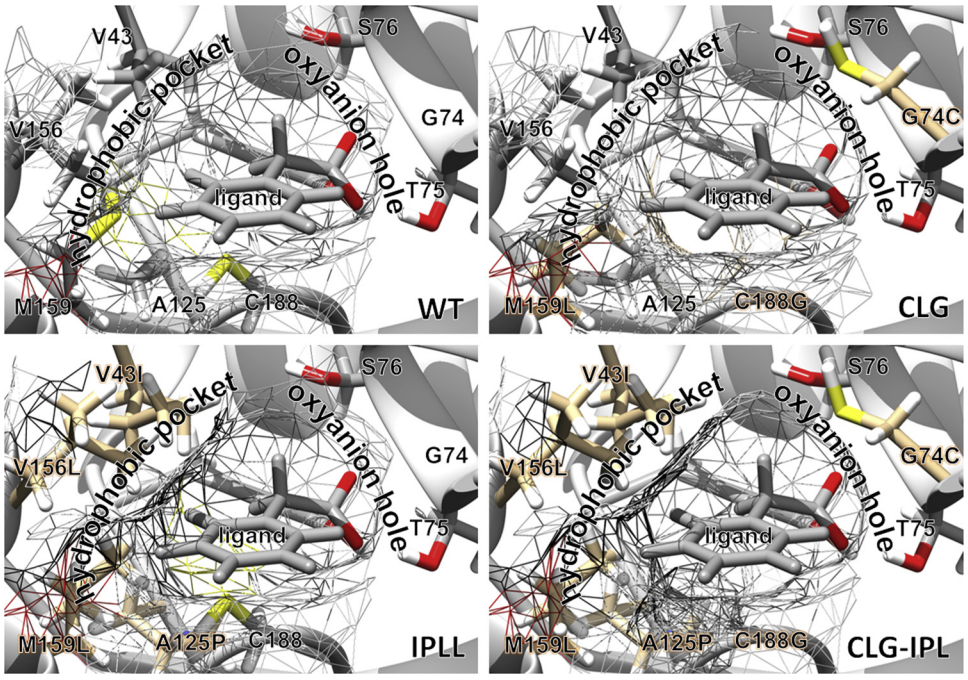


Fig. 1. Illustrations of the substrate binding sites of the four investigated AMDase variants. The graphics are based on the AMDase protein structure PDB 3IXL [13] with phenylacetate as ligand. Amino acid sequences were adapted manually and energy-minimized (*cf.* supporting information). Top left, (*R*)-selective AMDase wt; Bottom left, (*R*)-selective improved variant AMDase IPLL; Top right, (*S*)-selective variant AMDase CLG; Bottom right, (*S*)-selective improved variant AMDase CLG-IPL.

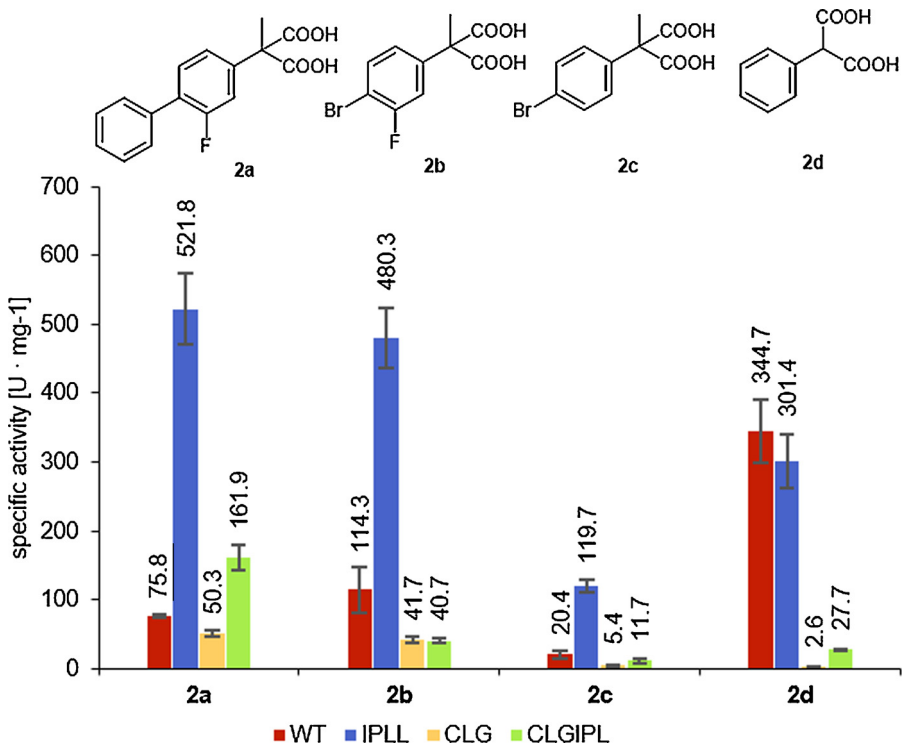


Fig. 2. Specific activities of the four investigated AMDase variants towards the model substrates **2a**-**d**.

available purity grade. As analytical product standard, *rac*-flurbiprofen *rac*-**3a** (100% purity, ChemicalPoint, Deisenhofen, Germany) was purchased. Palladium on charcoal (Pd/C, 10 wt-%, 50% water wet) was purchased from Acros Organics. Solvents for anhydrous procedures were purchased in its anhydrous form in sealed flasks. Light petroleum (b.p. 40–65 °C) and ethyl acetate (EtOAc) used for synthesis were of technical grade.

Compounds **1c**^{Bn}, **1e**^{Bn}, **1f**^{Bn}, **1h**^{Bn}, **2c**, **3c** as well as *rac*-**3b** and *rac*-**3c** as racemic standards for enantiopurity determination were prepared as described in the supporting information. Preparations of compounds **2a** from **1a**^{Bn} and of enantiopure (*R*)- resp. (*S*)-**3a** from **2a** in preparative scale have been reported earlier[3].

General analytic conditions

NMR analyses were carried out on Bruker Avance DPX-200 (200.1/50.3 MHz for $^{1}H/^{13}C\{^{1}H\}$) or DRX-400 (400.13/100.61 MHz for $^{1}H/^{13}C\{^{1}H\}$) spectrometers. Data is given relative to TMS and referenced to the solvent residual signal. Signal assignment was confirmed by H,H-COSY, HSQC and HMBC experiments. FTIR spectra (ATR) were recorded on a Bruker Tensor 27 device, equipped with a Pike MIRacle micro ATR unit. HPLC chromatograms and mass spectra were obtained

from LC–MS (ESI) runs, using a Shimadzu LCMS-8030 device, equipped with a SPD-M20 A diode array detector (scan range: 200–800 nm) and using a Phenomenex Kinetex C18 reversed-phase column (2.6 µm, 100 A, 100 × 2.1 mm) including a Phenomenex SecurityGuard ULTRA C18 guard column. HPLC runs were performed at an isocratic flow of 39.95% water, 0.1% formic acid and 59.95% acetonitrile at a flow rate of 0.5 mL · min^{-1}. MS experiments were performed using both, positive and negative, electrospray ionization. The following standard instrument parameters were used: Nebulizing gas flow 3 L · min^{-1}, dilution line temperature 250 °C, heat block temperature 400 °C, drying gas flow 15 L · min^{-1}, CID gas 230 kPa, ion gauge vacuum 1.7·10^{-3} Pa, Interface voltage 4.5 kV. Mass detection occurred in scan mode (scan range: (±) 150–350 or 300-600 *m/z*, depending on analyte) and in single ion mode (SIM) set to *m/z* values of the $[M+H]^+$/$[M+2+H]^+$ and $[M-H]$-/[M +2-H]- ions of the respective analytes. For high-resolution mass data (HRMS), samples were dissolved in 50% acetonitrile with (for positive ionization) or without (for negative ionization) 0.1% formic acid and directly injected into a Synapt G2-S-HDMS^E mass spectrometer (Waters, Milford, Massachusetts, USA) in combination with an ESI-LockSpray™-Source (Waters). Spectra were recorded for 3 min in positive or negative high-resolution mode with the following settings: capillary voltage, 3 or 2 kV, respectively; cone voltage, 40 V; source temperature, 100 °C; cone gas flow, 50 L · h^{-1}; desolvation gas flow, 500 L · h^{-1}; desolvation temperature, 150 °C. MS spectra were recorded within a mass range of 50–800 m/z with a scan time of 1 s. Leucine-encephalin was injected every 60 s as a lock mass using a capillary voltage of 3 kV. Data were recorded and analyzed using the MassLynx™ software (Waters).

Dibenzyl-2-(4-bromo-3-fluorophenyl)-malonate 1g^{Bn}

A Schlenk flask was charged with dibenzylmalonate **8^{Bn}** (2.88 g, 10.13 mmol), 1-bromo-2-fluoro-4-iodobenzene **6** (3.00 g, 10.13 mmol) and Cs_2CO_3 (9.90 g, 30.39 mmol), degassed, secured and dissolved in 1,4-dioxane (50 mL, anhydrous). Simultaneously, picolinic acid (124.34 mg, 1.01 mmol) and CuI were mixed and dissolved in 1,4-dioxane (10 mL, anhydrous) in another Schlenk flask, upon which an intensive red color appeared. The catalyst solution was added to the reaction mixture and stirred at room temperature for 24 h. During that time, the mixture's color changed from an initial red to yellow or green. To quench the reaction, NH_4Cl (50 mL, 10% aqueous solution) was added and residual Cs_2CO_3 neutralized by addition of HCl (37%, dropwise). The intensively stained red reaction mixture was extracted with EtOAc (3 × 30 mL) and the combined organic phases extracted with $NaHCO_3$ (saturated solution, 3 × 30 mL), dried over anhydrous Na_2SO_4, filtered and the solvents evaporated. The resulting yellowish oil slowly crystallized at room temperature. Residual impurities (mainly educts) were removed by washing the crude crystals with a mixture of ice-cold EtOAc and hexane (1:1 v/v). Finally, the product was re-crystallized from boiling EtOAc. Isolated yield: 3.39 g (73%), white crystals. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 7.56 (dd, $^3J_{HH}$ = 8.3, 7.1 Hz, 1H, Ar-H), 7.46 – 7.18 (m, 11H, Ar-H), 7.09 (m, 1H, Ar-H), 5.21 (d, J_{HH} = 1.6 Hz, 4H, Ar − CH_2), 4.72 (s, 1H, CH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 166.9 (COOR), 160.2, 157.7 (Ar-C^q), 134.9 (Ar-C^q), 133.5 (Ar-C), 128.8 (Ar-C), 127.1 (Ar-C), 126.3 (Ar-C), 117.8 (Ar-C), 109.4, 109.2 (Ar-C^q), 100.0 (Ar-C^q), 67.7 (Bn − CH_2), 56.9 (CH); IR (ATR, $\tilde{\nu}$ cm^{-1}): 3067.2, 3037.4, 2945.3, 1748.7, 1719.8, 1577.5, 1496.5, 1483.0, 1455.6, 1423.4, 1381.4, 1318.1, 1294.0, 1228.5, 1191.5, 1153.3, 1141.5, 1081.8, 1042.5, 1000.3, 952.8, 937.2, 904.6, 889.6, 868.4, 837.8, 819.7, 789.4, 761.8, 742.6, 72.6, 692.8, 640.7, 619.0; HRMS (ESI +, *m/z*): found 457.0458 $[M+H]^+$, calcd. 457.0451; *rp*-HPLC (C18): t_R = 1.47 min (> 98%).

Dibenzyl-2-(4-bromo-3-fluorophenyl)-2-methylmalonate 1b^{Bn}

A Schlenk flask was charged with NaH (272 mg, 6.78 mmol, 60 wt-% in petroleum) and **1g^{Bn}** (2.00 g, 4.37 mmol), dissolved in DMF (30 mL, anhydrous), was added dropwise under nitrogen atmosphere whilst cooling the system to 0 °C in an ice bath. After stirring the reaction mixture for 1 h at 0 °C, iodomethane (815 µL, 13.1 mmol) was added and the reaction mixture stirred for additional 3 h, while the temperature was allowed to reach room temperature. The reaction mixture was quenched by addition of NH_4Cl (30 mL, 10% aqueous solution) and subsequently extracted with EtOAc (3 × 30 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and the solvents evaporated. Finally, the resulting colorless oil was purified by silica gel column chromatography (mobile phase: light petroleum/EtOAc 20:1 v/v, R_f(**1b^{Bn}**) ≈ 0.3). Isolated yield: 2.03 g (99%), colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 7.37 (dd, *J* = 8.5, 7.3 Hz, 1H, Ar-H), 7.27 – 7.11 (m, 10H, Ar-H), 7.06 (dd, $^3J_{HH}$ = 10.2, $^2J_{HH}$ = 2.3 Hz, 1H, Ar-H), 7.00 – 6.82 (m, 1H, Ar-H), 5.07 (s, 4H, Ar − CH_2), 1.78 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 170.4 (COOR), 159.9, 157.7 (Ar-C^q), 139.5 (Ar-C^q), 134.8, (Ar-C^q), 132.9 (Ar-C), 128.8 (Ar-C), 124.5 (Ar-C), 127.9 (Ar-C), 115.9 (Ar-C), 108.6 (Ar-C^q), 67.5 (Bn − CH_2), 58.4 ((C^q), 22.2 (CH_3). IR (ATR, $\tilde{\nu}$ cm^{-1}): 3034.1, 2997.4, 2950.4, 1727.4, 1673.2, 1623.5, 1577.8, 1483.9, 1455.0, 1414.0, 1383.9, 1321.9, 1210.8, 1178.8, 1108.9, 1074.8, 1038.2, 952.0, 926.1, 889.4, 877.7, 842.7, 802.0, 764.4, 725.5, 695.2, 632.6, 620.4. HRMS (ESI +, *m/z*): found 471.0591 $[M+H]^+$, calcd. 471.0607. *rp*-HPLC (C18): t_R = 1.76 min (> 92%).

2. -(4-bromo-3-fluorophenyl)-2-methylmalonic acid 2b

For cleavage of the benzyl esters, **1b^{Bn}** (1.89 g, 4 mmol) was dissolved in EtOAc (10 mL) and mixed with Pd/C (189 mg, 0.02 mol-eq., 10 wt-%, 50% water wet). The reaction mixture was stirred under an atmosphere of H_2 (1 bar) at room temperature for 5 h. The Pd/C catalyst was removed by filtration over a pad of diatomaceous earth and the solvent evaporated under reduced pressure at room temperature. The reddish powder was purified by crystallization from EtOAc and light petroleum. Isolated yield: 873 mg (75%), white crystals. ^{1}H NMR (400 MHz, MeOD, δ ppm) 7.59 (dd, $^3J_{HH}$ = 8.5, 7.4 Hz, 1H, Ar-H), 7.34 (dd, $^3J_{HH}$ = 10.7, $^2J_{HH}$ = 2.2 Hz, 1H, Ar-H), 7.21 (dd, $^3J_{HH}$ = 8.5, $^2J_{HH}$ = 2.3 Hz, 1H, Ar-H), 1.84 (s, 3H, CH_3); ^{13}C NMR (100 MHz, MeOD, δ ppm) 174.2 (COOH), 161.1, 158.7 (Ar-C^q), 142.6 (Ar-C^q), 134.0 (Ar-C), 126.0 (Ar-C), 116.1 (Ar-C), 108.6, 108.4 (Ar-C^q), 59.2 (C^q), 22.7 (CH_3); IR (ATR, $\tilde{\nu}$ cm^{-1}): 2993.3, 2877.7, 2627.0, 2529.9, 1697.9, 1575.4, 1487.4, 1463.5, 1405.2, 1286.6, 1268.4, 1247.1, 1179.6, 1132.5, 1115.8, 1077.3, 1035.5, 911.5, 853.2, 808.9, 781.8, 746.8, 724.8, 696.3, 685.5, 643.1; HRMS (ESI-, *m/z*): found 290.9659 [M − H]-, calcd. 290.9668; *rp*-HPLC (C18): t_R = 0.68 min (> 99%).

(S)- and (R)-2-(4-bromo-3-fluorophenyl)-propanoic acid (S)-3b and (R)-3b by AMDase catalysis in preparative scale

A 250 mL round bottom flask was charged with Tris − HCl buffer (Tris(hydroxymethyl)-aminomethan − HCl, 43 mL, 50 mM Tris − HCl pH 8.5, 300 mM NaCl). **2b** (291 mg, 1 mmol) and NaOH (1 M aqueous solution, 2 mL, 2 mmol) were added stepwise in an alternating manner. Subsequently the pH of the solution was re-adjusted to pH 8.5 with NaOH (1 M aqueous solution). After addition of excess crude cell extract containing AMDase (5 mL, *Escherichia coli* (*E. coli*) BL21 (DE3) transformed with pET28a AMDase IPLL resp. pET28a AMDase CLG-IPL; *cf.* below for expression procedure), the reaction mixture was stirred for 1 h at 30 °C. The reaction was quenched by addition of HCl (37%, 20 mL) and the mixture extracted with EtOAc (3 × 10 mL), the combined organic phases dried over anhydrous Na_2SO_4 and the solvents evaporated. Finally, the resulting brownish powder was crystallized from EtOAc and light petroleum. Isolated yields: (*S*)-**3b**: 235 mg (95%), > 99%*ee*, cream colored crystals; (*R*)-**3b**, 247 mg (99%), > 99% *ee*, cream colored crystals; ^{1}H NMR (400 MHz, MeOD, δ ppm) 7.55 (dd, $^3J_{HH}$ = 8.3, $^3J_{HH}$ = 7.3 Hz, 1H, Ar-H), 7.18 (m, 1H, Ar-H), 7.06 (m, 1H, Ar-H), 3.77 (q, $^3J_{HH}$ = 21.0, $^3J_{HH}$ = 7.2 Hz, 1H, CH), 1.47 (d,

$^3J_{HH}$ = 7.2, $^2J_{HH}$ = 1.1 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, MeOD, δ ppm) 175.3 (COOH), 173.5 (Ar-C^q) 159.9, 157.0 (Ar-C^q), 142.5 (Ar-C^q), 133.0 (Ar-C), 124.1 (Ar-C), 114.0 (Ar-C), 107.2 (Ar-C^q), 44.3 (CH), 17.1 (CH_3); IR (ATR, $\tilde{\nu}$ cm^{-1}): 2984.7, 2941.3, 2883.2, 2612.5, 2543.3, 2232.6, 2058.2, 1697.0, 1602.8, 1577.5, 1484.2, 1461.9, 1421.8, 1384.3, 1336.6, 1278.4, 1241.5, 1210.7, 1165.0, 1151.3, 1079.1, 1061.8, 1041.7, 1008.9, 947.2, 924.7, 869.2, 853.7, 830.3, 816.9, 794.5, 743.6, 714.3, 706.4, 674.8, 646.1, 634.3; MS (ESI-, *m/z*): 245/247 [M−H]-/[M+2-H]-; *Rp*-HPLC (C18): t_R((*S*)-**3b**) = 0.75 min (> 99%); t_R((*R*)-**3b**) = 0.76 min (> 99%).

(S)- and (R)-2-(2-fluoro-biphenyl-4-yl)-propanoic acid (S)-3a and (R)-3a

Depending on the desired product, (*S*)-**3b** or (*R*)-**3b** (200 mg, 0.8 mmol) were dissolved in water (15 mL) together with sodium tetraphenylborate (139 mg, 0.4 mmol), Na_2CO_3 (172 mg, 1.6 mmol). After addition of Pd/C (85 mg, 0.05 mol-eq., 10 wt-% 50% water wet), the reaction mixture was stirred for 2 h under reflux conditions. The reaction was quenched and **3a** precipitated by addition of HCl (37%, 10 mL). The suspension was filtered on a pad of diatomaceous earth and the precipitated **3a** re-dissolved by washing with EtOAc, followed by precipitation with light petroleum. Isolated yields: (*S*)-**3a**: 196 mg (98%), > 99%*ee*, cream colored crystals; (*R*)-**3a**, 199 mg (99%), > 99% *ee*, cream colored crystals; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 7.56 – 6.98 (m, 8H, Ar-H), 3.72 (q, $^3J_{HH}$ = 7.2 Hz, 1H, CH), 1.49 (d, $^3J_{HH}$ = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 179.3 (COOH), 160.9, 158.5 (Ar-C^q), 140.9 (Ar-C^q), 135.4 (Ar-C^q), 130.9 (Ar-C), 128.9 (Ar-C^q), 128.6 (Ar-C), 127.8 (Ar-C), 123.5 (Ar-C), 115.4 (Ar-C), 44.8 (CH), 18.1 (CH_3); IR (ATR, $\tilde{\nu}$ cm^{-1}): 3166.6, 1730.6, 1696.7, 1621.2, 1580.7, 1560.9, 1509.4, 1482.5, 1460.3, 1419.0, 1389.9, 1269.8, 1233.3, 1218.7, 1176.1, 1144.7, 1129.9, 1091.0, 1070.8, 1039.6, 1009.4, 924.2, 915.6, 884.4, 847.0, 794.7, 761.4, 732.3, 722.6, 696.8, 646.0; MS (ESI-, *m/z*): 243 [M−H]-; *rp*-HPLC (C18): t_R((*S*)-**3a**) = 0.81 min (> 99%); t_R((*R*)-**3a**) = 0.81 min (> 99%).

Enzyme preparation

E. coli BL21 (DE3) cells bearing a pET28a vector with an *N*-terminal His-tag sequence and the desired AMDase mutants were cultivated in LB-medium (200 mL) containing 30 μg · mL^{-1} kanamycin at 37 °C in a 1 L shake flask. After reaching an OD_{600} of 0.5, IPTG (isopropyl-*β*-D-thiogalactopyranoside,1 mM) was added and the cells were cultivated for another 12 h at 30 °C. Cells were harvested by centrifugation (20 min, 4 °C, 5000 × g) and washed twice with Tris−HCl buffer (20 mL, 50 mM, pH 7.5, 300 mM NaCl). Cells were resuspended in Tris−HCl buffer (5 mL) containing 10 mM imidazole and were disrupted by sonication. Cell debris was removed by centrifugation (30 min, 4 °C, 8000 × g). AMDase variants were, when required, purified by His-tag purification by using a Ni sepharose spin coloumn (Thermo Fischer) according to the instructions of the manufacturer. The protein was eluted with 250 mM imidazole solution. The enzyme solution was prepared by washing the protein in centricon tubes (10 kDa membrane, Thermo Fischer) with Tris−HCl buffer (50 mM, pH 7.5, 300 mM NaCl).

Determination of specific activities for the enantioselective decarboxylation of arylmalonates 2a-2d by AMDase mutants

Reaction buffer solutions (50 mM Tris−HCl, 300 mM NaCl) were prepared freshly and the pH adjusted to pH 8.5 with NaOH at the reaction temperature (30 °C) to ensure the proper conditions throughout the reactions. Substrate stock solution (250 μL, 20 mM substrate in reaction buffer) and pure buffer (240 μL) were mixed in a small glass vial and pre-incubated at 30 °C and 600 rpm for 5 min. Afterwards, enzyme solution (10 μL, 12.5 μg · mL^{-1}, 50 mM Tris−HCl/NaOH pH 7.5 at 25 °C, c_{end} = 0.25 μg · ml^{-1} for AMDase wt and IPLL; c_{end} = 0.50 μg·mL^{-1} for AMDase CLG and CLG-IPL) was added to start the biocatalysis. The reactions were quenched by addition of acetonitrile (250 μL) either after 2 min or 5 min. Simultaneously, negative control samples without enzyme solution were withdrawn after 0 min and 5 min.

Afterwards samples were measured by HPLC without any additional preparation. HPLC chromatograms were obtained from a Knauer Azura HPLC system (Knauer, Berlin, Germany). A NUCLEODUR C18 Pyramid column (5 μm; 4.6 × 250 mm; Macherey-Nagel, Düren, Germany) was used for the stationary phase. As mobile phases, HPLC-grade solvents (Fisher Scientific) were used. Mobile phase A consisted of acetonitrile with 0.05% TFA and mobile phase B of ultrapure water with 0.05% TFA. All runs were monitored at a detection wavelength of 254 nm. HPLC methods were optimized according to the substrate/product pairs' requirements as given in detail in the supporting information. Enzymatic activities were calculated by linear regression of the area values for the product **3** including all time points with a conversion rate of less than 10%. The concentration of product **3** was corrected by the value of the respective negative control and plotted *versus* the time. For detailed data, *cf.* the Supporting information.

Determination of optical purity of compounds 3

Isolated product powders (ca. 1 mg) were dissolved in 600 μL MTBE. 100 μL Methanol and 25 μL trimethylsilyl-diazomethane were added and the mixture incubated at room temperature for 30 min. 5 μL Acetic acid were added, the solvents evaporated and the residue re-dissolved in 200 μL ethyl acetate. Samples were then analyzed by GC-FID using a Shimadzu GC Plus 2010 device; using a FS-Hydrodex-β-6TBDM column (Macherey-Nagel, 25 m × 0.25 mm ID), with methods having a temperature profile optimized for each compound (*cf.* supporting information) with an injection split of 1/20. A peak difference of at least 0.5 min was achieved to ensure baseline separation. The *ee* values were determined from the relative peak areas according with standard procedures. Errors of *ee* value determination were extrapolated from the area difference obtained from the respective racemic authentic standard. From this, the precision of *ee* values was found to be ± 0.3 %.

Acknowledgements

The Federal Ministry for Innovation, Science and Research of North Rhine-Westphalia (grant number PtJ-TRI/1411ng006) is gratefully acknowledged for financial support. R.K. and F.B. thank the European Union's Horizon 2020 MSCA ITN-EID program (BIOCASCADES under Grant Agreement No. 634200) for financial support. Sina Schäkermann and Julia Bandow (Ruhr-University Bochum) are gratefully acknowledged for recording high-resolution mass spectra.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mcat.2019.01.024.

References

[1] K. Faber (Ed.), Biotransformations in Organic Chemistry, Springer-Verlag Berlin Heidelberg, Heidelberg, Dordrecht, London, New York, 2011.
[2] R. Kourist, P.D. de María, K. Miyamoto, Green Chem. 13 (2011) 2607–2618.
[3] S.K. Gaßmeyer, J. Wetzig, C. Mügge, M. Assmann, J. Enoki, L. Hilterhaus, R. Zuhse, K. Miyamoto, A. Liese, R. Kourist, ChemCatChem 8 (2016) 916–921.
[4] K. Miyamoto, R. Kourist, Appl. Microbiol. Biotechnol. 100 (2016) 8621–8631.
[5] R. Lewin, M. Goodall, M.L. Thompson, J. Leigh, M. Breuer, K. Baldenius, J. Micklefield, Chem. Eur. J. 21 (2015) 6557–6563.
[6] K. Okrasa, C. Levy, M. Wilding, M. Goodall, N. Baudendistel, B. Hauer, D. Leys, J. Micklefield, Angew. Chem. Int. Ed. 48 (2009) 7691–7694.
[7] K. Miyamoto, Y. Yatake, K. Tamura, Y. Terao, H. Ohta, J. Biosci. Bioeng. 104 (2007) 263–267.
[8] Y. Yatake, K. Miyamoto, H. Ohta, Appl. Microbiol. Biotechnol. 78 (2008) 793–799.
[9] Y. Ijima, K. Matoishi, Y. Terao, N. Doi, H. Yanagawa, H. Ohta, Chem. Commun. (2005) 877–879.

[10] T.A. Hylton, J.A. Walker, Process for Preparing Therapeutic 2-Arylpropionic Acids and Esters, and Novel Arylmethylmalonate Esters, (1981) EP0032620 (A1).
[11] G. Lu, R. Franzén, X.J. Yu, Y. jun Xu, Chin. Chem. Lett. 17 (2006) 461–464.
[12] Y. Terao, Y. Ijima, K. Miyamoto, H. Ohta, J. Mol. Catal. B Enzym. 45 (2007) 15–20.
[13] R. Obata, M. Nakasako, Biochemistry (Mosc.) 49 (2010) 1963–1969.
[14] S. Yoshida, J. Enoki, R. Kourist, K. Miyamoto, Biosci. Biotechnol. Biochem. 79 (2015) 1965–1971.
[15] Y. Miyauchi, R. Kourist, D. Uemura, K. Miyamoto, Chem. Commun. 47 (2011) 7503–7505.
[16] Y. Terao, K. Miyamoto, H. Ohta, Appl. Microbiol. Biotechnol. 73 (2006) 647–653.
[17] M. Aßmann, C. Mügge, S.K. Gaßmeyer, J. Enoki, L. Hilterhaus, R. Kourist, A. Liese, S. Kara, Front. Microbiol. 8 (2017) 448.
[18] T. Mizushima, M. Otsuka, Y. Okamoto, N. Yamakawa, 2-Fluorophenylpropionic Acid Derivative, (2014) EP2799424 (A1).
[19] Y. Terao, Y. Ijima, H. Kakidani, H. Ohta, Bull. Chem. Soc. Jpn. 76 (2003) 2395–2397.
[20] (a) R. Morrone, G. Nicolosi, A. Patti, M. Piattelli, Tetrahedron Asymmetry 6 (1995) 1773–1778;
(b) L. Qian, S. Chen, B. Shi, Biocatal. Biotransform. 25 (1) (2007) 29–34.
[21] R. Zuhse, J. Wetzig, R. Kourist, S. Gassmeyer, Verfahren Zur Herstellung von 2-Aryl-2-Alkyl-Malonsäuren, (2015) DE102014006224 (A1).
[22] G. Lu, R. Franzén, Q. Zhang, Y. Xu, Tetrahedron Lett. 46 (2005) 4255–4259.
[23] S.F. Yip, H.Y. Cheung, Z. Zhou, F.Y. Kwong, Org. Lett. 9 (2007) 3469–3472.
[24] R. Kourist, Y. Miyauchi, D. Uemura, K. Miyamoto, Chem. Eur. J. 17 (2011) 557–563.
[25] M.E.S. Lind, F. Himo, ACS Catal. 4 (2014) 4153–4160.
[26] K. Okrasa, C. Levy, B. Hauer, N. Baudendistel, D. Leys, J. Micklefield, Chem. Eur. J. 14 (2008) 6609–6613.
[27] K. Matoishi, M. Ueda, K. Miyamoto, H. Ohta, J. Mol. Catal. B Enzym. 27 (2004) 161–168.