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Arylmalonate Decarboxylase-Catalyzed Asymmetric Synthesis of Both Enantiomers of Optically Pure Flurbiprofen

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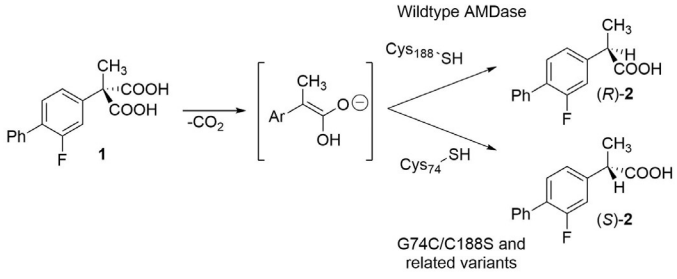
The bacterial decarboxylase (AMDase) catalyzes the enantioselective decarboxylation of prochiral arylmalonates with high enantioselectivity. Although this reaction would provide a highly sustainable synthesis of active pharmaceutical compounds such as flurbiprofen or naproxen, competing spontaneous decarboxylation has so far prevented the catalytic application of AMDase. Here, we report on reaction engineering and an alternate protection group strategy for the synthesis of these compounds that successfully suppresses the side reaction and provides pure arylmalonic acids for subsequent enzymatic conversion. Protein engineering increased the activity of the synthesis of the (*S*)- and (*R*)-enantiomers of flurbiprofen. These results demonstrated the importance of synergistic effects in the optimization of this decarboxylase. The asymmetric synthesis of both enantiomers in high optical purity (>99%) and yield (>90%) can be easily integrated into existing industrial syntheses of flurbiprofen, thus providing a sustainable method for the production of this important pharmaceutical ingredient.

Introduction

Enzymatic asymmetric synthesis is considered a sustainable strategy as it allows the full conversion of a prochiral substrate into an enantiomerically pure product. The bacterial arylmalonate decarboxylase (AMDase) from *Bordetella bronchiseptica* allows the synthesis of optically pure carboxylic acids by enantioselective decarboxylation of prochiral malonates. The enzyme shows outstanding enantioselectivity and has been successfully applied for the preparation of optically pure aryl,[1] alkenyl,[2] aliphatic, and hydroxycarboxylic acids.[3] The high selectivity of the enzyme and the mild reaction conditions offer significant advantages in terms of sustainability. The arylpropionates flurbiprofen (*S*)-**2a** and naproxen (*S*)-**2b** are widely used as non-steroidal anti-inflammatory agents (NSAIDS) and belong to the most common family of over-the-counter drugs.[4] Moreover, (*R*)-**2a** is under investigation for the treatment of Alzheimer's disease[5] and has been shown to reduce proliferation of gastric cancer cells.[6] The distinctive biological activity of both enantiomers of **2a** makes the synthesis of their optically pure enantiomers a desirable goal.

So far, all known wildtype AMDases have strict selectivity for the formation of the (*R*)-enantiomer.[7] As several arylpropionates, such as the non-steroidal anti-inflammatory drugs ibuprofen, naproxen and ketoprofen, exert their anti-inflammatory activity only in the (*S*)-form, the successful inversion of the enzyme's enantiopreference greatly expanded the synthetic potential of the enzyme (Scheme 1).[8] Several rounds of structure-inspired saturation mutagenesis increased the activity of



Scheme 1. Mechanism of the AMDase-catalyzed asymmetric synthesis of optically pure (*R*)- and (*S*)-flurbiprofen, (*R*)- and (*S*)-**2a**. The rate-determining step is the cleavage of the pro-(*R*)-carboxylate.

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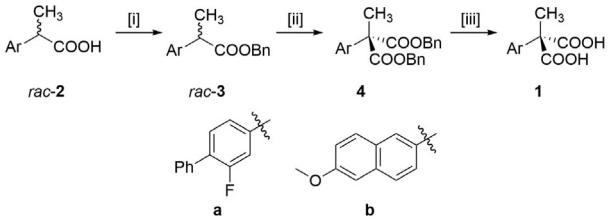
the (*S*)-selective variant considerably, the best variant with six substituted amino acids converting the malonate precursors naproxen and ibuprofen with 0.6 and 1.2-fold relative activity compared with the (*R*)-selective wildtype.[9] Although an asymmetric reaction employing AMDase would be simple and elegant, its broad commercial application has been hampered so far by the limited stability of several arylmalonic acids. The saponification of the commonly used diethyl malonates bears the risk of spontaneous decarboxylation during work-up, resulting in racemic arylpropionic acids. These spontaneously formed products "dilute" the enantiomeric excess of the otherwise highly enantiopure product of the enzymatic reaction. Their removal, however, entails laborious work-up and thus makes an up-scaling very difficult. Avoiding this side reaction is particularly challenging for substrates such as **2a**, where electron-withdrawing substituents at the aryl unit increase their tendency to undergo spontaneous decarboxylation even at neutral pH. For industrial synthesis of *rac*-**2a**, the methylmalonic acid is generated by cleavage of the respective diethyl ester under acidic conditions, followed by decarboxylation of the free malonic acid at high temperatures.[10] An enzymatic decarboxylation under mild reaction conditions (neutral pH, only slightly elevated temperatures, aqueous solvents) would clearly present an environmentally friendly alternative and directly lead to the optically pure product in 100% maximal yield.[11] A previous attempt to integrate the AMDase-catalyzed decarboxylation step into the industrial synthesis route towards **2a** showed the feasibility of the approach. Unfortunately, the occurrence of spontaneous decarboxylation upon protolytic ester cleavage limited the enantiomeric excess in the AMDase-catalyzed synthesis of (*R*)-**2a** to a maximum of 92% *ee*,[12] which is too low for pharmaceutical applications. As the enzymatic mechanism is strictly stereospecific, suppression of the side reaction can be expected to increase the optical purity considerably. In this paper, we report an approach to circumvent the side reaction by an alternative protection group strategy and optimization of the reaction conditions. Protein engineering and immobilization strategies are used to increase the total turnover number of the enzyme considerably and thus should lead to appreciable yields of optically pure compounds **2**.

Results and Discussion

We reasoned that an alternative deprotection procedure might avoid deviations of the pH value and thus suppress the nondesired side reaction.[13] The benzyl protection group (Bn) was chosen to enable hydrogenolysis instead of saponification to isolate arylmethyl malonic acids **1**. Dibenzyl arylmethyl malonates **4a**/**b** are thus key intermediates to this alternate synthetic route.

To prove the principle of this approach, compounds **4a**/**b** were generated from the respective profenes **2** via their benzyl-protected monoesters **3** (Scheme 2). Although **4a** is an oil, compound **4b** was obtained as crystals suitable for single crystal X-ray analysis (Figure S1 in the Supporting Information) and proves the presence of all the desired structural features.



Scheme 2. Preparation of arylmethylmalonic acids **1** as the starting material for asymmetric enzymatic synthesis by deprotection of benzylmalonic esters **4** as key intermediates for asymmetric synthesis. Reaction conditions: [i] a) 1.5 equiv. benzyl alcohol, cat. toluenesulfonic acid, toluene, reflux, b) OH^-, RT; [ii] a) 1.2 equiv. diisopropylamine, 1.2 equiv. *n*-butyllithium, 1.5 equiv. benzyl chloroformate, THF, −20 °C to RT, b) H^+; [iii] 0.5% Pd/C, H_2, MTBE, 0 °C.

The deprotection of **4a** and **4b** by hydrogenolysis proceeded smoothly with a conversion of >99% (as judged by TLC controls) and produced only minute amounts (<0.5%) of side-product *rac*-**2** (Figure S8 in the Supporting Information). After one round of recrystallization, 58% of **1a** was obtained, which is a promising starting point for further optimization. Interestingly, the analogous deprotection of **4b** did not require further purification and produced **1b** with excellent yield (99%). The new route using hydrogenolysis thus proved to be a practicable and scalable substitute to provide the starting material for asymmetric decarboxylation.

Once **1a** and **1b** were successfully isolated, they could be used for enzymatic conversion. However, spontaneous decarboxylation of **1** competes with the stereoselective enzymatic reaction once the substrate is dissolved in aqueous medium. Suppression of this background reaction is essential to achieve a pharmaceutically relevant optical purity. We qualitatively assessed the rate of decarboxylation of **1a** at pH 6 and 9 over time and found that upon dissolving the powder in buffered solutions, spontaneous decarboxylation sets in. By increasing the pH from 6 to 9, we were able to slow down this side reaction considerably (see the Supporting Information). The data suggests that a biocatalytic reaction at the pH activity maximum of AMDase (pH 8.5)[7c] with a reaction time below 1 h should yield compounds **2** in satisfactory enantiopurity, given sufficient activity of the enzyme. To this aim, the conversion rates of both (*R*)- and (*S*)-selective AMDase variants should be optimized in the next step.

The preparation of a biocatalyst requires a considerable amount of energy and is thus one of the key factors for sustainability. Increasing the total turnover number is, in addition to the aforementioned issues, therefore an important objective for a catalytic process as it allows the use and preparation of minimized amounts of biocatalyst. This can be achieved by the complementary methods of enzyme and reaction engineering.

For AMDase, both (*R*)-selective and (*S*)-selective variants are available.[1,2,9,14] Previous work showed that the effect of amino acid substitutions is highly substrate-specific, and at the same time synergistic effects of different mutations affect the activity of the enzyme strongly.[9] The rate-determining step of enzymatic decarboxylation by AMDase is the destabilization of the pro-(*R*) carboxylate, which depends on the stereoelectronic ef-

fects of the substrate and its spatial fit into the enzyme active site. Although naproxen malonate **1b** has a much higher stability than flurbiprofen malonate **1a**, wildtype AMDase decarboxylates **1b** four times faster than **1a**.[12] In contrast, (*S*)-selective AMDase variants convert **1a** faster than **1b** (Figure 1). The variant AMDase G74C with racemizing activity shows a fourfold preference for naproxen.[14b] Binding of **1a** inside the enzymatic active center is expected to decrease the degrees of freedom for rotation of the second phenyl ring, which might result in an unfavorable entropy. This example shows that for an efficient catalysis, a good fit of the substrate in a productive binding mode inside the active center is a decisive parameter for conversion. The enzymatic decarboxylation is initiated by cleavage of the pro-(*R*) carboxylate group (Scheme 1). Semiempiric calculations confirmed that the resulting planar enedionate intermediate is protonated by a cysteine residue, which decides the stereoselective outcome of the reaction.[8b] Transferring the catalytic cysteine 188 to the opposite side of the active center switches the enzyme's enantiopreference towards the formation of the (*S*)-enantiomer.[8a,15] The active site of AMDase consists of a so-called dioxyanion hole to accommodate the pro-(*S*)-carboxylate, a hydrophobic pocket for cleavage of the pro-(*R*)-carboxylate and the catalytic C188 residue (Figure 2). In the (*S*)-selective AMDase, the catalytic residue is transferred to the opposite side of the substrate. It should be noted that the stereoconfiguration of the rate-determining decarboxylation remains the same. This means that the active sites of the variants with (*R*)- and (*S*)-selectivities do not represent mirror images, and that the binding of substrates might differ considerably in such variants.[14b] Taking into account the importance of synergistic effects that multiple mutations have on AMDase activity, the same amino acid substitution may affect (*R*)- and (*S*)-selective variants very differently.

To optimize the activity of AMDase in the synthesis of (*S*)-**2**, we chose variant G74C/M159L/C188G[1] as a starting point (Figure 1). Micklefield et al. showed that the double mutant AMDase P14V/P15G leads to a 12-fold activity improvement over the (*R*)-selective wildtype.[2] Although the mutation P14V/P15G was reported to have an improved (*R*)-activity,[2] a combination of this double mutant with the (*S*)-selective variant to give G74C/M159L/C188G/**P14V/P15G** resulted in an inactive mutant. Variation of the hydrophobic pocket proved to be a more successful approach. The single substitutions V43I and V156L led to a fairly measurable activity increase. Their combination into variant G74C/M159L/C188G/**V43I/V156I**, however, doubled their specific activity towards (*S*)-**2**. An additional replacement of A125 by P led to an activity of 55 U mg^{-1}, which is a further 3.7-fold improvement compared with the previous variant. A125 is situated on a sharp loop between a β-strand and an α-helix in the vicinity of the catalytic C74. Although all known natural AMDases possess this alanine, the closely related maleate *cis–trans* isomerases contain a proline at this posi-

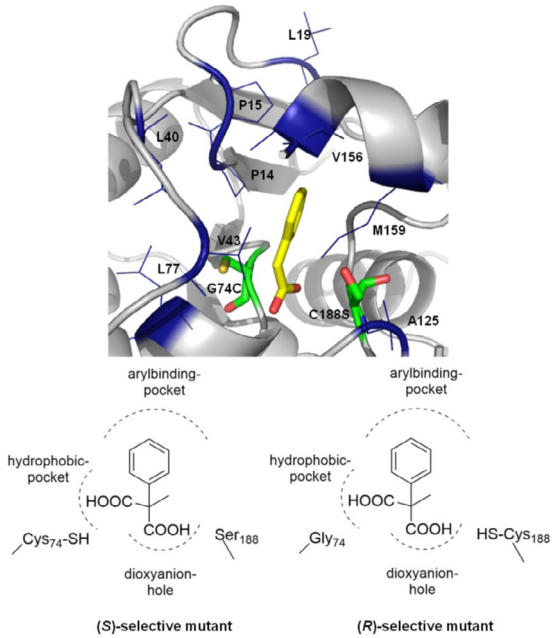


Figure 2. Configuration of the active site of (*S*)-selective and (*R*)-selective AMDase variants.

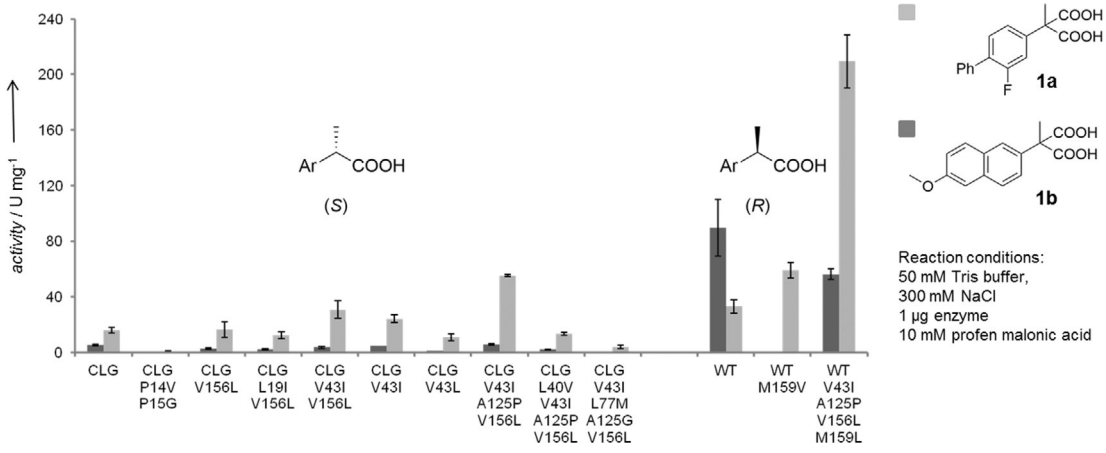


Figure 1. Activity improvement of (*S*)-selective and (*R*)-selective AMDase variants in the enzymatic decarboxylation of substrates **1a** and **1b**. Mutants given on the left part of the graph are based on the CLG variant and are (*S*)-selective, whereas mutants given on the right part are based on the wildtype (WT) enzyme and are thus (*R*)-selective.

tion. The substitution A125P proved to be beneficial for the (*S*)-activity towards **1b**[9] and the racemizing variant AMDase G74C.[14b] The variant G74C/M159L/C188G/**V43I**/**A125P**/**V156I** (CLG-IPL) was previously found to have a higher activity in the synthesis of naproxen.[9] In our studies, this variant showed a four-fold activity increase towards **1a** with respect to the starting variant G74C/M159L/C188G, which nicely confirms its increased activity towards α-methyl arylmalonic acids. Interestingly, it converts **1a** even faster than the (*R*)-selective wildtype.

In a pH-based quick assay, the (*R*)-selective AMDase variant M159V was reported by Micklefield and co-workers to have a 43-fold increased activity in the decarboxylation of phenylmalonate.[2] It was shown in a later publication that the activity of AMDase M159V towards pro-chiral malonates is actually increased two-fold.[3] We were pleased to find that this also holds true for the decarboxylation of **1a** with a decarboxylation activity of 59 U mg^{-1}. AMDase P14V/P15G did not show any measurable activity in the decarboxylation of **1a**. Interestingly, simultaneous introduction of the substitutions A125P, V43I, V156L, and M159L, which were found to be favorable for increasing the (*S*)-activity, led to a further activity increase to 209 U mg^{-1}. AMDase V43I/A125P/V156L/M159L (WT-IPLL) has a six-fold higher activity than the wildtype, which is the highest activity improvement for the (*R*)-selectivity of AMDase in the decarboxylation of prochiral malonates.

The results from protein engineering show that steric and electronic effects play a distinctive role in the different AMDase variants. CLG-IPL showed higher activity in the synthesis of ibuprofen than the wildtype.[9] Interestingly, this is also the case with **1a**, whereas CLG-IPL converts **1b** markedly slower than the wildtype. Owing to its electron-withdrawing phenyl substituent, **1a** has a stronger tendency to decarboxylate than **1b**. It is known that for structurally similar substrates, electronic effects determine the activity of AMDase.[16] The preference of wildtype AMDase for **1b** over **1a** indicates that in the case of malonates with a large aryl substituent, steric effects dominate the efficiency of substrate decarboxylation. The fact that the improved (*R*)-selective WT-IPLL and the (*S*)-selective CLG-IPL both favor **1a** as a substrate strongly indicates that the activity increase is based on a reduction of steric hindrances as a result of the varied space-fills of the exchanged amino acids, which in turn lead to an improved binding of the bulky biaryl system.

In addition to an activity increase by protein engineering, optimization of enzyme expression and immobilization to enable easy recycling are strategies to increase the productivity as well as to minimize the consumption of the biocatalyst. In consequence, costs and effort for catalyst preparation are reduced, which in turn improves the sustainability of the process. By substituting the vector pBAD with pET28a, the expression yield of AMDase was increased from 100 mg L^{-1} to 160 mg L^{-1} cultivation volume. In immobilization processes, the loss of catalyst during the immobilization procedure is compensated by an increased operational stability, the possibility to reuse the enzyme, and, most importantly, by significant savings in downstream processing. Covalent binding onto a C2 amino acrylate carrier was chosen as a robust and established method.[17] Affinity immobilization showed a very high immobilization yield of 80.7 mg g^{-1} carrier and a high activity yield of about 19.8% towards a solution of purified free AMDase. After a slight initial activity loss, the carrier could be reused for ten consecutive batches without a marked decrease in yield per batch (Figure 3). A half-life of the biocatalyst preparation of 16.5 h was determined in this application, resulting in an overall total turnover number of 20.103 for the AMDase.

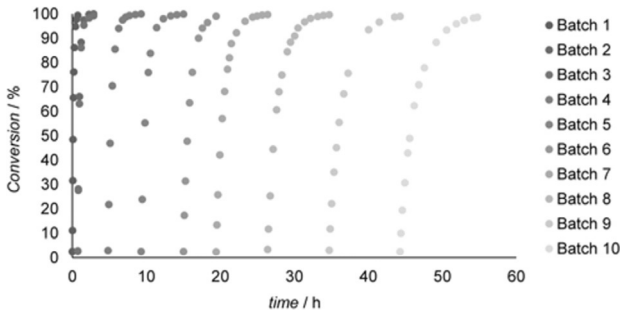


Figure 3. Enzymatic decarboxylation of **1a** catalyzed by covalently immobilized AMDase variant CLG-IPL on C2 amino acrylate in repeated batches. Reaction conditions: 1 mL reaction volume, 30 °C, 1500 rpm, 20 mM **1a**, 10 mM Tris buffer, 20.3 mg immobilized enzyme (loading: 80.7 mg g^{-1} carrier).

The improved AMDase variants were then used in the synthesis of both enantiomers of **2a**. Either cell-free extracts or immobilized proteins could successfully be applied in preparative-scale procedures (Table 1). In all cases, spontaneous decarboxylation could be suppressed efficiently. After complete conversion, (*R*)-**2a** and (*S*)-**2a** were isolated in high yield (>96%) and excellent optically purity (>98%) from cell-free extracts. The synthetic usefulness of the method was demonstrated by using 150 mg of **1a**, which was converted by immobilized AMDase to (*S*)-**2a** with excellent yield (99%) and enantiopurity (>99%).

Table 1. Synthesis of optically pure **2a** under optimized reaction conditions.

AMDase Variant[a]	Product	Yield [%]	Optical purity[b] *ee* [%]
WT-IPLL (CFE)	(*R*)-**2a**	99	98.2
CLG-IPL (CFE)	(*S*)-**2a**	96	98.1
CLG-IPL (imm.)	(*S*)-**2a**	99	99.5

[a] CFE = cell-free extract; imm. = immobilized enzyme. [b] Determined by chiral gas chromatography, ±0.3% accuracy.

Overall, an integrated procedure for the preparation of profenes **2**, by using a new protection group strategy for the chemical synthesis of arylmalonates, combined with reaction engineering, demonstrated that bacterial arylmalonate decarboxylase is a highly efficient biocatalyst for the conversion of arylmalonate substrates of limited stability. The chosen pathway of dibenzylmalonic ester cleavage by hydrogenolysis avoided unwanted deviations of the pH value during work-up

and efficiently suppressed the non-desired spontaneous decarboxylation as a side reaction. The deprotection of dibenzyl malonates **4** proceeded smoothly and led to full conversion; the initial yields of pure arylmethyl malonic acids **1** after work-up are promising and have good potential for optimization. Protein engineering provided two highly active enzyme variants for the synthesis of both enantiomers of **2a**. For the first time, this has made the AMDase-catalyzed synthesis of optically pure (*R*)-**2a** and (*S*)-**2a** possible in high yields and pharmaceutically relevant enantiopurity (>98% *ee*).

Conclusions

The results presented here demonstrate that successful incorporation of biocatalysts into synthetic pathways needs to consider all aspects of a synthetic route, including the provision of the starting material. After this first proof-of-concept, the new protecting group strategy can be easily integrated into the industrial synthesis of flurbiprofen[12] or other arylpropionates. The possibility to produce optically pure **2a** without increasing the number of reaction steps is expected to significantly improve the sustainability of the production of this important pharmaceutical agent.

Experimental Section

Compounds **1**, **2**, **3**, and **4** were prepared and characterized as described in the Supporting Information.

Enzyme preparation

Escherichia coli BL21 (DE3) cells bearing a pET28a-Vector with an *N*-terminal His-Tag sequence and the desired AMDase mutant 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 (15 min, 4 °C, 5000 g) and washed twice with Tris buffer (tris(hydroxymethyl)-aminomethane, 20 mL, 50 mM, pH 8.0). Cells were resuspended in Tris buffer (5 mL) containing 10 mM imidazole and were disrupted by sonication. Cell debris was removed by centrifugation (15 min, 4 °C, 8000 g). AMDase variants were, if applicable, purified by His-tag purification by using a Ni sepharose spin column (ThermoFischer) according to the instructions of the manufacturer. The protein was eluted with 250 mM imidazole. The enzyme solution was prepared by washing the protein in centricons (10 kDa membrane, ThermoFischer) with Tris buffer (50 mM, pH 8.0).

Immobilization on amino C2 acrylate resin

Amino C2 acrylate resin (Iris Biotech) was equilibrated in water for 20 min on a shaker and filtered. The resin was preactivated by incubation with 2% (211.75 mM) glutaraldehyde solution in a ratio of 1:4 (w/w resin/2% glutaraldehyde) for 1 h. After preactivation, the resin was carefully washed with water and filtered. The preactivated resin (350 mg) was added to the purified enzyme solution (8 mL, 4 mg mL^{-1}) or cell lysate (8 mL) and shaken (8 rpm, overhead shaker) for 16 h at 20 °C. Afterwards, the resin was washed with 0.5 M aqueous NaCl solution and water. The concentration of the obtained protein was determined by Bradford Assay.[18] The immobilized enzyme was stored at 4 °C until use.

Synthesis of (*R*)-2a and (*S*)-2a by using cell-free extract of different AMDase variants

For the synthesis of optically pure **2a**, the cell-free extract containing the appropriate AMDase mutant was used. **1a** (0.347 mmol, 100 mg) was dissolved carefully in Tris buffer (50 mM) to a final concentration of 10 mM. To avoid spontaneous decarboxylation, the pH was kept at pH 8.5. The reaction mixture was incubated over 20 min at 30 °C and then stopped by addition of hydrochloric acid (2 M). Complete conversion was confirmed by thin layer chromatography. After extraction with MTBE (methyl-*tert*-butyl ether) and drying with $MgSO_4$, the analytical purity of **2a** was determined by HPLC (Knauer HPLC Azura; Nucleodur C18 Pyramid Column, Macherey–Nagel, isocratic with MeCN/H_2O/trifluoroacetic acid (TFA) 60:40:0.05), and ^{1}H NMR spectroscopy.

(*S*)-**2a**: Using the (*S*)-selective AMDase variant G74C/M159L/C188G/V43I/A125P/V156L, (*S*)-**2a** (81 mg, 0.331 mmol, 95% yield) was isolated with an optical purity of 98.1% *ee*.

^{1}H NMR (400 MHz, Chloroform-*d*): δ = 7.58–7.50 (m, 2H), 7.47–7.34 (m, 4H), 7.21–7.13 (m, 2H), 3.80 (q, J = 7.2 Hz, 1H), 1.57 ppm (d, J = 7.2 Hz, 3H).

(*R*)-**2a**: Using the (*R*)-selective AMDase variant V43I/A125P/V156L/M159L, (*R*)-**2a** (84 mg, 0.344 mmol, 99% yield) was isolated with an optical purity of 98.2% *ee*.

^{1}H NMR (400 MHz, Chloroform-*d*): δ = 7.59–7.51 (m, 2H), 7.49–7.34 (m, 4H), 7.23–7.13 (m, 2H), 3.82 (q, J = 7.2 Hz, 1H), 1.59 ppm (d, J = 7.2 Hz, 3H).

Determination of enzyme activity for immobilized AMDase CLG-IPL in repeated batch experiments

For activity measurements, the concentration of substrate **1a** was set to 20 mM in 10 mM Tris buffer and the pH was adjusted to 8.5 with 1 M NaOH. Experiments were carried out in 2 mL round bottom microreaction tubes on a Biometra thermo shaker at 1500 rpm and 30 °C. Immobilized purified enzyme (20.3 mg) or immobilized cell lysate (122.7 mg) was mixed with 1 mL of 20 mM **1a** and the reaction progress monitored by reverse-phase HPLC. Samples were taken in duplicate over the course of the reaction (12 μL sample to 6 μL MeCN, ten-fold dilution with water/MeCN (1:1)) and analyzed by using a C18 column (Nucleodur C18 pyramid 250/4.6, Macherey–Nagel) with a mobile phase of MeCN/H_2O/TFA (59.025:39.025:0.05) at isocratic conditions and a flow rate of 0.8 mL min^{-1} for 9 min. Compounds **1a** and **2a** eluted at 4.9 and 7.0 min, respectively, as observed by a diode array detector at 245 nm. Between the batches, the enzyme-loaded carrier was carefully washed with water and stored at 4 °C until next usage.

Preparation of compound 1a by chemocatalytic hydrogenolysis of benzyl esters

Dibenzyl 2-(2-fluoro-[1,1′-biphenyl]-4-yl)-2-methylmalonate **3a** (63.4 g, 135.3 mmol) was dissolved in methyl-*tert*-butyl ether (MTBE, 510 mL) and cooled to 0 °C. After adding palladium (10% on activated carbon, 7.2 g, 6.8 mmol), the mixture was stirred under an atmosphere of hydrogen at 0 °C for 5 h. The crude material was filtered through Celite, washed with MTBE, and the solvent

removed by cold evaporation. Pure **1a** (22.7 g 58%) was obtained by crystallization from *n*-hexane/ethyl acetate as white solid.

^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 13.17$ (s, b, 1H, COO*H*), 7.76–7.07 (m, 8H, H$_{ar}$), 1.76 ppm (s, 3H, CH$_3$).

Conversion of compound 1a with immobilized AMDase CLG-IPL on a preparative scale

Compound **1a** (152.2 mg, 526.9 mmol, 26.35 mM) was dissolved in 20 mL water containing 10 mM Tris buffer and the pH was adjusted to 8.5 by using 1 M NaOH. Immobilized cell lysate (250.9 mg) was added and the suspension mixed in a water-tempered stirred tank reactor (30 °C, 600 rpm). Samples were taken to follow the reaction course by reverse-phase HPLC as described above. After complete conversion (22 h), the reaction solution was separated from the immobilized cell lysate by centrifugation. The supernatant was isolated, acidified with 6 M HCl (5 mL), extracted with ethyl acetate (3× 15 mL), and dried by addition of $MgSO_4$. The combined organic solutions were evaporated to dryness to afford (*S*)-flurbiprofen (*S*)-**2a** (127.9 mg, 523.6 mmol, 99.1% isolated yield, >99% *ee*) as an off-white powder.

Determination of the optical purity of compounds 2a and 2b

Isolated product powders (ca. 1 mg) were dissolved in MTBE (600 μL). Methanol (100 μL) and trimethylsilyl diazomethane (25 μL) were added and the mixture incubated at room temperature for 30 min. Acetic acid (5 μL) was added, the solvents evaporated to dryness, and the residue re-dissolved in ethyl acetate (200 μL). Samples were then analyzed by chiral gas chromatography on a Shimadzu GC Plus 2010 device; using a FS-Hydrodex-β-6TBDM column (Macherey–Nagel), with a method operating isothermically at 170 °C with an injection split of 1:20. The elution order was (1) (*S*)-**2a**, (2) (*R*)-**2a** with baseline separation and a peak difference of 0.5 min. The *ee* values were determined from the relative peak areas according to 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 the *ee* values was found to be ±0.3%.

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Keywords: asymmetric synthesis · biocatalysis · immobilization · lyase · protein engineering

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