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Asymmetric Amination of α-Chiral Aliphatic Aldehydes *via* Dynamic Kinetic Resolution to Access Stereocomplementary Brivaracetam and Pregabalin Precursors

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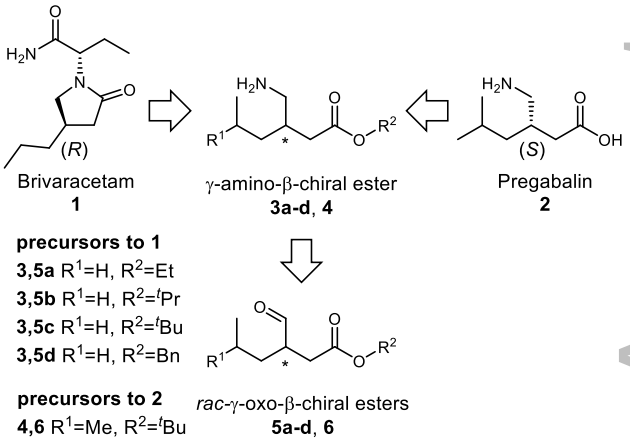
Abstract. Over the last decades biocatalysis has emerged as an indispensable and versatile tool for the asymmetric synthesis of active pharmaceutical ingredients (APIs). In this context, especially transaminases (TAs) have been successfully used for the preparation of numerous α-chiral, optically pure amines, serving as important building blocks for APIs. Here we elaborate on the development of transaminases recognizing the α-chiral centre adjacent to an aldehyde moiety with aliphatic residues, opening up concepts for novel synthetic routes to the antiepileptic drugs Brivaracetam and Pregabalin. The transformation proceeded *via* dynamic kinetic resolution (DKR) based on the bio-induced racemisation of the aldehyde enantiomers, enabling the amination of the racemic substrates with quantitative conversions. Medium, substrate as well as enzyme engineering gave access to both (*R*)- and (*S*)-enantiomers of the amine precursors of the stereocomplementary drugs in high optical purity, representing a short route to mentioned APIs.

Keywords: amines; asymmetric catalysis; transaminases; biotransformations; β-chiral amines

Introduction

Both Brivaracetam **1** and Pregabalin **2** (Scheme 1) are γ-amino carboxylic acids exhibiting remarkable physiological activity on the central nervous system (CNS).[1-2] Consequently, they represent highly valuable pharmaceuticals for the treatment of various neurological diseases. Pregabalin for instance, is marketed by Pfizer under the trade name Lyrica® and made it into the 20 top selling drugs worldwide in 2013.[3-4] Brivaracetam in turn is a relatively new and promising anticonvulsant drug developed by UCB pharma and has recently been approved as adjunctive therapeutic for the treatment of epilepsy.[5] UCB pharma has developed several synthetic routes for the preparation of the 2-oxo-pyrrolidine derivative,[6-8] however, these exclusively chemical methods come along with poor stereoselectivity and hence elaborate chromatography steps in order to obtain the far more active (2*S*,4*R*)-stereoisomer in pure form. The formation of side products and/or the unwanted enantiomer is also an issue regarding the classical manufacturing route towards Pregabalin developed by Pfizer,[9] where the amine is prepared in racemic form and the targeted (*S*)-enantiomer is obtained *via*



Scheme 1. Brivaracetam **1** and Pregabalin **2**: β-chiral amines **3** and **4** were identified as precursors accessible by reductive amination of corresponding α-chiral aldehydes **5** and **6**.

diastereomeric salt formation with mandelic acid. This process has been improved in terms of yield and economical aspects by replacing chemical resolution by a lipase-catalysed one.[10-11]

Recently, biocatalysis has contributed significantly to the synthesis of APIs[12-16] and a vast number of methods involving ene-reductases,[17-19] nitrilases[20-22] and tautomerases[23] were established serving as alternative enzymatic routes towards Pregabalin *via* asymmetric synthesis or resolution of enantiomers. Interestingly, despite of their popularity for the synthesis of chiral amines used for APIs and their successful application also on industrial scale,[24-31] transaminases (TA)[32-51] have not yet been considered as the biocatalyst of choice to prepare key intermediates of Pregabalin and Brivaracetam by deracemisation of the corresponding aldehydes. Reports of transaminase mediated amination of α-chiral aldehydes[52-55] prior to this work are scarce and focus on substrates bearing the α-stereocentre in benzylic position. These compounds allow racemisation of the aldehyde in aqueous medium due to the acid proton in benzylic position as well as in α-position to the aldehyde, consequently enabling dynamic kinetic resolution (DKR). In the most recent example a transaminase mediated DKR of a cyclic α-chiral aldehyde represented the key step in the chemo-enzymatic synthesis of the heart-rate reducing agent Ivabradine.[55]

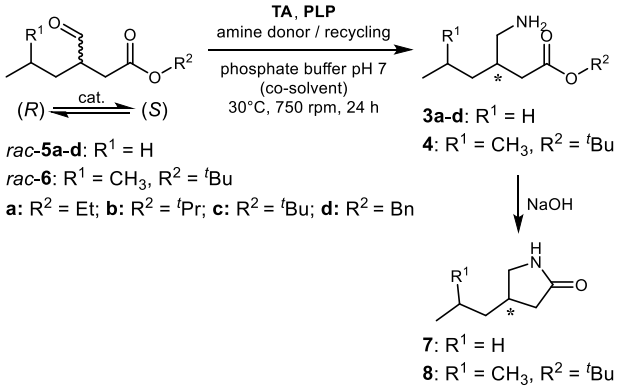
In this study, we focused on the stereoselective amination of racemic aldehydes **5** and **6** (Scheme 1), bearing aliphatic residues at the chiral centre in α-position to the aldehyde moiety.[56-57] The TA-catalysed bioamination gives access to chiral amines **3** and **4**, being precursors for the anticonvulsant agents Brivaracetam **1** and Pegabalin **2**. Since these chiral compounds display an absolute configuration opposite to each other, stereocomplementary transaminases are required for their selective preparation.

Results and Discussion

We started with the synthesis of oxo esters **5** serving as precursors for Brivaracetam **1** and tested them in the biocatalytic amination reaction. Then we intended to employ the most promising biocatalysts and reaction conditions for related oxo ester **6** leading to Pregabalin **2**. The ester form of precursor **5** and **6** was preferred due to its easier handling; additionally, the size of the ester moiety might allow tuning of chiral recognition by the enzyme and therefore the optical purity of the product obtained. Consequently, ethyl-, *i*-propyl-, *t*-butyl and benzyl esters of Brivaracetam precursor **5** were prepared.[58-59] The asymmetric amination of aldehydes **5a-d** was first tested with a library of wild-type transaminases (TAs), showing both (*R*)- and (*S*)-selectivity for the amination of ketones (Table 1). In the first experiments, alanine was used as amine donor together with the AlaDH-recycling system[60] to push the equilibrium towards the product side (Scheme 2). Since γ-amino esters **3a-d** were converted to the corresponding lactam **7** by ring closure due to basic work-up conditions, analytical methods were established and optimised for product **7**. Racemic reference material was synthesised by chemical means and the absolute configuration was assigned by comparison of elution order on a chiral HPLC phase with literature.[8] Most of the enzymes tested successfully converted the chiral oxo esters **5a-d** to the corresponding amino esters in enantioenriched form (Table 2). While eleven wild type enzymes formed the (*R*)-amine (entry 1-11), only four transaminases gave the (*S*)-enantiomer with mostly poor stereoselectivity. In general, the highest *ee* values of product **7** were reached when the *t*-butyl ester **5c** was applied as substrate, probably due to steric reasons. In many cases the racemic substrates were converted quantitatively to product **7**, which indicated that the reaction proceeded *via* a dynamic kinetic resolution (DKR) involving racemisation of the aliphatic aldehyde substrate. Actually, a racemisation of oxo esters **5a-d** was not expected due to the aliphatic residue at the chiral centre and a proton being less acidic compared to benzylic examples, which do not simply racemise in buffer. Therefore, racemisation was investigated in more detail later.

Table 1. Library of investigated wild-type TAs from different organisms

Source Organism	Abbr.	Select.	Ref.
Arthrobacter sp. KNK168	ArR	(*R*)	[61]
Aspergillus terreus	AT	(*R*)	[62]
Giberella zeae	GZ	(*R*)	[62]
Hyphomonas neptunium	HN	(*R*)	[62]
Neosartorya fischeri	NF	(*R*)	[62]
Arthrobacter citreus	ArS	(*S*)	[63]
Bacillus megaterium	BM	(*S*)	[63]
Chromobacterium violaceum	CV	(*S*)	[63]
Ochrobactrum antropi	OA	(*S*)	[64]
Paracoccus dentrificans	PD	(*S*)	[65]
Pseudomonas fluorescens	PF	(*S*)	[66]
Pseudomonas putida KT2440	PP1	(*S*)	[67]
Pseudomonas putida KT2440	PP2	(*S*)	[67]
Silicibacter pomeroyi	SP	(*S*)	[68]
Vibrio fluvialis	VF	(*S*)	[66]



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Scheme 2. Transaminase-catalysed bioamination of Brivaracetam precursors **5a-d** and Pregabalin precursor **6.**.

Table 2. (*R*)- and (*S*)-selective bioamination of Brivaracetam precursors **5a-d**.[a)]

		***rac*-5a**		***rac*-5b**		***rac*-5c**		***rac*-5d**	
entry	TA	conv.[b)] (%)	*ee* **7**[c)] (%)	conv.[b)] (%)	*ee* **7**[c)] (%)	conv.[b)] (%)	*ee* **7**[c)] (%)	conv.[b)] (%)	*ee* **7**[c)] (%)
1	PP1	>99	*rac*	>99	4 (*R*)	>99	22 (*R*)	81	8 (*R*)
2	PP2	99	22 (*R*)	86	14 (*R*)	78	42 (*R*)	60	24 (*R*)
3	PD	>99	12 (*R*)	>99	*rac*	>99	56 (*R*)	88	30 (*R*)
4	ArS	>99	46 (*R*)	>99	8 (*R*)	80	52 (*R*)	82	34 (*R*)
5	CV	>99	7 (*R*)	>99	10 (*R*)	97	17 (*R*)	91	4 (*R*)
6	PF	>99	22 (*R*)	>99	32 (*R*)	>99	54 (*R*)	73	20 (*R*)
7	VF	>99	10 (*R*)	>99	8 (*R*)	99	62 (*R*)	82	26 (*R*)
8	HN	75	70 (*R*)	73	90 (*R*)	73	92 (*R*)	78	80 (*R*)
9	SP	n.d.[d)]	n.d.[d)]	n.d.[d)]	n.d.[d)]	98	14 (*R*)	n.d.[d)]	n.d.[d)]
10	OA	n.d.[d)]	n.d.[d)]	n.d.[d)]	n.d.[d)]	80	52 (*R*)	n.d.[d)]	n.d.[d)]
11	NF	n.d.[d)]	n.d.[d)]	n.d.[d)]	n.d.[d)]	15	11 (*R*)	n.d.[d)]	n.d.[d)]
12	AT	>99	14 (*S*)	98	30 (*S*)	70	16 (*S*)	62	4 (*S*)
13	ArR	>99	20 (*R*)	92	20 (*S*)	95	28 (*S*)	67	32 (*S*)
14	BM	50	10 (*S*)	71	14 (*S*)	98	*rac*	n.d.[d)]	n.d.[d)]
15	GZ	n.d.[d)]	n.d.[d)]	n.d.[d)]	n.d.[d)]	70	55 (S)	n.d.[d)]	n.d.[d)]

[a)] Reaction conditions: substrate **5** (50 mM), TA (lyophilised cells of *E. coli*, 20 mg/mL) in phosphate buffer (100 mM, pH 7, 1 mM PLP) at 30 °C, 120 rpm, 24 h, L-or D-alanine (250 mM), AlaDH (12 U), FDH (11 U), NAD^+ (1 mM) and ammonium formate (150 mM). [b)] Conversion of **5** to **7** determined from area by GC-FID measurement on an achiral stationary phase. [c)] Optical purity was measured by GC-FID on a chiral phase. [d)] not determined

The most promising amination results concerning conversion were obtained with HN-TA, affording the product with 73% conversion and 92% *ee* (*R*). While the (*R*)-amine is required for the production of Brivaracetam, the identification of enantio-complementary transaminases leading to the (*S*)-enantiomer was of importance to access also Pregabalin intermediates in due course. For this purpose, the most selective TA was the one from *Giberella zeae* forming the amine (*S*)-**3c** with 70% conversion and 55% *ee* (Table 2, entry 15). Interestingly, ArR-TA showed a switch in enantiopreference depending on the ester moiety of the substrate. Thus, it preferred the (*R*)-enantiomer of **5a** and the (*S*)-enantiomer of **5b-d** (Table 2, entry 13). It may also be noted, that the stereopreference for the amination of aldehydes does not correlate with the one observed for the amination of ketones, as indicated before.[54] Since it has been shown in previous work[54] that reaction parameters such as temperature, pH and especially organic co-solvents can have a significant effect on the biocatalytic amination of α-chiral aldehydes, Brivaracetam precursor **5c** was chosen for a detailed investigation, employing selected enzymes (ArR, ArS, PD, PF, VF) under varying reaction conditions (for detailed data see SI). While reaction temperature did not significantly affect the stereoselectivity of the enzymes with exception of a switch in enantiopreference of ArR, the addition of organic co-solvents such as DMSO, DMF and DME positively influenced *ee*-values obtained. However, (*S*)-selective enzymes like GZ, which would be of special interest for the preparation of Pregabalin, lost their activity upon co-solvent addition. In order to identify further efficient transaminases yielding the (*S*)-product in higher optical purity, bioamination of substrates **5a**, **5c** and **5d** was tested with a variant of ArR-TA, namely ArRmut11, which has been evolved for the synthesis of Sitagliptin.[24] Furthermore, three variants of ArR-TA and six ones of ArRmut11-TA were designed to achieve higher activity and improved optical purity of the amine (Table 3). With these enzymes 2-propylamine (IPA) was employed as amine donor and DMSO (20 vol%) was added as co-solvent. Using mentioned reaction conditions, imine formation between aldehyde **5** and either IPA or product amine **3**, respectively, was observed in several samples. Nevertheless, many reactions went to completion. The variants of ArR-TA were designed based on the presented approach in previous studies[24] in order to raise the activity of the enzyme. From the three tested ArR-TA variants only the double variant (ArR-G132F/S218P, entry 4) transformed the tested aldehydes to the corresponding (*S*)-amines with high conversion (90 to >99%), albeit with moderately improved *ee*'s (up to 34%) compared to the wild-type (ArR-WT). The results also showed that the enantiopreference can be easily inverted at low *ee*, since with variant ArR-G132F the (*R*)-enantiomer was obtained, whereas the double variant led to the (*S*)-enantiomer. Since ArRmut11 only led to racemic amine for two of the oxo esters and 20% *ee* in the best case (entry 5), further variants were designed. Originally, ArRmut11 has been evolved to enable the transformation of sterically demanding ketones as well as to increase enzyme stability and activity.[24] To ideally maintain the high

stability of the peptide backbone, ArRmut11 was taken as starting point for further variations with the idea to reduce the size of one of the two binding pockets. The mutations M117F and G279A were expected to make the binding pocket tighter, however only variant ArRmut11-G279A led to

Table 3. Bioamination of Brivaracetam precursors **5a**, **5c** and **5d** employing variants of ArR-TA and ArRmut11.[a)]

		***rac*-5a**		***rac*-5c**		***rac*-5d**	
entry	TA	**7**[b)] (%)	*ee* **7**[c)] (%)	**7**[b)] (%)	*ee* **7**[c)] (%)	**7**[b)] (%)	*ee* **7**[c)] (%)
1	ArR-WT	>99	20 (*R*)	95	20 (*S*)	62	4 (*S*)
2	ArR-S218P	<1	n.d.[e)]	<1	n.d.[e)]	8	n.d.[e)]
3	ArR-G131F	51	26 (*R*)	<1	n.d.[e)]	25	40 (*R*)
4	ArR-G131F/S218P	>99	34 (*S*)	>99	20 (*S*)	90	33 (*S*)
5	ArRmut11	>99	*rac*	90	20 (*S*)	81	*rac*
6	ArRmut11-M117F	>99	*rac*	>99	6 (*S*)	80	*rac*
7	ArRmut11-G279A	>99	35 (*S*)	>99	20 (*S*)	65	30 (*S*)
8	ArRmut11-M117F/G279A	56	12 (*R*)	<1	n.d.[e)]	91	10 (*R*)
9	ArRmut11-A60V/M117F	>99	*rac*	97	8 (*S*)	93	*rac*
10	ArRmut11-A60V/M117F/G279A	>99	40 (*S*)	98	23 (*S*)	68	32 (*S*)
11	ArRmut11-A60V/M117F/G279V	>99	50 (*S*)	75	74 (*S*)	52	50 (*S*)
12	ArRmut11-A60V/M117F/G279V[d)]	n.d.[f)]	n.d.[f)]	55	80 (*S*)	n.d.[f)]	n.d.[f)]

[a)] Reaction conditions: substrate **5** (50 mM), TA (lyophilised cells of *E. coli*, 20 mg/mL) in phosphate buffer (100 mM, pH 7, 1 mM PLP), 20 vol% DMSO if not indicated otherwise, 2-propylamine (500 mM) at 45 °C, 120 rpm, 24 h. [b)] Determined from area by GC-FID measurement on an achiral stationary phase. The value for **7** represents the percentage of **7** in the mixture of **5**/**7**/imines. [c)] Optical purity was measured by GC-FID on a chiral phase. [d)] 20 vol% DMF. [e)] not determined due to low conversion. [f)] not determined.

an improvement, allowing transformation of the three esters **5a**,**c**,**d** with low stereo-recognition (entry 7, *ee* 20-35%). Interestingly, the variant bearing both mutations (ArRmut11-M117F/G279A) turned out to be useless due to the decrease of both conversion and product *ee*. Comparing the wild type (ArR) and the new variants, the additional amino acid exchange A60V was suspected to improve the compactness of the structure, especially at the dimer interface. Indeed, variant ArRmut11-A60V/M117F/G279A led to >99% conversion and slightly improved *ee* (entry 9). Since position 279 seemed to have significant impact on the *ee*, a more demanding amino acid (valine) was introduced at this position instead of alanine. This triple variant (ArRmut11-A60V/M117F/G279, entry 11) enabled the highest *ee* (up to 74%) obtained so far for the (*S*)-enantiomer. Addition of 20 vol% DMF further improved the optical purity to 80% (entry 12), making this variant a promising candidate for the preparation of Pregabalin.

Since in the amination of **5a-d** the unreacted substrate was detected in racemic form in every experiment and several transformations went to completion with high *ee* for the product, it became obvious that the reaction proceeded *via* a dynamic kinetic resolution[69-70] involving racemisation of the substrate. Notably, substrates **5** do not racemise if only applied in buffer at pH 7-9, as it would be the case for substrates bearing a benzylic proton.[52-54] Investigating the racemisation of enantioenriched precursor **5c** in the presence of various components of the transaminase reaction system allowed to elucidate the reason for the observed racemisation and subsequent DKR. Enantioenriched **5c** was prepared by enantioselective reduction of *rac*-**5c** in a kinetic resolution, transforming one enantiomer to the corresponding alcohol, catalysed by the alcohol dehydrogenase ADH-A from *Rhodococcus ruber*[71-73] (overexpressed in *E. coli* and partially purified by heat precipitation, please note that here DKR was not observed).

Actually, no significant racemisation of optically enriched **5** (within 2-4 hours) was observed neither in buffer (phosphate, pH 7, 100 mM), nor in buffer supplemented with pyridoxamine phosphate (PMP, 0.5 mM), the aminated form of the cofactor pyridoxal phosphate (PLP) used by TAs. Racemisation was not promoted by amino acids such as L-proline or L-lysine (5 mM) either. However, when the aldehyde was incubated in buffer together with elevated concentration of amine donor *rac*-alanine (250 mM) clearly detectable racemisation was observed within 4 hours (but too slow for a DKR). While racemisation was not induced by the TA (ArRmut11, partially purified by heat treatment) itself in buffer, it was slightly increased by addition of PMP as well as the presence of L-alanine (250 mM). It turned out that amines, e.g. IPA efficiently caused racemisation, which was even doubled when enzyme and PLP (1 mM) were present in addition to IPA (50 mM). Nevertheless, also crude *E. coli* extract significantly provoked racemisation, most likely due to the amine compounds present. Therefore, it was assumed that the substrate bearing aliphatic moieties at the chiral centre in α-position to the aldehyde moiety were racemised *via* Schiff-base formation (Scheme 3).[74]

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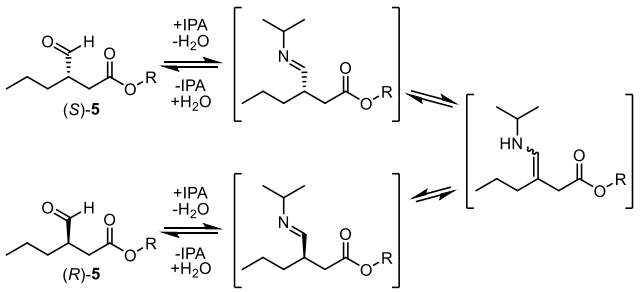

Scheme 3. Proposed mechanism for the racemisation of substrate **5c** *via* imine/enamine formation exemplified with 2-propylamine (IPA).

In a next step, the focus was set on Pregabalin. Since in the case of Brivaracetam precursors the *tert*-butyl ester **5c** gave the best results with respect to optical purity in most experiments, *tert*-butyl ester **6** was chosen as precursor substrate for Pregabalin. It was prepared similarly as described in literature,[58-59] however, since the starting material was not commercially available, the route was expanded by one additional step (for further information check SI). The optical purity to be obtained with used TAs was assumed to be higher than for Brivaracetam precursors **5** due to the additional methyl group and consequently, the higher steric demand. The asymmetric bioamination of aldehyde **6** (Scheme 2) was performed using the most promising biocatalysts and reaction conditions identified previously for aldehyde **5c**. Consistent with the behaviour of amino esters **3a-d** the formed Pregabalin intermediate **4** was transformed to the corresponding lactam **8** during basic work-up. Racemic reference material was synthesised by transformation of Pregabalin to its corresponding methyl ester, followed by subsequent spontaneous cyclisation. Two of the tested TAs did not convert aldehyde **6** at all (Table 4) and in accordance with the results obtained for Brivaracetam precursor **3**, the (*R*)-enantiomer of **8** was rather easily accessible in high optical purity (entry 2-3).

Table 4. Bioamination of Pregabalin precursor **6**.[a)]

		***rac*-6**	
entry	TA	**8**[b)] (%)	*ee* **8**[c)] (%)
1	PF[d)]	30	n.d.[g)]
2	VF[d)]	70	90 (*R*)
3	HN	50	94 (*R*)
4	AT	<1	n.d.[h)]
5	GZ	40	n.d.[g)]
6	ArR-WT	<1	n.d.[h)]
7	ArRmut11[e)]	85	12 (*S*)
8	ArRmut11[f)]	65	20 (*S*)
9	ArRmut11-A60V/M117F/G279A[f)]	48	50 (*S*)
10	ArRmut11-A60V/M117F/G279V[e)]	46	64 (*S*)
11	ArRmut11-A60V/M117F/G279V[f)]	27	76 (*S*)

[a)] Reaction conditions: AlaDH-system: substrate (50 mM), TA (lyophilised cells of *E. coli*, 20 mg/mL) in phosphate buffer (100 mM, pH 7, 1 mM PLP) at 30 °C, 120 rpm, 24 h, L-or D-alanine (250 mM), AlaDH (12 U), FDH (11 U), NAD^+ (1 mM) and ammonium formate (150 mM); 2-propylamine: substrate (50 mM), TA (lyophilised cells of *E. coli*, 20 mg/mL) in phosphate buffer (100 mM, pH 7, 1 mM PLP), 2-propylamine (500 mM) at 45 °C, 120 rpm, 24 h (1 mM). [b)] Determined from area by GC-FID measurement on an achiral stationary phase. The value for **8** represents the percentage of **8** in the mixture of **6**/**8**/imines. [c)] Optical purity was measured by GC-FID on a chiral phase. [d)] in presence of 20 vol% DME. [e)] in presence of 20 vol% DMSO. [f)] in presence of 20 vol% DMF. [g)] not determined due to quantitative imine formation. [h)] not determined due to low conversion.

The anticipated effect of increased stereoselectivity caused by the additional methyl functionality of **6** was not found, except when ArRmut11-A60V/M11F/G279A was used as biocatalyst (entry 9). In this case, the *ee* of product **8** was doubled compared to lactam **7** obtained from Brivaracetam precursor *rac*-**5c** (Table 3, entry 10). In general, the *ee* values obtained for product **8** were comparable to the ones of product **7**, albeit conversions to **8** were significantly lower, exceeding 50% only in few examples. In case of the desired (*S*)-product an increase of optical purity was again achieved by employing ArRmut11 variants. The smaller the binding pocket by substitution of glycine at position 279 with a bigger amino acid residue, the better the *ee*, however going in hand with a loss of conversion. Thus, ArRmut11-A60V/M117F/G279V transformed *rac*-**6** with moderate conversion (27%) and 76% *ee* (entry 11) into the (*S*)-amine.

To further improve the stereoselectivity of the most promising enzymes and to increase the optical purity of aminated product (*S*)-**4**, more variants were constructed. The fact that only a slightly reduced size of the binding pocket of ArRmut11 had significant impact on the selectivity, encouraged the study of further mutations at this position. However, replacing glycine 279 by bulkier amino acid residues such as leucine, isoleucine and phenylalanine led to variants with decreased efficiency and negligibly improved stereoselectivity (see SI). Thus, a different approach for developing variants was pursued, involving the generation of a homology model based on the structure of the wild type enzyme ArR-TA. At first we aimed for understanding why an enlargement of the amino acid residue at position 279 from glycine to alanine and then to valine successively increased the stereoselectivity of ArRmut11-A60V/M117F, but a substitution to even bigger residues such as Leu, Ile or Phe did not have beneficial effects. Based on the homology model (see SI), we envisioned that these residues were rather flexible and thereby able to adapt conformations which did not result in interaction with the substrate molecule. Since the TA from *Giberella zeae* (GZ) allowed the formation of (*S*)-**3c** with 70% conversion and 55% *ee*, this enzyme was chosen as second candidate to be engineered. Sharing a sequence identity of 35% with ArRmut11, it was also comprised in the homology modelling approach, leading to active site models of both enzymes (Figure 1). To identify amino acid residues

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which are important for the stereoselective transformation of substrates investigated, residues from the active site of GZ-TA were replaced with the ones in equivalent position of ArRmut11-A60V/M117F/G279V (for sake of easier reading designated as ArRmut*) and *vice versa* (for more detailed information see SI). Hence, five mutants of ArRmut* and six of GZ-TA were constructed and tested for the amination of substrates *rac*-**5c** and *rac*-**6**. Two of the prepared ArRmut* variants converted Brivaracetam precursor **5c** quantitatively with 77% *ee* and 56% *ee* (*S*), respectively (Table 5), representing a significant improvement of conversion, while preserving the optical purity achieved by the template enzyme. In case of Pregabalin precursor **6** only the ArRmut*-S277T variant gave the (*S*)-amine with low conversion and mediocre optical purity (58% *ee*). Even though most of the developed GZ-TA variants either produced the desired products with lower or equal optical purity as the template enzyme or did not

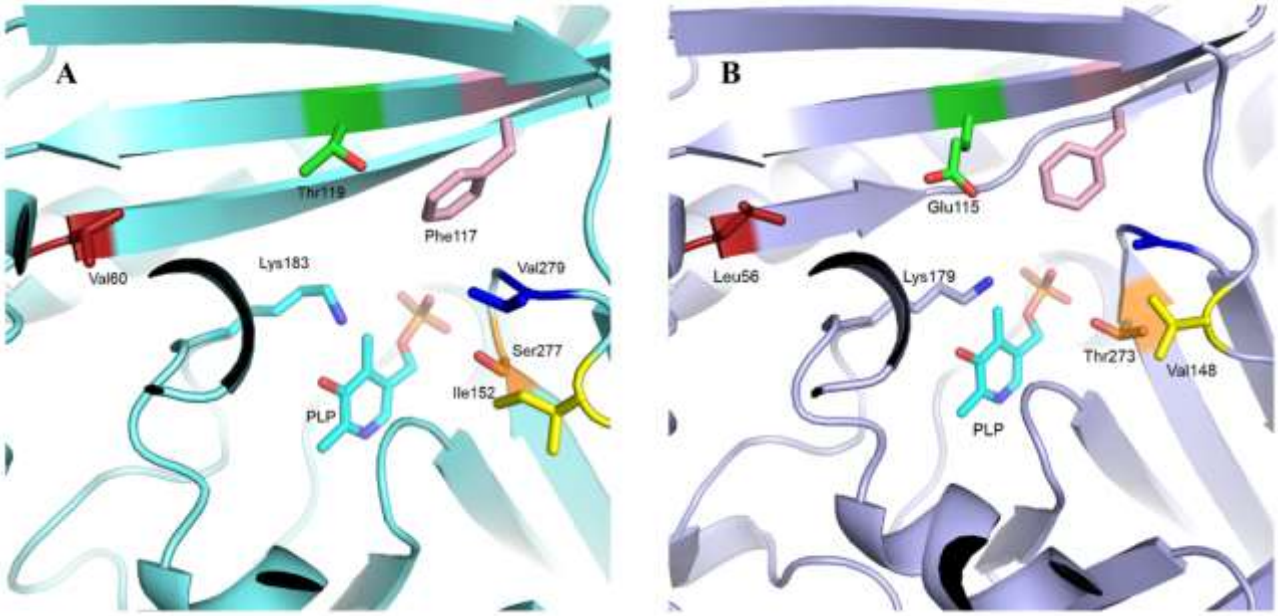


Figure 1. Active site models of ArRmut11-A60V/M117F/G279V (A) and GZ-TA (B).

Table 5. Bioamination of precursors **5c** and **6** using variants of ArRmut*-TA[a)] and GZ-TA[b)].

			***rac*-5c**		***rac*-6**	
entry	TA	analogy of mutation number for ArRmut*	**7**[c)] (%)	*ee* **7**[d)] (%)	**8**[c)] (%)	*ee* **8**[d)] (%)
1	ArRmut*		54	80 (*S*)	27	76 (*S*)
2	ArRmut*-T64V		<1	n.d.[e)]	<1	n.d.[e)]
3	ArRmut*-T119E		<1	n.d.[e)]	<1	n.d.[e)]
4	ArRmut*-I152V		>99	77 (*S*)	<1	n.d.[e)]
5	ArRmut*-S277T		>99	56 (*S*)	15	58 (*S*)
6	ArRmut*-S277N		<1	n.d.[e)]	10	n.d.[f)]
7	GZ		70	55 (*S*)	40	n.d.[f)]
8	GZ-L56V	L60V (red)	62	48 (*S*)	63	80 (*S*)
9	GZ-V60T	V64T	48	rac	3	42 (*S*)
10	GZ-E115T	E119T (green)	86	6 (*S*)	60	15 (*S*)
11	GZ-V148I	V152I (yellow)	<1	n.d.[e)]	<1	n.d.[e)]
12	GZ-T273S	T277S (orange)	94	34 (*S*)	65	72 (*S*)
13	GZ-T273N	T277N (orange)	<1	n.d.[e)]	25	n.d.[f)]
14	GZ-L56V-T273S	L60V-T277S	97	25 (*S*)	92	60 (*S*)

[a)] Reaction conditions: substrate (50 mM), ArRmut*-TA=ArRmut11-A60V/M117F/G279V (lyophilised cells, 20 mg mL^{-1}) in phosphate buffer (100 mM, pH 7, 1 mM PLP) containing 20 vol% DMF and 2-propylamine (500 mM) at 30 °C, 120 rpm, 24 h. [b)] Reaction conditions: substrate (50 mM), GZ-TA (lyophilised cells, 20 mg mL^{-1}) in phosphate buffer (100 mM, pH 7, 1 mM PLP) at 30 °C, 120 rpm, 24 h; D-alanine (250 mM), AlaDH (12 U), FDH (11 U), NAD^+ (1 mM) and ammonium formate (150 mM). [c)] Determined from area by GC-FID measurement on an achiral stationary phase. The value for **7** represents the percentage of **7** in the mixture of **5**/**7**/imines. The value for **8** represents the percentage of **8** in the mixture of **6**/**8**/imines. [d)] Optical purity was measured by GC-FID on a chiral phase. [e)] not determined due to low conversion. [f)] not determined due to quantitative imine formation.

convert the substrates at all, two variants attracted attention. Pregabalin precursor *rac*-**6** was transformed to the desired (*S*)-product with high optical purity and conversions of up to 65% using GZ-L56V (80% *ee*) and GZ-T273S (72% *ee*), respectively. Mutation L56V in GZ relates to mutation A60V in ArRmut*, which has already been introduced before and improved the stereoselectivity significantly. According to the homology model of GZ (Figure 1B, red residue) this position is not located directly in the active site, but close to the dimer interface. A further amino acid that has been identified to have an impact both on the stereoselectivity and activity of the enzymes was the one in position 273 of GZ-TA and the equivalent position 277 of ArRmut* (see Figure 1, orange). When the serine in ArRmut* was replaced by a threonine, being the respective amino acid in GZ, stereoselectivity dropped. In contrast, substituting T273 in GZ to serine led to an increased optical purity of (*S*)-**4**, while an asparagine at the same position implied complete loss of activity. As a final attempt to further increase the optical purity of (*S*)-**4**, a variant was prepared bearing both beneficial mutations L56V and T273S. This constructed enzyme aminated substrates **5c** and **6** with high conversions, however, formed (*S*)-**4** with mediocre 60% *ee*.

Finally, the TA-mediated bioamination of aldehyde substrates being precursors for Brivaracetam and Pregabalin was performed on preparative scale (200 mg, 20 mL), employing the enzymes which gave highest conversions on an analytical scale. Thus, PD-TA was used to aminate Brivaracetam precursor **5c** and Pregabalin precursor **6** was converted by VF-TA. By replacing the aqueous NaOH solution (10 N) in the basic workup procedure by aqueous saturated $NaHCO_3$ the formation of lactam was prevented and the *tert*-butyl-ester **3c** was obtained in 65% isolated yield with a very good *ee* of 90% (*R*). Similarly, Pregabalin precursor **4** was isolated in 55% yield, however, with 60% *ee* (*R*). Subsequently, the *tert*-butyl ester moiety of obtained GABA derivatives was cleaved under acidic conditions resulting in the Brivaracetam acid intermediate (55% yield) and Pregabalin **2** (63% yield) as HCl salt.

Conclusion

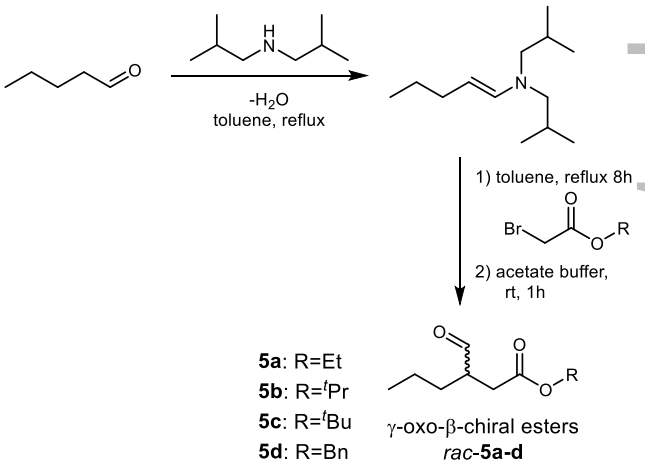
A novel chemo-enzymatic route towards Brivaracetam precursors and Pregabalin has been developed, involving a transaminase-catalysed amination of an α-chiral aldehyde moiety as the biocatalytic asymmetric key step. This stereoselective reaction was run *via* DKR, enabled by efficient racemisation of the substrate induced by the transaminase reaction system. The application of (*R*)- and (*S*)-selective enzymes provided access to both enantiomers of the desired chiral amines in optically enriched form. By substrate and medium engineering as well as the design of transaminase variants, the Brivaracetam precursor **3c** was formed with 92% *ee* (*R*) and 80% *ee* (*S*) and the Pregabalin precursor **4** was prepared with 94% *ee* (*R*) and 80% *ee* (*S*). Finally, in order to confirm the results obtained on an analytical scale, the biotransformation was successfully carried out on a preparative scale, leading to isolated GABA derivatives **3c** and **4** which were further transformed to the targeted anticonvulsant APIs. The herein developed process represents a short method for the preparation of Pregabalin and a Brivaracetam precursor by amination *via* dynamic kinetic resolution of the aldehyde moiety.

Experimental Section

General Information

All chemicals/reagents were purchased from commercial suppliers and used as received unless stated otherwise. Reference compounds (*S*)- and *rac*-Pregabalin were obtained from Sandoz. NMR spectra were recorded on a 300 MHz Bruker NMR unit at 293 K; chemical shifts are given in ppm relative to Me_4Si or the corresponding solvent signal (^{1}H: $CDCl_3$ = 7.26 ppm, CD_3OD = 3.31 ppm; ^{13}C: $CDCl_3$ = 77.0 ppm, CD_3OD = 49.00 ppm). Plasmids for overexpression of transaminases were obtained from GeneArt AG (Regensburg, Germany), cloned and transformed into *E. coli* BL21(DE3) as reported previously.[63] Purified recombinant L-alanine dehydrogenase was prepared as described before.[61] Biocatalytic small scale reactions were carried out in a thermo-shaker Eppendorf Comfort. Preparative scale reactions were performed in an Infors Unitron shaker. For analytical methods, NMR-spectra, DNA- and amino acid sequences of enzymes see electronic Supporting Information.

Preparation of Oxo Esters 5a-d

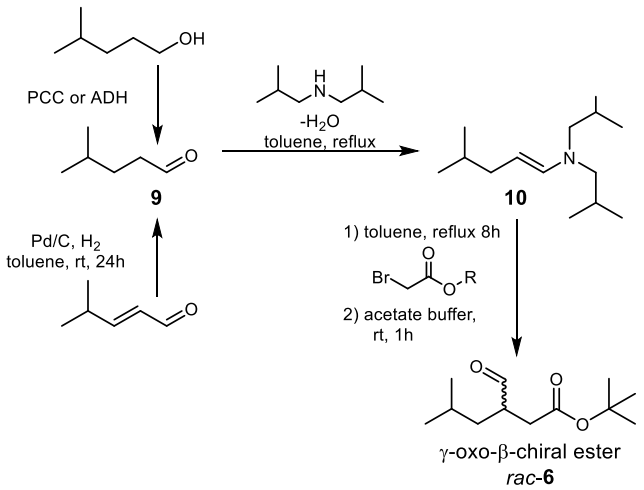


Scheme 4. Synthesis of Brivaracetam precursors *rac*-**5a-d**.

Valeraldehyde (30 mM) and diisobutylamine (30 mM) were dissolved in toluene (120 mL) and refluxed for 8 hours in a Dean Stark apparatus. Molecular sieve was added and the mixture was stirred at 21 °C overnight. Molecular sieve was removed by filtration over Celite® and the appropriate bromo acetate ester (45 mM) was added and the reaction was refluxed for 8 h. Acetate buffer (70 mL; 35 g acetic acid, 35 g sodium acetate, 70 mL water) was added, the suspension was stirred for 1 hour at 21 °C and the organic phase was washed with Na_2CO_3 saturated solution. The organic phase was dried over Na_2SO_4 and the solvent was evaporated.[59] The product was purified by column chromatography (silica, eluent: hexane/EtOAc 97:3). For a more detailed procedure, obtained yields and NMR data see SI.

10.1002/adsc.201701449

Preparation of Oxo Ester 6



Scheme 5. Synthesis of Pregabalin precursors *rac*-**6**.

The starting material 4-methyl-pentanal **9** was synthesised according to three different methods. One the one hand aldehyde **9** was formed by PCC- or ADH-mediated oxidation of 4-methyl-1-pentanol. On the other hand it was synthesised *via* hydrogenation of 4-methyl-2-pentenal. Regardless of which procedure was used, intermediate **9** was isolated as the corresponding enamine **10** after addition of diisobutylamine. The following reaction steps were continued as described before for Brivaracetam precursors, yielding in oxo ester *rac*-**6**. For detailed procedures of all steps, related yields and NMR data see SI.

General Procedure for Biotransformations

All experiments were performed in duplicates on a one millilitre scale using 2 mL Eppendorf tubes as reaction vessels.

Biotransformations using alanine as amine donor: D-alanine was used as amine donor together with ArR-TA, AT-TA, NF-TA, GZ-TA and HN-TA because these transaminases show (*R*)-selectivity for the amination of ketones. When using other TAs, L-alanine was applied as donor. Lyophilised cells of *E. coli* containing overexpressed transaminase (20 mg) were rehydrated in phosphate buffer (1 mL, pH 7, 100 mM) containing PLP (1 mM) and NADH free acid (1 mM) at 30 °C and 120 rpm for 30 min. AlaDH (15 µL, 12 U), FDH (5 mg, 11 U), ammonium formate (9.5 mg, 150 mM) and alanine (22.3 mg, 250mM) as well as the substrate (50 mM) were added. Transamination was carried out at 30 °C in a thermo shaker (750 rpm) for 24 h. The reaction was quenched by addition of aqueous NaOH 10 N solution (200 µL). After extraction with EtOAc (2x 500 µL) the combined organic phases were dried over Na_2SO_4 and analysed *via* gas chromatography. For medium engineering respective parameters were changed.

Biotransformations using 2-propylamine as amine donor: Lyophilised cells of *E. coli* overexpressing transaminase (20 mg) were rehydrated in phosphate buffer (800 µL, pH 7, 100 mM) containing PLP (1 mM) at 30 °C and 750 rpm for 30 min. DMSO (200 µL) and 2-propylamine (50 µL, 500mM) as well as the substrate (9 µL, 50 mM) were added. Transamination was carried out at 30 °C in a thermo shaker (750 rpm) for 24 h. The reaction was quenched by addition of aqueous NaOH 10 N solution (200 µL). After extraction with EtOAc (2x 500 µL) the combined organic phases were dried over Na_2SO_4 and analysed *via* gas chromatography. For medium engineering respective parameters were changed.

Racemisation of α-Chiral Aldehydes

Preparation of enantioenriched *t*-butyl-3-formyl-hexanoate 5c: Purified (heat pulse) ADH-A (20 µL, 0.3 U) and aldehyde *rac*-**5c** (9 µL, 50 mM) were added to sodium phosphate buffer (1 mL, 100 mM, pH 7.0, 1mM NAD free acid) containing 2-propanol (30 µL). The reduction was performed at 30 °C and 750 rpm for 15-20 min. After extraction with EtOAc (2x 500 µL) the combined organic phases were dried over Na_2SO_4 and analysed *via* gas chromatography.

Racemisation: The organic solvent was removed under air flow and enantioenriched aldehyde **5c** was incubated in phosphate buffer containing lyophilised *E. coli* BL21 (DE3) cells (20 mg), 2-propylamine (50 mM), ArRmut11-TA (100 µL, 1.5 U, purified by heat treatment), PMP (1 mM) or PLP (1 mM) or appropriate mixtures of these compounds. Racemisation was performed at 30 °C and 750 rpm. Samples were taken after 1 min, 10 min, 30 min, 1 h, 2 h and 4 h. After extraction with EtOAc (2x 500 µL) the combined organic phases were dried over Na_2SO_4 and analysed *via* chiral gas chromatography.

Isolation of γ-Amino Esters 3c and 4 and cleavage of *tert.*-Butyl-Group

Lyophilised cells of *E. coli* containing overexpressed TA (400 mg) and FDH (100 mg, 220 U) were rehydrated in phosphate buffer (18 mL, pH 7, 100 mM) containing PLP (1 mM) and NADH free acid (1 mM) at 30 °C and 120 rpm for 30 min. AlaDH (300 µL, 240 U), ammonium formate (180 mg, 150 mM), alanine (446 mg, 250 mM) and co-solvent 1,2-dimethoxyethane (200 mL) as well as the substrate (1 mmol) were added. Transamination was carried out at 30 °C in an orbital shaker (120 rpm) for 24 h. Cell material was removed by centrifugation and the supernatant was basified by addition of aqueous saturated $NaHCO_3$ solution (3.5 mL) and extracted with *t*-butyl methyl ether (7 × 10 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was evaporated to yield the product.

^{t}Bu-3-(aminomethyl)hexanoate **3c**: Obtainend from ^{t}Bu-3-formylhexanoate **5c** (201 mg, 1 mmol), using lyophilised cells of *E. coli* containing overexpressed PD-TA (400 mg). The product was isolated as a yellow oil (130 mg, 65% yield) and was used without further purification. NMR: ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H [ppm] = 0.92 (t, $^3J_{6,5}$ = 6.9 Hz, 3 H, Me), 1.20-1.48 (m, 6 H, H-4, H-5, NH_2), 1.45 (s, 9 H, ^{t}Bu), 1.86 (m_c, 1 H, 3-C), 2.14-2.22 (dd, $^3J_{2a,3}$= 6.3 Hz, $^2J_{2a,2b}$= 14.6 Hz, 1 H, 2-H$_a$), 2.20-2.30 (dd, $^3J_{2b,3}$= 7.1 Hz, $^2J_{2b,2a}$= 14.6 Hz, 1 H, 2-H$_b$), 2.56-2.65 (dd, $^3J_{NCHa,3}$= 6.3 Hz, $^2J_{NCHa,\ NCHb}$= 12.9 Hz, 1 H, NCH_2), 2.65-2.75 (dd, $^3J_{3,NCHb}$ = 5.6 Hz, $^2J_{NCHb,\ NCHa}$= 12.9 Hz, 1 H, NCH_2). ^{13}C-NMR (75MHz, $CDCl_3$): δ_C [ppm] = 14.43(-*C*H$_3$), 19.99 (5-C), 28.23 (-*C*H(*C*H$_3$)$_3$), 34.22 (3-C, 4-C), 38.44 (2-C), 45.52 (N*C*H$_2$), 80.31 (*C*O), 172.95 (O*C*O).

^{t}Butyl-3-(aminomethyl)-5-methylhexanoate **4***:* Prepared from ^{t}Bu-3-formyl-5-methylhexanoate **6** (220 mg, 1 mmol), using lyophilised cells of *E. coli* containing overexpressed VF-TA (400 mg). The product was isolated as a yellow oil, purified by column chromatography (silica, CH_2Cl_2 : MeOH 100/0 to 94/6) and isolated as an orange oil (108 mg, 0.4 mM, 50% yield). ^{1}H-NMR (300 MHz, CD_3OD): δ_H [ppm] = 0.89 (d, $^3J_{6,5}$ = 6.5 Hz, 3 H, Me), 0.90 (d, $^3J_{6,5}$ = 6.5 Hz, 3 H, Me), 1.14 (m_c, 2H, 4-H), 1.44 (s, 9H, ^{t}Bu), 1.64 (m_c, 1H, 5-H), 1.69 (bs, 2H, NH_2), 1.93 (m_c, 1H, 3-H), 2.13-2.21 (dd, $^3J_{2a,3}$ = 6.1 Hz, $^2J_{2a,b}$= 14.6 Hz, 1 H, 2-H$_a$), 2.21-2.30 (dd, $^3J_{2b,3}$ = 7.3 Hz, $^2J_{2b,a}$= 14.6 Hz, 1 H, 2-H$_b$), 2.55-2.65 (dd, $^3J_{NCHa,3}$= 6.5 Hz, $^2J_{NCHa,\ NCHb}$= 12.9 Hz, 1 H, NCH_2), 2.66-2.76 (dd, $^3J_{NCHb,3}$= 5.1 Hz, $^2J_{NCHb,\ NCHa}$= 12.9 Hz, 1 H, NCH_2).^{13}C-NMR (75MHz, CD_3OD): δ_C [ppm] = 22.82(Me), 23.06 (5-C), 25.30 (^{t}Bu), 28.40 (^{t}Bu), 36.25 (3-C), 38.61 (2-C), 41.52 (4-C), 45.71 (N*C*H$_2$), 80.40 (*C*O), 172.91 (O*C*O).

General procedure for ester hydrolysis: ^{t}Bu-ester **3c** and **4**, respectively, were diluted with aqueous HCl (0.3 M), the suspension was heated to 65 °C for 5-12 h and after cooling to room temperature the solvent was evaporated. The remaining brown oil was dissolved in a small amount of

water and extracted with EtOAc (3x 0.5-1 mL). The aqueous phase was removed under vacuum to give the product as a yellow oil.

3-(Aminomethyl)hexanoic acid hydrochloride: Prepared from ^{t}Bu-3-(aminomethyl)hexanoate (40 mg, 0.20 mmol), using aqueous HCl solution (4 mL, 0.3 M). The product was isolated as brown oil (20 mg, 0.11 mmol, 55% yield). NMR: ^{1}H-NMR (300 MHz, CD_3OD): δ_H [ppm] = 0.95 (m_c, 3H, Me), 1.29-1.51 (m, 4H, 4-H, 5-H), 2.17 (m_c, 1H, C*H*), 2.46 (d, $^3J_{2,3}$= 6.4 Hz, 2 H, 2-H), 2.97 (d, $^3J_{NCH,3}$= 6.5 Hz, 2 H, NCH_2). ^{13}C-NMR (75MHz, CD_3OD): δ_C [ppm] = 14.32 (Me), 20.51 (5-C), 34.61(4-C), 34.74 (3-C), 36.71 (2-C), 44.04 (NCH_2), 174.33 (OCOH).

3-(Aminomethyl)-5-methylhexanoic acid **1** *hydrochloride:* Prepared from ^{t}Bu-3-(aminomethyl)-5-hexanoate (140 mg, 0.65 mmol), using aqueous HCl solution (5 mL, 0.3 M). The product was isolated as brown oil (104 mg, 0.41 mmol, 63% yield). ^{1}H-NMR (300 MHz, CD_3OD): 0.93 (d, $^3J_{6,5}$ = 6.7 Hz, 3 H, Me), 0.95 (d, $^3J_{6,5}$ = 6.7 Hz, 3 H, Me), 1.27 (t, ^3J = 7.2 Hz, 2 H, 4-H), 1.70 (m_c, 1H, 5-H), 2.22 (m_c, 1H, 3-H), 2.45 (d, $^3J_{2,3}$ = 6.2 Hz, 2 H, 2-H), 2.97 (d, $^3J_{2,3}$ = 6.3 Hz, 2 H, NCH_2). ^{13}C-NMR (75MHz, $CDCl_3$): δ_C [ppm] = 22.52 (Me), 23.18 (Me), 26.14 (5-C), 32.60 (3-C), 37.11 (2-C), 42.03 (4-C), 44.50 (NCH_2), 175.79 (OCO). NMR spectra are in agreement with literature.[75-77]

Molecularbiological and microbiological methods

Construction of TA variants: Models of the enzyme variants were generated using YASARA 13.6.16 and the dimeric structure of ArR-TA as template. Primers were designed using the Agilent QickChange primer design tool and mutagenesis was performed using Agilent QickChange Mutagenesis Kit. For detailed information about used plasmids, primers and conditions see supporting information.

Overexpression of enzymes: To one shot of *E. coli* BL21 (DE3) competent cells plasmid solution (10-100 ng) was added and the cells were incubated on ice for 30 min. Then cells were heat-shocked at 42 °C for 30 sec, preheated SOC medium (250 µL) was added and incubated at 37 °C and 350 rpm for 1 h. The cells were streaked out on LB agar and incubated at 37 °C overnight. For the preculture 10 mL of LB medium containing ampicillin (100 mg/mL) were inoculated with one colony and incubated at 30 °C and 120 rpm overnight. Main cultures (330 mL LB medium) containing the antibiotic ampicillin were inoculated with 2-5 mL of the preculture. After growing the cells at 30 °C and 120 rpm until an OD_{600} = 0.6 – 0.8 was reached, expression was induced and shaking was continued at 120 rpm and 20 °C overnight. Cells were harvested by centrifugation (20 min, 8000 rpm), washed with phosphate buffer (100 mM, pH 7, 0.5 mM PLP), resuspended in phosphate buffer (100 mM, pH 7, 0.5 mM PLP) and lyophilised. For detailed information about plasmids, inducer concentration, and enzyme activities see SI.

Acknowledgements

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