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(54) **Titre : TRANSAMINASES MUTANTES AINSI QUE LEURS PROCEDES ET LEURS UTILISATIONS**
(54) **Title: MUTANT TRANSAMINASES AS WELL AS METHODS AND USES RELATING THERETO**

(57) **Abrégé/Abstract:**
The present invention relates to a mutant transaminase with increased transaminase activity relative to the wild-type transaminase, a fusion protein comprising the transaminase, a polynucleotide coding for the transaminase, a host cell comprising the polynucleotide, mutant transaminase and/or fusion protein, a method of producing an amine with the mutant transaminase or fusion protein and the use of the mutant transaminase or fusion protein for the production of an amine.

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(54) **Title**: MUTANT TRANSAMINASES AS WELL AS METHODS AND USES RELATING THERETO

(57) **Abstract**: The present invention relates to a mutant transaminase with increased transaminase activity relative to the wild-type transaminase, a fusion protein comprising the transaminase, a polynucleotide coding for the transaminase, a host cell comprising the polynucleotide, mutant transaminase and/or fusion protein, a method of producing an amine with the mutant transaminase or fusion protein and the use of the mutant transaminase or fusion protein for the production of an amine.

Mutant transaminases as well as methods and uses relating thereto

The present invention relates to a mutant transaminase with increased transaminase activity relative to the wild-type transaminase, a fusion protein comprising the transaminase, a polynucleotide coding for the transaminase, a host cell comprising the polynucleotide, mutant transaminase and/or fusion protein, a method of producing an amine with the mutant transaminase or fusion protein and the use of the mutant transaminase or fusion protein for the production of an amine.

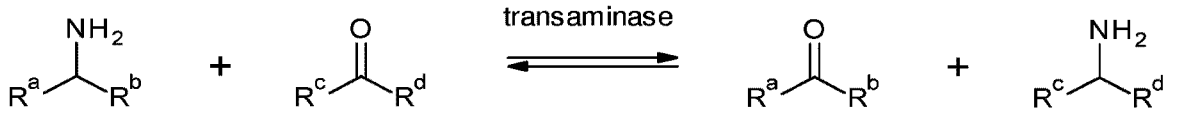
Transaminases (also referred to as aminotransferases) catalyze a transamination reaction, i.e. the transfer of an amino group from an amine donor to an amine acceptor, particularly the amination of a ketone along with the deamination of an amine, wherein the NH_2 group on one molecule or domain is exchanged with the =O group on the other molecule or domain (cf. Scheme 1).

Amine compounds such as chiral amines are significant building blocks for pharmaceutical, agrochemical or chemical industry. In many of these applications, it is of significant importance that only one optically pure form is used. Chemical synthesis has been established for the production of such compounds (for instance asymmetric hydrogenation using transition metals) however, these chemical processes are far from being perfect with respect to yield, purity and waste generation. Transaminase-catalyzed production of amines is an often advantageous alternative to classical methods, therefor alternative or improved transaminases are of interest in the field.

The production of chiral amines via enzyme-mediated transamination is beneficial compared to established chemical asymmetric synthesis, as it was nicely presented in the production of the drug sitagliptin by using an engineered *(R)*-

selective transaminase (Savile et al., 2010; US 2015/0037869). In this case, an initially inactive amine transaminase (ATA) towards the desired substrate was extensively engineered via directed evolution methods. Further mutants of transaminases are described in the art (Steffen-Munsberg et al., 2013; Nobili et al., 2015 Deszcz et al., 2015). Also other enzymes can be used for the production of optically pure chiral amines and amino acids, such as mono amine oxidases, imine reductases, amino acid dehydrogenases, ammonia lyases or amino mutases. Although transaminases can be used for the kinetic resolution of racemic amines, the asymmetric synthesis from keto acids, ketones or aldehydes is of most importance, as they can potentially lead to a theoretical 100 % conversion, compared to only 50 % theoretical yield in the kinetic resolution mode (cf. Scheme 1 illustrating synthesis as well as kinetic resolution).

Scheme 1:



amine donor amine acceptor

The residues R^a, R^b, R^c and R^d have been applied for illustrative purpose only and shall represent common organic residues.

However, the directed evolution approaches do not provide us with an insight for the molecular reasons e.g. with respect to the acceptance of the bulkier substrates. The high enantioselectivity of transaminases fueled the theory of the big and small binding pocket, however, enlarging the small binding pocket to accommodate bulky substrates is a target not easily attained, as illustrated by the fact that by far most of the successful examples found in literature are transaminases converting methylketones.

The target of the present invention was to design mutant transaminases with increased transaminase activity relative to the wild-type transaminase. Preferably, they should be capable of accepting a wide spectrum of substrates, particularly bulky substrates, especially while maintaining stereoselectivity, as well as to use

iso-propylamine as amine donor. Accordingly, an increased activity is of particular relevance for bulky substrates (an exemplified selection is depicted in Schemes 2 and 3, see below) as well as for the amine donor *iso*-propylamine. These stereoselective transaminases can be used for the asymmetric synthesis of chiral amines from the respective ketones as well as for kinetic resolution.

Surprisingly, it has been found that a mutant transaminase comprising an amino acid sequence that is at least 65 % identical to the amino acid sequence of SEQ ID NO: 1 (*Ruegeria sp.* TM1040 transaminase; referred to as 3FCR) and having at least two amino acid substitutions relative to the wild-type transaminase, wherein the amino acid at the position corresponding to position 59 of SEQ ID NO: 1 is substituted with Trp or Phe (Trp59 or Phe59, respectively) and the amino acid at the position corresponding to position 231 of SEQ ID NO: 1 is substituted with Ala or Gly (Ala231 or Gly231, respectively) shows an increased transaminase activity relative to the wild-type transaminase.

As shown in the Examples, several amino acid residues in transaminases were identified whose substitution increases the transaminase activity of the mutant transaminase relative to the respective wild-type transaminase. Particularly, it could be shown that a double mutant of 3FCR, namely 3FCR with the substitution of Tyr with Trp at position 59 (Y59W) and the substitution of Thr with Ala at position 231 (T231A) of SEQ ID NO:1 (referred to as Y59W/T231A), had an increased activity towards substrates accepted by the wild-type (amines 3a and 4a as shown in Scheme 2), but also a low activity (~ 5 mU/mg) was determined towards amine 1a (see Tables 3 and 4). The results obtained with mutants of transaminase 3FCR could be confirmed with mutants of the transaminase of *Mesorhizobium loti maff303099* (referred to as 3GJU) (see Table 3). Moreover, 3FCR mutants with the additional substitution of Tyr with Phe at position 87 and/or the additional substitution of Tyr with Phe at position 152 of SEQ ID NO:1 (referred to as Y87F and Y152F) had an increased activity towards substrates with two aromatic rings (i.e. amines 1a, 3a and 4a) (see Y59W/Y87F/T231A and Y59W/Y87F/Y152F/T231A in Table 4). At the same time mutation Y152F increased the stability of the mutant significantly (Figure 1). An even higher increase in activity and the range of substrates accepted could be obtained by the

further substitution of Pro with His at position 423 of SEQ ID NO: 1 (referred to as P423H) in transaminases of 3FCR (see Tables 3 and 4). It could be shown that mutants with substitutions Y59W, Y87F, Y152F, T231A and P423H are particularly active on bulky substrates (such as amines 1a, 3a and 4a). Again, the results obtained with mutants of transaminase 3FCR could be confirmed with mutants of 3GJU (see Table 3). Moreover, mutant transaminases of ATA-3, ATA-5, ATA-6, ATA-7, ATA-8 and ATA-9 with mutations Y59W/Y87F/Y152F/T231A were shown to be active towards amines 1a, 3a, 4a and 6a (see Table 5). Please note that the wild-type sequences ATA-3 to ATA-9 have sequence identities to wild-type 3FCR in the range of from 65-70 % to approximately 90 %. Thus, it can be concluded that the concept of the present invention can be transferred to all transaminase having a sequence identity to the sequence of 3FCR (SEQ ID NO: 1) of 65 % or more. Additionally, 3FCR and 3GJU mutants which have an aliphatic hydrophobic amino acid in the position 87, such as Leu or Val (Y87L or Y87V, respectively), were found to have increased activity and were particularly effective for the acceptance of tertiary carbon substituents such as amine 2a (see Table 6). Mutants that have a phenylalanine in position 59 (Y59F) and/or a glycine in position 231 (T231G) show increased activity towards bicyclic compounds such as amine 5a (see Table 7). Moreover, 3FCR mutants with the substitution of Ile with Phe or Met at position 234 (referred to as I234F and I234M, respectively) had an increased activity towards amine 5a (see Table 7) and the amine donor *iso*-propylamine (see Table 8). Maintained stereoselectivity and suitability of the mutants in asymmetric synthesis and kinetic resolution was proven in Examples 5 to 11 (see Tables 9 to 10).

Accordingly, in a first aspect the present invention provides a mutant transaminase with increased transaminase activity relative to the wild-type transaminase, wherein the mutant transaminase comprises an amino acid sequence that is at least 65 % identical to the amino acid sequence of SEQ ID NO: 1 (*Ruegeria sp.* TM1040 transaminase; referred to as 3FCR) and wherein the mutant transaminase has at least two amino acid substitutions relative to the wild-type transaminase, wherein the amino acid at the position corresponding to position 59 of SEQ ID NO: 1 is substituted with Trp or Phe (Trp59 or Phe59, respectively) and

the amino acid at the position corresponding to position 231 of SEQ ID NO: 1 is substituted with Ala or Gly (Ala231 or Gly 231, respectively).

The term "transaminase" (classified as EC 2.6.1.XX by the Enzyme Commission of the International Union of Biochemistry; known also as aminotransferases) generally means an enzyme that catalyses the transfer of an amine group from an amine donor to the carbonyl group of an amine acceptor (transamination). Transaminases are pyridoxal-5'-phosphate dependent (PLP-dependent) enzymes. As seen in Scheme 1, the amine donor provides the amino group to the amine acceptor – so that the desired amine is synthesized – and a corresponding ketone is formed. As transaminases often posses a high stereoselectivity, the transamination reaction can provide the desired amine by asymmetric reduction and/or the remaining amine donor by resolution via oxidative deamination as enantiomerically enriched amine. ω-Transaminases (ω-TAs) are of particular interest in the present invention, as they are able to convert ketones without adjacent carboxylic function into chiral amines – in contrast to amino acid transaminases known for the synthesis of α-amino acids from α-keto acids. Note that the term amine transaminase (ATA) is preferred over ω-TA, as these enzymes were named for their ability to synthesize, e.g., L-lysine from the corresponding ε-aldehyde. (*S*)-selective ATAs have been already known since more than a decade (Coffen et al, 1994). The transaminase of the present invention may be either non-selective or selective, such as (*S*)-selective or (*R*)-selective, especially a (*S*)-selective transaminase. Suitable examples of these transaminases are also given in Table 1.

The term "wild-type transaminase" relates to a transaminase as it typically occurs in nature. Examples of naturally occurring wild-type transaminases are given below, in Table 1 and as SEQ ID NOs: 1, 3, and 5 to 11 (amino acid sequences). If one or more mutations are introduced a mutant transaminase is obtained. Therefore, the term "mutant transaminase" relates to a transaminase whose amino acid sequence is different from the wild-type sequence. With respect to the mutant transaminase of the present invention it is noted that the mutant is functionally active. This means that the mutant has maintained its biological function, i.e. its enzymatic activity of a transaminase. In accordance with the present invention, the

transaminase activity of the mutant is increased relative to the transaminase activity of the wild-type. The wild-type transaminase corresponding to a mutant is that wild-type transaminase which differs from the mutant by a minimal number of mutations. Exemplary wild-type transaminases which may be mutated according to the present invention are listed in the following:

- Aminotransferase (*Ruegeria sp.* TM1040) (GI: 499859271; referred to as 3FCR; SEQ ID NO: 1)
- Transaminase (*Mesorhizobium loti maff303099*) (GI: 499217058; referred to as 3GJU; SEQ ID NO: 3)
- Transaminase (*Oceanicola granulosus*) (GI: 494465841; referred to as ATA-3; SEQ ID NO: 5)
- Transaminase (*Jannaschia Sp CCS1*) (GI: 499773242; referred to as ATA-4; SEQ ID NO: 6)
- Transaminase (*Xanthobacteraceae*) (GI: 517199618; referred to as ATA-5; SEQ ID NO: 7)
- *Rhodobacteraceae bacterium* RB2150_13166) (GI: 126705951; referred to as ATA-6; SEQ ID NO: 8)
- Transaminase (*Martelella mediterranea* DSM 17316) (GI:516720233; referred to as ATA-7; SEQ ID NO: 9)
- Transaminase (*Rugeria pomeroyi* DSS-3) (GI:56677770; referred to as ATA-8; SEQ ID NO: 10)
- Transaminase (*Sagittula stellata* E-37) (GI:126711082; referred to as ATA-9; SEQ ID NO: 11)

Particularly preferred wild-type transaminases in which the mutations described herein may be introduced in accordance with the present invention are those specified in SEQ ID NO: 1, 3 and 5 to 11, particularly in SEQ ID NO: 1 and 3, especially SEQ ID NO: 1.

According to the present invention, the mutant transaminase has increased transaminase activity relative to the respective wild-type transaminase without mutation. Enzyme activity is a measure of the activity of enzyme. The SI unit for enzyme activity is katal (1 katal = 1 mol s^{-1}) A more practical and commonly used value is enzyme unit (U) = 1 µmol min^{-1}. 1 U corresponds to 16.67 nanokatals. One U is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute. The conditions when measuring the activity are usually standardized: one usually takes a temperature of 25 °C or 30 °C (as in the Examples) and the pH value and substrate concentration that yields the maximal substrate conversion rate. The specific activity of an enzyme is the

activity of an enzyme per milligram of total protein (expressed in µmol $min^{-1}mg^{-1}$). It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of total protein. Specific activity is equal to the rate of reaction multiplied by the volume of reaction divided by the mass of total protein. The SI unit is katal kg^{-1}, but a more practical unit is µmol min^{-1} mg^{-1}. Specific activity is a measure of enzyme processivity, at a specific (usually saturating) substrate concentration, and is usually constant for a pure enzyme. If the molecular weight of the enzyme is known, the turnover number, or µmol product sec^{-1} $µmol^{-1}$ of active enzyme, can be calculated from the specific activity. The turnover number can be visualized as the number of times each enzyme molecule carries out its catalytic cycle per second.

The activity may be determined in an enzyme assay measuring either the consumption of substrate or production of product over time. A large number of different methods of measuring the concentrations of substrates and products exist and many enzymes can be assayed in several different ways as known to the person skilled in the art. In the present invention, the transaminase in question is incubated with as suitable amine donor and a suitable amine acceptor under conditions and for a time conducive to the transamination.

Enzyme assays can be split into two groups according to their sampling method: continuous assays, where the assay gives a continuous reading of activity, and discontinuous assays, where samples are taken, the reaction stopped and then the concentration of substrates/products determined.

In a preferred embodiment, the transaminase of the present invention is stereoselective, such as (*S*)-selective or (*R*)-selective, particularly (*S*)-selective. "Stereoselectivity" refers to the preferential formation in a chemical or enzymatic reaction of one stereoisomer over another. Stereoselectivity can be partial, where the formation of one stereoisomer is favored over the other, or it may be complete where only one stereoisomer is formed. When the stereoisomers are enantiomers, the stereoselectivity is referred to as enantioselectivity. It is commonly reported in the art (typically as a percentage) as the enantiomeric excess (e.e.) calculated therefrom according to the formula [major enantiomer - minor enantiomer] / [major

enantiomer + minor enantiomer]. Alternatively, the enantiomeric ratio or er (S:R) may be used to characterize stereoselectivity. The enantiomeric ratio is the ratio of the percent of one enantiomer (e.g. the (*S*)-enantiomer) in a mixture of enantiomers to that of the other enantiomer (e.g. the (*R*)-enantiomer). Where the stereoisomers are diastereoisomers, the stereoselectivity is referred to as diastereoselectivity, the fraction (typically reported as a percentage) of one diastereomer in a mixture of two diastereomers, commonly alternatively reported as the diastereomeric excess (d.e.). Enantiomeric excess and diastereomeric excess are types of stereomeric excess.

Enantiomerically enriched in this context stands for an enantiomeric ratio of the desired chiral amine relative to the undesired chiral amine of more than 50:50, preferably at least 70:30, even more preferably at least 95:5, most preferably at least 99.5:0.5 or likewise for an enantiomeric excess greater than 0%, preferably at least 40%, even more preferably at least 90%, most preferably of at least 99%.

Surprisingly, it was found that the mutations as defined herein in the context of the present invention, which are introduced in wild-type transaminases, increase the transaminase activity of the enzyme relative to the wild-type enzyme. As detailed herein, this is of particular interest for the production of amines, particularly for the production of enantiomerically enriched chiral amines

either by method

a) a kinetic resolution of a racemic amine in the presence of an amine acceptor and a mutant transaminase or the fusion protein

or by method

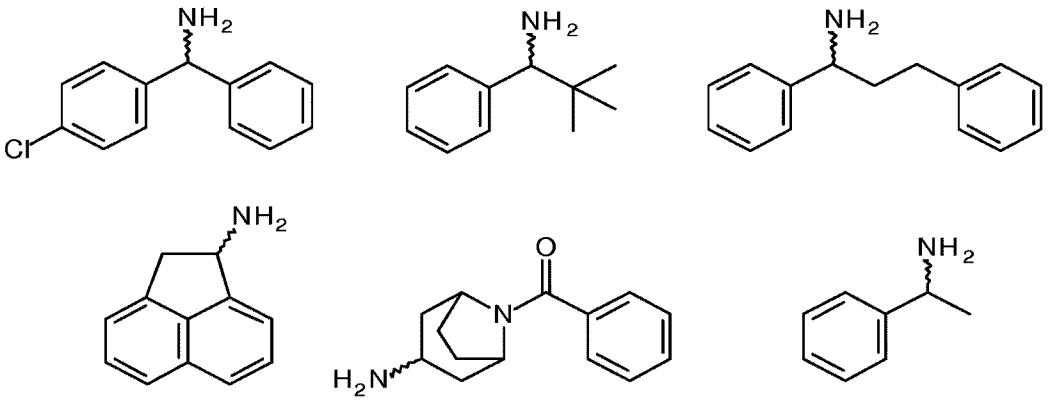
b) an asymmetric transamination of a prochiral ketone in the presence of an amine donor and a mutant transaminase or the fusion protein,

wherein the mutant transaminase or the fusion protein is stereoselective.

Particularly, the activity for selected compounds, e.g. bulky substrates, which increases the spectrum of substrates accepted by the transaminase, and/or the amine donor *iso*-propylamine is increased. This is important in the present invention, as it allows for broader industrial applicability especially in the transaminase-mediated production of amines such as chiral amines.

It was found that the transaminase showed increased activity, particularly on bulky substrates. Illustrative examples for bulky (racemic) amines according to method a) are shown in Scheme 2 below.

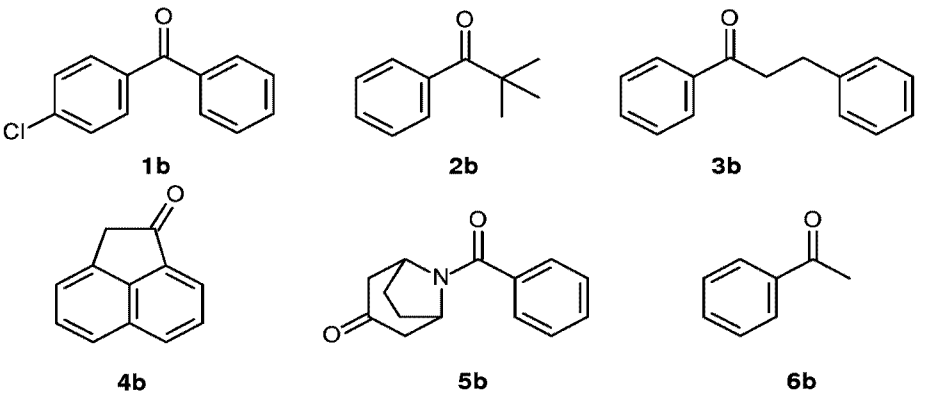
Scheme 2:



Their chemical names are 1-(4-chlorophenyl)-1-phenyl-1-aminomethane (1a), 2,2-dimethyl-1-phenyl-1-amino propane (2a), 1,3-diphenyl-1-aminopropane (3a), 1,2-dihydroacenaphthylen-1-amine (4a), 3-amino-8-benzoyl-8-azabicyclo[3.2.1]octane (5a) and 1-phenylethylamine (6a).

Illustrative examples for bulky prochiral ketones according to method b) are shown in Scheme 3 below.

Scheme 3:



Their chemical names are (4-chlorophenyl)-phenyl-methanone (1b), 2,2-dimethyl-1-phenyl-propan-1-one (2b) 1,3-diphenylpropan-1-one (3b), 2H-acenaphthylen-1-one (4b), 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one (5b) and 1-phenylethanone (6b).

There are various methods of determining enzyme activity well-known in the art. These include without limitation spectrometric assays, fluorometric assays, calorimetric assays, chemiluminescence assays, assays involving light scattering, radiometric assays, and chromatographic assays. Assays are usually performed under well controlled conditions including e.g. pH value, temperature, salt, buffers, and substrate concentration. Suitable tests for studying transaminase activity of an enzyme are well-known to the skilled person. A suitable test is described by Schätzle et al., in Analytical Chemistry (2009). This direct photometric assay is based on the significant difference in the absorption spectrum of the amine and its corresponding ketone. In general, the absorption spectrum of the conjugated aromatic ketones is distinct due to the electron delocalization of their benzoyl moiety, which is not existent in the corresponding amines. This difference is mirrored in a difference in the absorption spectrum, and the production of ketone can be monitored at the wavelength of the highest absorption difference between the ketone and the amine, which can be easily determined from someone trained in the art. This assay is particularly suitable for substrates 1, 2, 3, 4 and 6 [i.e. amines 1a, 2a, 3a, 4a and 6a (see Scheme 2) or ketones 1b, 2b, 3b, 4b and 6b (see Scheme 3)]. For substrates, for which the aforementioned assay may not be suitable, like substrate 5a/b or *iso*-propylamine, a suitable test was developed in order to screen amine donors; its principle was described by Weiß et al. in Analytical Chemistry (2014). In this test, glyoxylate is used as amine acceptor. The transaminase uses a desired amine donor to produce glycine by the amination of glyoxylate, and the glycine is subsequently oxidized by a glycine oxidase, producing also hydrogen peroxide. The hydrogen peroxide is used by horseradish peroxidase to oxidize phenol to 1,4-benzoquinone, which spontaneously reacts with 4-aminoantipyrine with a condensation reaction to form the quinone imine dye, which is detectable in visible wavelength (498 nm). The phenol can be substituted by vanillic acid, which undergoes an oxidative decarboxylation and oxidation mediated by the horseradish peroxidase to 2-methoxy-1,4-

benzoquinone. Apart from these two tests, standard chromatographic methods known to the person trained in the field, such as high performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis were also used.

Furthermore, an exemplary test is also described in the Examples (see section B2) and particularly suitable substrates are substrates 1 to 5 [as amines 1a to 5a (see Scheme 2) or ketones 1b to 5b (see Scheme 3)] or the amine donor *iso*-propylamine (2-propylamine).

Moreover, the person skilled in the art knows statistical procedures to assess whether or not one value is increased relative to another such as Student's t-test or chi-square test. It is evident for the skilled person that any background signal has to be subtracted when analyzing the data. In specific embodiments, the increase in enzyme activity is at least about 10 %. In other embodiments, the increase is at least 20 %, 30 %, 40 %, 50 % or 100 %, especially 150 %, 200 %, 250 %, or 300 %. Percentage increase in activity may be determined as [activity (mutant) / activity (wild-type) – 1] * 100.

In accordance with the present invention the mutant transaminase comprises an amino acid sequence that is at least 65 % identical to the amino acid sequence of SEQ ID NO: 1.

The term "SEQ ID NO: 1" as referred to herein denotes the amino acid sequence as shown in SEQ ID NO: 1 and represents the amino acid sequence of a wild-type aminotransferase from *Ruegeria sp.* TM1040 (referred to as 3FCR). In particular, the term "SEQ ID NO.: 1" refers to the amino acid sequence as set forth below: (please note that preferred substitution sites according to the present invention are indicated by bold/underline and specified by *position numbers*):

```
        10         20         30         40         50         60
MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGIK LLDAFAGLYC
                                                                59

        70         80         90        100        110        120
VNVGYGRQEI AEAIADQARE LAYYHSYVGH GTEASITLAK MILDRAPKNM SKVYFGLGGS
                           87
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       130        140        150        160        170        180
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI
                                 152
       190        200        210        220        230        240
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG TGGIVPPPAG
                                                    231  234
       250        260        270        280        290        300
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS

       310        320        330        340        350        360
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN

       370        380        390        400        410        420
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR

       430        440        450
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG
423
```

(SEQ ID NO: 1)

The term "at least 65 % identical" or "at least 65 % sequence identity" as used herein means that the sequence of the mutant transaminase according to the present invention has an amino acid sequence characterized in that, within a stretch of 100 amino acids, at least 65 amino acids residues are identical to the sequence of the corresponding wild-type sequence. Sequence identity according to the present invention can, e.g., be determined by methods of sequence alignment in form of sequence comparison. Methods of sequence alignment are well known in the art and include various programs and alignment algorithms which have been described in, e.g., Pearson and Lipman (1988). Moreover, the NCBI Basic Local Alignment Search Tool (BLAST) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Percentage of identity of mutants according to the present invention relative to the amino acid sequence of SEQ ID NO: 1 is typically characterized using the NCBI Blast blastp with standard settings. Alternatively, sequence identity may be determined using the software GENEious with standard settings. In the present invention, alignment results presented are derived from the Software Geneious (version R8), using the global alignment protocol with free end gaps as alignment type, and Blosum62 as a cost matrix. Identity of wild-type proteins towards to the SEQ ID NO: 1 (3FCR),

without tags included have been determined for 3GJU as 71.3 %, for ATA-3 as 72.2 %, ATA-4 as 70.7 %, for ATA-5 as 69.6 %, for ATA-6 as 71.8 %, for ATA-7 as 77.9 %, for ATA-8 as 90.8 %, and for ATA-9 as 80.3 %.

In accordance with the present invention, the mutant transaminase has at least two amino acid substitutions relative to the wild-type transaminase, wherein the amino acid at the position corresponding to position 59 of SEQ ID NO: 1 is substituted with Trp or Phe (Trp59 or Phe59, respectively) and the amino acid at the position corresponding to position 231 of SEQ ID NO: 1 is substituted with Ala or Gly (Ala231 or Gly231, respectively).

In a preferred embodiment, the mutant transaminase of the present invention has one or more further mutations, namely:

- the amino acid at the position corresponding to position 87 of SEQ ID NO: 1 is substituted with a hydrophobic amino acid (HYaa87), particularly wherein the hydrophobic amino acid is Leu (Leu87) or Val (Val87) or Phe (Phe87); and/or
- the amino acid at the position corresponding to position 152 of SEQ ID NO: 1 is substituted with Phe (Phe152), and/or
- the amino acid at the position corresponding to position 234 of SEQ ID NO: 1 is substituted with Phe (Phe234) or Met (M234), and/or
- the amino acid at the position corresponding to position 423 of SEQ ID NO: 1 is substituted with His (His423).

Positions of mutations are identified in the present invention with reference to SEQ ID NO: 1, i.e. the amino acid sequence of *Ruegeria sp.* TM1040 transaminase (referred to as 3FCR). The corresponding mutation sites of transaminases other than 3FCR can be identified by performing an amino acid alignment as detailed above (e.g. by using BLAST; Basic Local Alignment Search Tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome with standard settings) or by comparison of the structures, if available, and identifying the corresponding amino acid. Examples of corresponding positions are indicated in Figures 2 to 4 and in the following Table:

Transaminase	Position					
3FCR	59	87	152	231	234	423
3GJU	60	88	153	232	235	423
ATA-3	59	87	152	231	234	422
ATA-4	59	87	152	231	234	426
ATA-5	64	92	157	236	239	427
ATA-6	59	87	152	231	234	422
ATA-7	59	87	152	231	234	422
ATA-8	59	87	152	231	234	423
ATA-9	59	87	152	231	234	422

In one embodiment of the present invention, the mutant transaminase according to the present invention may comprise one or more amino acid deletion(s), particularly small (e.g. up to 10 amino acids) N- and/or C-terminal deletions.

In one embodiment, the sequence of the mutant transaminase according to the present invention may comprise, in addition to the substitutions specified herein one or more additional amino acid substitution(s), particularly one or more conservative amino acid substitutions. "Conservative amino acid substitution" refers to a substitution of a residue with a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid, e.g., alanine, valine, leucine, and isoleucine; an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain, e.g., serine and threonine; an amino acid having aromatic side chains is substituted with another amino acid having an aromatic side chain, e.g., phenylalanine, tyrosine, tryptophan, and histidine; an amino acid with a basic side chain is substituted with another amino acid with a basic side chain, e.g., lysine and arginine; an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain, e.g., aspartic acid or glutamic acid; and a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively. Examples of conservative amino acid substitutions include those listed below:

Original Residue	Conservative Substitutions
Ala, Leu, Val, Ile	Other aliphatic (Ala, Leu, Val, Ile) Other non-polar (Ala, Leu, Val, Ile, Gly, Met)
Gly, Met	Other non-polar (Ala, Leu, Val, Ile, Gly, Met)
Asp, Glu	Other acidic (Asp, Glu)
Lys, Arg	Other basic (Lys, Arg)
Asn, Gln, Ser, Thr	Other polar (Asn, Gln, Ser, Thr)
His, Tyr, Trp, Phe	Other aromatic (His, Tyr, Trp, Phe)
Cys, Pro	None

In one embodiment of the present invention, the mutant transaminase according to the present invention may comprise one or more amino acid addition(s), particularly small (e.g. up to 10 amino acids) internal amino acid additions.

In another embodiment, the sequence of the mutant transaminase according to the present invention may comprise, in addition to the substitutions specified herein a combination of one or more deletion(s), substitution(s) or addition(s) as defined above.

Yet, in another preferred embodiment, the mutant transaminase of the present invention has at least the mutations

- Trp59 and Ala231, or
- Trp59, Phe87 and Ala231; or
- Trp59, Leu87 and Ala231; or
- Trp59, Val87 and Ala231; or
- Trp59, Phe87, Ala231 and His423; or
- Trp59, Phe87, Phe152 and Ala231; or
- Trp59, Leu87, Phe152 and Ala231; or
- Trp59, Val87, Phe152 and Ala231; or
- Trp59, Phe87, Phe152, Ala231 and His423, or
- Trp59 and Gly231; or
- Trp59, Phe87 and Gly231; or
- Trp59, Leu87 and Gly231; or

- Trp59, Val87 and Gly231; or
- Trp59, Phe87, Phe152 and Gly231; or
- Trp59, Phe87, Phe152, Gly231 and His423; or
- Phe59, and Ala231; or
- Phe59, Phe87, and Gly231; or
- Trp59, Ala231 and Phe234; or
- Trp59, Ala231 and Met234; or
- Trp59, Phe87, Ala231 and Phe234; or
- Trp59, Leu87, Ala231 and Phe234; or
- Trp59, Val87, Ala231 and Phe234; or
- Trp59, Phe87, Phe152, Ala231 and Phe234; or
- Trp59, Phe87, Phe152, Ala231, Phe234 and His423, or
- Trp59, Gly231 and Phe234; or
- Trp59, Phe87, Gly231 and Phe234; or
- Trp59, Leu87, Gly231 and Phe234; or
- Trp59, Val87, Gly231 and Phe234; or
- Trp59, Phe87, Phe152, Gly231 and Phe234; or
- Trp59, Phe87, Phe152, Gly231, Phe234 and His423; or
- Phe59, Phe87, Gly231 and Phe234, or
- Trp59, Ala231 and Met234; or
- Trp59, Phe87, Ala231 and Met234; or
- Trp59, Leu87, Ala231 and Met234; or
- Trp59, Val87, Ala231 and Met234; or
- Trp59, Phe87, Phe152, Ala231 and Met234; or
- Trp59, Phe87, Phe152, Ala231, Met234 and His423, or
- Trp59, Gly231 and Met234; or
- Trp59, Phe87, Gly231 and Met234; or
- Trp59, Leu87, Gly231 and Met234; or
- Trp59, Val87, Gly231 and Met234; or
- Trp59, Phe87, Phe152, Gly231 and Met234; or
- Trp59, Phe87, Phe152, Gly231, Met234 and His423; or
- Phe59, Phe87, Gly231 and Met234.

More preferably, the mutant transaminase of the present invention differs from the corresponding wild-type transaminase only by the following mutations

- Trp59 and Ala231, or
- Trp59, Phe87 and Ala231; or
- Trp59, Leu87 and Ala231; or

- Trp59, Val87 and Ala231; or
- Trp59, Phe87, Ala231 and His423; or
- Trp59, Phe87, Phe152 and Ala231; or
- Trp59, Leu87, Phe152 and Ala231; or
- Trp59, Val87, Phe152 and Ala231; or
- Trp59, Phe87, Phe152, Ala231 and His423, or
- Trp59 and Gly231; or
- Trp59, Phe87 and Gly231; or
- Trp59, Leu87 and Gly231; or
- Trp59, Val87 and Gly231; or
- Trp59, Phe87, Phe152 and Gly231; or
- Trp59, Phe87, Phe152, Gly231 and His423; or
- Phe59, and Ala231; or
- Phe59, Phe87, and Gly231; or
- Trp59, Ala231 and Phe234; or
- Trp59, Ala231 and Met234; or
- Trp59, Phe87, Ala231 and Phe234; or
- Trp59, Leu87, Ala231 and Phe234; or
- Trp59, Val87, Ala231 and Phe234; or
- Trp59, Phe87, Phe152, Ala231 and Phe234; or
- Trp59, Phe87, Phe152, Ala231, Phe234 and His423, or
- Trp59, Gly231 and Phe234; or
- Trp59, Phe87, Gly231 and Phe234; or
- Trp59, Leu87, Gly231 and Phe234; or
- Trp59, Val87, Gly231 and Phe234; or
- Trp59, Phe87, Phe152, Gly231 and Phe234; or
- Trp59, Phe87, Phe152, Gly231, Phe234 and His423; or
- Phe59, Phe87, Gly231 and Phe234, or
- Trp59, Ala231 and Met234; or
- Trp59, Phe87, Ala231 and Met234; or
- Trp59, Leu87, Ala231 and Met234; or
- Trp59, Val87, Ala231 and Met234; or
- Trp59, Phe87, Phe152, Ala231 and Met234; or
- Trp59, Phe87, Phe152, Ala231, Met234 and His423, or
- Trp59, Gly231 and Met234; or
- Trp59, Phe87, Gly231 and Met234; or
- Trp59, Leu87, Gly231 and Met234; or
- Trp59, Val87, Gly231 and Met234; or

- Trp59, Phe87, Phe152, Gly231 and Met234; or
- Trp59, Phe87, Phe152, Gly231, Met234 and His423; or
- Phe59, Phe87, Gly231 and Met234.

In an even more preferred embodiment, the mutant transaminase of the present invention has at least the mutations

- Trp59, Phe87, Phe152 and Ala231, or
- Trp59, Phe87, Phe152, Ala231 and Met234, or
- Trp59, Phe87, Phe152, Ala231 and H423, or
- Phe59, Phe87 and Gly231.

More preferably, the mutant transaminase of the present invention differs from the corresponding wild-type transaminase only by the following mutations

- Trp59, Phe87, Phe152 and Ala231, or
- Trp59, Phe87, Phe152, Ala231 and Met234, or
- Trp59, Phe87, Phe152, Ala231 and H423, or
- Phe59, Phe87 and Gly231.

Preferred and specific amino acid sequences of mutant transaminase are those comprising or consisting of the sequence as defined in any of SEQ ID NO: 30 to 38, 43 to 51, 53, 55 and 58-64 (see Figures 3, 4 and 5) and 57 (see below section "SEQUENCES").

As detailed above, the mutant transaminase of the present invention comprises an amino acid sequence that is at least 65 % identical to the amino acid sequence of SEQ ID NO: 1. In a preferred embodiment, the mutant transaminase comprises an amino acid sequence that is at least 70 %, 75 %, 80 %, 85 %, 90 %, 95 %, 96 %, 97 %, 98 %, or 99 % identical to the amino acid sequence of SEQ ID NO: 1. Sequence identity may be determined as described above. In a preferred embodiment of the present invention, the mutant transaminase comprises an amino acid sequence that is at least 90 %, 95 %, 96 %, 97 %, 98 %, or 99 % identical to the amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 3 and 5 to 11.

In accordance with the present invention, the mutant transaminase has an increased transaminase activity relative to the corresponding wild type enzyme. As detailed above, this is of particular interest with bulky substrates as well as the amine donor *iso*-propylamine. Accordingly, increased activity for bulky substrates/*iso*-propylamine is preferred in the context of the present invention. Suitable bulky test substrates for determining whether activity for these is increased are substrates 1 to 5 include those shown in Scheme 2, namely 1-(4-chlorophenyl)-1-phenyl-1-aminomethane (1a), 2,2-dimethyl-1-phenyl-1-amino propane (2a), 1,3-diphenyl-1-aminopropane (3a), 1,2-dihydroacenaphthylen-1-amine (4a), and 3-amino-8-benzoyl-8-azabicyclo[3.2.1]octane (5a) as well as those shown in Scheme 3 (4-chlorophenyl)-phenyl-methanone (1b), 2,2-dimethyl-1-phenyl-propan-1-one (2b) 1,3-diphenylpropan-1-one (3b), 2H-acenaphthylen-1-one (4b), and 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one (5b). The skilled person will understand that an increase in activity is already present if the activity with respect to at least one of substrate or *iso*-propylamine, particularly at least one of substrate 1 to 5 or *iso*-propylamine, is increased.

Accordingly, in a preferred embodiment of the present invention, the mutant transaminase has increased transaminase activity for transamination of at least one compound selected from the group consisting of (1-(4-chlorophenyl)-1-phenyl-1-aminomethane (1a), 2,2-dimethyl-1-phenyl-1-amino propane (2a), 1,3-diphenyl-1-aminopropane (3a), 1,2-dihydroacenaphthylen-1-amine (4a), and 3-amino-8-benzoyl-8-azabicyclo[3.2.1]octane (5a), 3(4-chlorophenyl)-phenyl-methanone (1b), 2,2-dimethyl-1-phenyl-propan-1-one (2b) 1,3-diphenylpropan-1-one (3b), 2H-acenaphthylen-1-one (4b), 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one (5b) and *iso*-propylamine.

In a preferred embodiment, the mutant transaminase has an at least 2-fold increased transaminase activity relative to the corresponding wild type enzyme, preferably an at least 2.5-fold increased transaminase activity, preferably an at least 3-fold increased transaminase activity, more preferably an at least 3.5-fold increased transaminase activity, and most preferably an at least 4-fold increased transaminase activity, particularly for at least one compound selected from the group consisting of 1-(4-chlorophenyl)-1-phenyl-1-aminomethane (1a), 2,2-

dimethyl-1-phenyl-1-amino propane (2a), 1,3-diphenyl-1-aminopropane (3a), 1,2-dihydroacenaphthylen-1-amine (4a), and 3-amino-8-benzoyl-8-azabicyclo[3.2.1]octane (5a), 3(4-chlorophenyl)-phenyl-methanone (1b), 2,2-dimethyl-1-phenyl-propan-1-one (2b) 1,3-diphenylpropan-1-one (3b), 2H-acenaphthylen-1-one (4b), 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one (5b) and *iso*-propylamine.

In a further aspect, the present invention relates to a fusion protein comprising the transaminase of the present invention.

Fusion proteins are proteins created by joining of two or more originally separate proteins or peptides. This procedure results in a polypeptide with functional properties derived from each of the original proteins. Accordingly, depending on the intended use of the transaminase it may be combined with a further peptide or protein into a fusion protein. The proteins may be fused via a linker or spacer, which increases the likelihood that that the proteins fold independently and behave as expected. Especially in the case where the linkers enable protein purification, linkers in protein or peptide fusions are sometimes engineered with cleavage sites for proteases or chemical agents that enable the liberation of the two separate proteins. Di- or multimeric fusion proteins can be manufactured through genetic engineering by fusion to the original proteins of peptide domains that induce artificial protein di- or multimerization (e.g., streptavidin or leucine zippers). Fusion proteins can also be manufactured with toxins or antibodies attached to them. Other fusions include the addition the addition of signal sequences, such a lipidation signal, sequence, a secretion signal sequence, a glycosylation signal sequence, a translocation signal peptide etc.

Preferably, the fusion protein of the present invention comprises a tag. Tags are attached to proteins for various purposes, e.g. in order to ease purification, to assist in the proper folding in proteins, to prevent precipitation of the protein, to alter chromatographic properties, to modify the protein or to mark or label the protein.

A number of (affinity) tags or (affinity) markers are known at present. These are usually divided into 3 classes according to their size: small tags have a maximum

of 12 amino acids, medium-sized ones have a maximum of 60 and large ones have more than 60. The small tags include the Arg-tag, the His-tag, the Strep-tag, the Flag-tag, the T7-tag, the V5-peptide-tag and the c-Myc-tag, the medium-sized ones include the S-tag, the HAT-tag, the calmodulin-binding peptide, the chitin-binding peptide and some cellulose-binding domains. The latter can contain up to 189 amino acids and are then regarded, like the GST- and MBP-tag, as large affinity tags. In order to produce especially pure proteins, so-called double tags or tandem tags were developed. In this case the proteins are purified in two separate chromatography steps, in each case utilizing the affinity of a first and then of a second tag. Examples of such double or tandem tags are the GST-His-tag (glutathione-S-transferase fused to a polyhistidine-tag), the 6xHis-Strep-tag (6 histidine residues fused to a Strep-tag (see below)), the 6xHis-tag100-tag (6 histidine residues fused to a 12-amino-acid protein of mammalian MAP-kinase 2), 8xHis-HA-tag (8 histidine residues fused to a haemagglutinin-epitope-tag), His-MBP (His-tag fused to a maltose-binding protein, FLAG-HA-tag (FLAG-tag fused to a hemagglutinin-epitope-tag), and the FLAG-Strep-tag. Most of these tags have been specifically developed for the purification of proteins produced by prokaryotic cells. Suitable tags are given in the description and include the one SEQ ID NO: 56 (SHHHHHH).

In a further aspect, the present invention relates to a nucleic acid encoding the transaminase of the present invention.

The term "nucleic acid" as used herein generally relates to any nucleotide molecule which encodes the mutant transaminase of the invention and which may be of variable length. Examples of a nucleic acid of the invention include, but are not limited to, plasmids, vectors, or any kind of DNA and/or RNA fragment(s) which can be isolated by standard molecular biology procedures, including, e.g. ion-exchange chromatography. A nucleic acid of the invention may be used for transfection or transduction of a particular cell or organism.

Nucleic acid molecule of the present invention may be in the form of RNA, such as mRNA or cRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA e.g. obtained by cloning or produced by chemical synthetic techniques or by

a combination thereof. The DNA may be triple-stranded, double- stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand. Nucleic acid molecule as used herein also refers to, among other, single- and double- stranded DNA, DNA that is a mixture of single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded, or a mixture of single- and double-stranded regions. In addition, nucleic acid molecule as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA.

Additionally, the nucleic acid may contain one or more modified bases. Such nucleic acids may also contain modifications e.g. in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "nucleic acid molecule" as that feature is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are nucleic acid molecule within the context of the present invention. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term nucleic acid molecule as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of nucleic acid molecule, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, inter alia.

Furthermore, the nucleic acid molecule encoding the mutant transaminase of the invention can be functionally linked, using standard techniques such as standard cloning techniques, to any desired sequence, such as a regulatory sequence, leader sequence, heterologous marker sequence or a heterologous coding sequence to create a fusion protein.

The nucleic acid of the invention may be originally formed *in vitro* or in a cell in culture, in general, by the manipulation of nucleic acids by endonucleases and/or

exonucleases and/or polymerases and/or ligases and/or recombinases or other methods known to the skilled practitioner to produce the nucleic acids.

The nucleic acid of the invention may be comprised in an expression vector, wherein the nucleic acid is operably linked to a promoter sequence capable of promoting the expression of the nucleic acid in a host cell.

As used herein, the term "expression vector" generally refers to any kind of nucleic acid molecule that can be used to express a protein of interest in a cell (see also above details on the nucleic acids of the present invention). In particular, the expression vector of the invention can be any plasmid or vector known to the person skilled in the art which is suitable for expressing a protein in a particular host cell including, but not limited to, mammalian cells, bacterial cell, and yeast cells. An expression construct of the present invention may also be a nucleic acid which encodes a transaminase of the invention, and which is used for subsequent cloning into a respective vector to ensure expression. Plasmids and vectors for protein expression are well known in the art, and can be commercially purchased from diverse suppliers including, e.g., Promega (Madison, WI, USA), Qiagen (Hilden, Germany), Invitrogen (Carlsbad, CA, USA), or MoBiTec (Germany). Methods of protein expression are well known to the person skilled in the art and are, e.g., described in Sambrook et al., 2000 (Molecular Cloning: A laboratory manual, Third Edition).

The vector may additionally include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication, one or more therapeutic genes and/or selectable marker genes and other genetic elements known in the art such as regulatory elements directing transcription, translation and/or secretion of the encoded protein. The vector may be used to transduce, transform or infect a cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell. The vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. Numerous types of appropriate expression vectors are known in the art for protein expression, by standard molecular biology techniques. Such vectors are selected from among conventional vector types including insects, e.g.,

baculovirus expression, or yeast, fungal, bacterial or viral expression systems. Other appropriate expression vectors, of which numerous types are known in the art, can also be used for this purpose. Methods for obtaining such expression vectors are well-known (see, e.g. Sambrook et al, supra).

As detailed above, the nucleic acid which encodes a mutant transaminase of the invention is operably linked to sequence which is suitable for driving the expression of a protein in a host cell, in order to ensure expression of the protein. However, it is encompassed within the present invention that the claimed expression construct may represent an intermediate product, which is subsequently cloned into a suitable expression vector to ensure expression of the protein. The expression vector of the present invention may further comprise all kind of nucleic acid sequences, including, but not limited to, polyadenylation signals, splice donor and splice acceptor signals, intervening sequences, transcriptional enhancer sequences, translational enhancer sequences, drug resistance gene(s) or alike. Optionally, the drug resistance gene may be operably linked to an internal ribosome entry site (IRES), which might be either cell cycle-specific or cell cycle-independent.

The term "operably linked" as used herein generally means that the gene elements are arranged as such that they function in concert for their intended purposes, e.g. in that transcription is initiated by the promoter and proceeds through the DNA sequence encoding the protein of the present invention. That is, RNA polymerase transcribes the sequence encoding the fusion protein into mRNA, which in then spliced and translated into a protein.

The term "promoter sequence" as used in the context of the present invention generally refers to any kind of regulatory DNA sequence operably linked to a downstream coding sequence, wherein said promoter is capable of binding RNA polymerase and initiating transcription of the encoded open reading frame in a cell, thereby driving the expression of said downstream coding sequence. The promoter sequence of the present invention can be any kind of promoter sequence known to the person skilled in the art, including, but not limited to, constitutive

promoters, inducible promoters, cell cycle-specific promoters, and cell type-specific promoters.

Another aspect of the present invention relates to a host cell comprising the transaminase of the present invention, the fusion protein of the present invention, or the polynucleotide or expression vector of the present invention. In one embodiment of the present invention, a host cell having transaminase activity is used in the context of the present invention. Particularly, a cell optionally lysed or otherwise permeabilized is used for transamination, e.g. in the methods and uses of the present invention. A "host cell" of the present invention can be any kind of organism suitable for application in recombinant DNA technology, and includes, but is not limited to, all sorts of bacterial and yeast strain which are suitable for expressing one or more recombinant protein(s). Examples of host cells include, for example, various *Bacillus subtilis* or *E. coli* strains. A variety of *E. coli* bacterial host cells are known to a person skilled in the art and include, but are not limited to, strains such as DH5-alpha, HB101, MV1190, JM109, JM101, or XL-1 blue which can be commercially purchased from diverse suppliers including, e.g., Stratagene (CA, USA), Promega (WI, USA) or Qiagen (Hilden, Germany). A particularly suitable host cell is also described in the Examples, namely *E. coli* BL21 (DE3) cells. *Bacillus subtilis* strains which can be used as a host cell include, e.g., 1012 wild type: leuA8 metB5 trpC2 hsdRM1 and 168 Marburg: trpC2 (Trp-), which are, e.g., commercially available from MoBiTec (Germany).

The cultivation of host cells according to the invention is a routine procedure known to the skilled person. That is, a nucleic acid encoding a mutant transaminase of the invention can be introduced into a suitable host cell(s) to produce the respective protein by recombinant means. These host cells can by any kind of suitable cells, preferably bacterial cells such as *E. coli*, which can be cultivated in culture. At a first step, this approach may include the cloning of the respective gene into a suitable plasmid vector. Plasmid vectors are widely used for gene cloning, and can be easily introduced, i.e. transfected, into bacterial cells which have been made transiently permeable to DNA. After the protein has been expressed in the respective host cell, the cells can be harvested and serve as the starting material for the preparation of a cell extract containing the protein of

interest. A cell extract containing the protein of interest is obtained by lysis of the cells. Methods of preparing a cell extract by means of either chemical or mechanical cell lysis are well known to the person skilled in the art, and include, but are not limited to, e.g. hypotonic salt treatment, homogenization, or ultrasonification.

In a further aspect, the present invention provides a method of producing an amine comprising reacting an amine acceptor with the mutant transaminase of the present invention or the fusion protein of the present invention in the presence of an amine donor.

The reaction of the present invention follows in principle the scheme presented in Scheme 1 (see above). The scheme illustrates that an amino group is transferred from the amine donor to the amine acceptor. The amine donor may be any molecule comprising a transferable amino group which is accepted by the mutant transaminase or fusion protein of the present invention. The amine acceptor may be any molecule to which the amino group may be transferred by the mutant transaminase or fusion protein of the present invention. In general, the amino group (NH_2) will be transferred by the transaminase from a primary amine (amine donor) to a carbonyl group (C=O) (amine acceptor). The terms "amine acceptor" and "amino acceptor" on the one hand as well as "amine donor" and "amino donor" on the other hand are used interchangeably.

In a preferred embodiment an enantiomerically enriched chiral amine is produced and

the method is either

a) a kinetic resolution of a racemic amine in the presence of an amine acceptor and a mutant transaminase or the fusion protein or

b) an asymmetric transamination of a prochiral ketone in the presence of an amine donor and a mutant transaminase or the fusion protein,

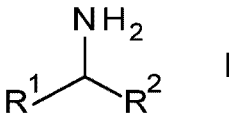
wherein the mutant transaminase or the fusion protein is stereoselective.

It is hereby understood that the mutant transaminase or fusion protein might be applied as solution, lyophilisate, immobilized or whole cell catalyst.

Method a)

In this method, referred to as kinetic resolution, the enantiomers of the racemic amine react with different reaction rates with the stereoselective transaminase or fusion protein (optionally in a host cell), resulting in an enantiomerically enriched composition of the less reactive enantiomer. Accordingly, the racemic mixture of amines is enantiomerically enriched for the desired enantiomer.

The racemic amine may have the formula



I

wherein

R^1 or R^2 independently of each other represent optionally substituted alkyl, aryl carbocyclyl or heterocyclyl; or

R^1 and R^2 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring.

In a preferred embodiment

R^1 is optionally substituted C_{1-12}-alkyl or aryl;

R^2 is optionally substituted aryl or heterocyclyl; or

R^1 and R^2 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring, wherein optional substituents are selected from C_{1-12}-alkyl, C_{1-12}-alkoxy, aryl, aryloxy, halogen, hydroxyl or cyano.

In a further preferred embodiment

R^1 is optionally substituted C_{1-7}-alkyl or phenyl;

R^2 is optionally substituted phenyl; or

R^1 and R^2 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring, wherein optional substituents are selected from C_{1-7}-alkyl, C_{1-7}-alkoxy, phenyl, phenyloxy, chlorine hydroxyl or cyano.

Illustrative examples of amines are shown in Scheme 2 (above).

Suitable amine acceptors for the kinetic resolution in method a) can be selected from ketones and keto carboxylic acids. Preferably, keto carboxylic acids such as the 2-keto carboxylic acids like 2-ketoglutaric acid, glyoxylic acid, pyruvic acid, oxaloacetic acid, and the like, as well as suitable salts thereof are used. Mostly used and therefore most preferred are the conjugate bases of the 2-keto carboxylic acids such as 2-ketoglutarate, pyruvate, glyoxylate or oxaloate.

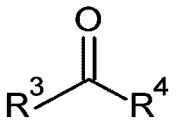
The kinetic resolution of the racemic amine with the mutant transaminase or fusion protein is expediently effected in an aqueous medium, suitably containing a physiological buffer such as the CHES buffer at a pH in the range from 5 to 11, in particular from 7.5 to 10 in a temperature range from 10° to 50°C, preferably from 20°C to 40°C.

The molar ratio of racemic amine to amine acceptor is, as a rule, selected in a molar ratio from 1.1 to 10, in particular from 1.1 to 2.5, when a 2-keto carboxylic acid is used as amine acceptor. When using ketones as amine acceptors the molar ratio can be significantly increased.

Method b)

Method b requires an asymmetric transamination of a prochiral ketone in the presence of an amine donor and a stereoselective mutant transaminase or the fusion protein (optionally in a host cell).

The prochiral ketone may have the formula



II

R^3 or R^4 independently of each other represent optionally substituted alkyl, aryl, carbocyclyl or heterocyclyl; or

R^3 and R^4 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring.

In a preferred embodiment
R^3 is optionally substituted C_{1-12}-alkyl or aryl;
R^4 is optionally substituted aryl or heterocyclyl; or
R^3 and R^4 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring, wherein optional substituents are selected from C_{1-12}-alkyl, C_{1-12}-alkoxy, aryl, aryloxy, halogen, hydroxyl or cyano.

In a further preferred embodiment
R^3 is optionally substituted C_{1-7}- alkyl or phenyl;
R^4 is optionally substituted phenyl; or
R^3 and R^4 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring, wherein optional substituents are selected from C_{1-7}-alkyl, C_{1-7}-alkoxy, phenyl, phenyloxy, chlorine hydroxyl or cyano.

Illustrative examples for prochiral ketones are shown in Scheme 3 (above).

Suitable amine donors may in general be selected from achiral or chiral amines or amino acids. Typical amines are primary aliphatic amines such as *iso*-propylamine, 1-phenylethylamine, 2-amino-4-phenylbutane, *o*-xylylene diamine or amino acids such as the alpha amino acids glycine, glutamic acid or alanine. Preferred amine donors are *iso*-propylamine and L-alanine.

In a preferred embodiment of the present invention, the amine acceptor and the amine donor are reacted with the mutant transaminase or fusion protein using a system that favors the equilibrium towards the desired enantiomerically enriched chiral amine.

Various biocatalytic strategies were employed to shift the equilibrium of the asymmetric transamination by removing the formed co-product, e.g. pyruvate, by a

second enzyme, e.g., lactate dehydrogenase, pyruvate decarboxylase, or recycling/removing via alanine dehydrogenase.

As multi enzymes systems are applied, the reaction conditions need to satisfy the requirements of all the involved enzymes. Expediently the reaction takes place in an aqueous medium, suitably containing a physiological buffer such as the CHES buffer at a pH in the range from 5 to 11, in particular from 7.5 to 10 in a temperature range from 10° to 50°C, preferably from 20°C to 40°C.

The molar ratio of the prochiral ketone and the amine donor is dependent on the second enzyme used and the individual reaction conditions. As a rule the molar ratio prochiral ketone and the amine donor can be selected in a range from 1:100 to 1:5, in particular from 1:50 to 1:10.

Applying a large excess of *iso*-propylamine as the preferred amine donor shifts the equilibrium in the asymmetric transamination to the formation of the target amine especially in combination with *in-situ* removal of the formed acetone, its corresponding ketone. The addition of organic co-solvents, water miscible and immiscible, or other specific additives might improve the reaction.

The terms as used herein for the racemic amine of formula I and for the prochiral ketone of formula II have the following meaning.

The term "halogen" denotes fluoro, chloro, bromo, or iodo, particularly chlorine.

The term "alkyl" denotes a monovalent linear or branched saturated hydrocarbon group of 1 to 12 carbon atoms. In particular embodiments, alkyl has 1 to 7 carbon atoms, and in more particular embodiments 1 to 4 carbon atoms. Examples of alkyl include methyl, ethyl, propyl, *iso*-propyl, *n*-butyl, *iso*-butyl, *sec*-butyl, or *tert*-butyl.

The term "alkoxy" refers to an alkyl group as defined above which is attached to an oxygen radical. Examples of alkoxy include methoxy, ethoxy, *iso*-propoxy, *n*-butoxy, *iso*-butoxy, *sec*-butoxy, or *tert*-butoxy.

The term "aryl" denotes a monovalent aromatic carbocyclic mono- or bicyclic ring system comprising 6 to 10 carbon ring atoms. Examples of aryl moieties include phenyl and naphthyl.

The term "aryloxy" denotes an aryl group as defined above which is attached to an oxygen radical. A suitable example is phenoxy or naphthyloxy.

The term "mono- or poly-cyclic ring" refers to a compound featuring one or more closed rings of atoms, primarily carbon. These ring substructures include cycloalkanes, aromatics, and other ring types. Though "poly" literally means "many", it also includes bicyclic, tricyclic, tetracyclic, etc.

The term "carbocyclic" refers to a saturated, partially unsaturated or unsaturated cyclic compound in which all of the ring members are carbon atoms, such as cyclohexane, decaline or 1,2-dihydronaphthalene. The compound may be aromatic or non-aromatic. Simple aromatic rings consist only of a conjugated planar ring system. Typical simple aromatic compounds are benzene, indole, and cyclotetradecaheptaene. Polycyclic aromatic hydrocarbons contain only carbon and hydrogen and are composed of multiple aromatic rings. Examples include naphthalene, anthracene and phenanthrene.

The term "heterocyclyl" refers to a saturated, partially unsaturated or unsaturated 5 to 6 membered monocyclic ring or 8 to 10 membered bicyclic ring which can comprise 1, 2 or 3 heteroatoms selected from nitrogen, oxygen and/or sulphur. The ring system may be aromatic or non-aromatic. Typical heterocyclyl residues are pyridinyl, piperidinyl, pyrrolidyl, pyrimidinyl, furanyl, pyranyl, benzoimidazolyl, quinolinyl or isoquinolinyl.

Optional substituents as used herein for the racemic amine of formula I and for the prochiral ketone of formula II can be selected from alkyl, alkoxy, aryl, aryloxy, halogen, hydroxyl or cyano. The preferences and examples as outlined above apply for the substituents as well.

In another aspect the present invention relates to the use of a mutant transaminase of to the present invention or the fusion protein of the present invention for the transamination of a ketone, particularly for the synthesis of a chiral amine having an asymmetric carbon atom bound to the transferred amino group.

With respect to the use of the present invention it is referred to the terms, examples and specific embodiments used in the context of the other aspects of the present disclosure, which are also applicable to this aspect. Particularly, the mutant transaminase or fusion protein according to the present invention may be used as detailed with respect to the methods of the present invention.

Unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the invention. Definitions of common terms in molecular biology can be found in Benjamin Lewin, Genes V, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1 -56081 -569-8).

The invention is not limited to the particular methodology, protocols, and reagents described herein because they may vary. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred methods, and materials are described herein. Further, the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Similarly, the words "comprise", "contain" and "encompass" are to be interpreted inclusively

rather than exclusively. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "plurality" refers to two or more.

The following Figures and Examples are intended to illustrate various embodiments of the invention. As such, the specific modifications discussed are not to be construed as limitations on the scope of the invention. It will be apparent to the person skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of the invention, and it is thus to be understood that such equivalent embodiments are to be included herein.

FIGURES

Figure 1: Thermal stability of 3FCR variants at 25ºC in HEPES buffer (50 mM) pH 7.5; 3FCR Y59W/T231A (open circle), 3FCR Y59W/Y87F/T231A (filled circle), 3FCR Y59W/Y87F/Y152F/T231A (triangle), 3FCR Y59W/Y87F/Y152F/T231A/P423H (square). Activity was evaluated in kinetic resolution mode as described in paragraph B2, using 6a as amine donor, and the activity prior to the incubation was defined as 100%.

Figure 2: Sequence alignment of preferred wild-type transaminases 3FCR, 3GJU, ATA-3, ATA-4, ATA5, ATA-6, ATA-7, ATA-8 and ATA-9. Sites for mutations Trp59, Phe87, Phe152, Ala231, Met234 and His423 according to the present invention are indicated in each transaminase by underline.

Figure 3: Sequence alignment of preferred specific mutant transaminases 3FCR, 3GJU, ATA-3, ATA-4, ATA5, ATA-6, ATA-7, ATA-8 and ATA-9 (each having 4 amino acids substituted as indicated). Mutations Trp59 (Y59W), Phe87 (Y87F), Phe152 (Y152F) and Ala231 (T231A) according to the present invention are indicated in each transaminase by underline.

Figure 4: Sequence alignment of preferred specific mutant transaminases 3FCR, 3GJU, ATA-3, ATA-4, ATA5, ATA-6, ATA-7, ATA-8 and ATA-9 (each having 5 amino acids substituted as indicated). Mutations Trp59 (Y59W), Phe87 (Y87F),

Phe152 (Y152F), Ala231 (T231A) and His423 (P423H) according to the present invention are indicated in each transaminase by underline.

Figure 5: Sequence alignment of preferred specific mutant transaminases 3FCR, 3GJU, ATA-3, ATA-4, ATA5, ATA-6, ATA-7, ATA-8 and ATA-9 (each having 5 amino acids substituted as indicated). Mutations Trp59 (Y59W), Phe87 (Y87F), Phe152 (Y152F), Ala231 (T231A) and Met234 (I234M) according to the present invention are indicated in each transaminase by underline.

EXAMPLES

GENERAL PROCEDURES

A Biocatalyst Production

A1 Transaminase Gene Acquisition and Construction of Expression Vectors

Transaminase (TA) open reading frames were designed and synthesized for expression in *Escherichia coli* (*E. coli*), based on the reported amino acid sequence of the transaminase (NCBI GI codes provided in Table 1), and the codon optimization algorithm of GenScript (Piscataway, U.S.A.), OptimumGene™. A C-terminal histidine tag, with a stop codon in the end was added in all cases, in order to facilitate the purification of the expressed TA, through a metal affinity chromatography (see respective paragraph). Restriction sites for the subsequent cloning in the vector of interest, pET22b, were added in the nucleotide sequence; NdeI restriction in the 5' end, and the BamHI restriction sequence in the 3' end. The starting codon (ATG) was incorporated in the restriction site of NdeI, so that there is only one methionine coded. The vector contains the *bla* coding sequence (beta-lactamase) for ampicillin resistance. According to the cloning strategy, the expression is under the control of a *lac* promoter. Resulting plasmids were transformed into *E. coli* BL21 (DE3) using standard methods. Sequences of the codon optimized genes and encoded polypeptides are provided in section "SEQUENCES".

The mutants used in the present invention were prepared with a modified version of the QuikChange® PCR method. Primers were designed with mismatches that provide the desired mutations. For each reaction, we used Pfu buffer, 0.2 mM dNTPs, 0.2 ng/µL parental plasmid, 0.2 µM of each primer, 2 % (w/w) DMSO and 0.2 µL of Pfu Plus! polymerase. The amplification was performed as follows:

a) 95°C, 5 min;

b) 20 cycles: 95°C, 45 sec; 60°C, 45 sec; 72°C, 7 min;

c) 72°C, 14 min

The PCR product was digested by DpnI (20 µL/mL) for 2 h at 37°C and then the restriction enzyme was deactivated by heating at 80ºC for 20 min. The digested PCR product was transformed into *E. coli* TOP-10 cells using standard methods. Plasmid isolation was performed from the clones transformed with the PCR product and the correct sequence was confirmed by DNA sequencing. The isolated plasmid with the correct mutations was transformed into *E. coli* BL21 (DE3) cells, using standard methods.

Table 1: Transaminases that were used in the present invention, and the NCBI gene identifier code (GI) of the wild type sequence.

Name	Species	NCBI (GI)
3FCR	*Ruegeria sp. TM1040*	499859271
3GJU	*Mesorhizobium loti maff303099*	499217058
ATA-3	*Oceanicola granulosus*	494465841
ATA-5	*Xanthobacteraceae*	517199618
ATA-6	*Rhodobacteraceae bacterium* RB2150_13166	126705951
ATA-7	*Martelella mediterranea* DSM 17316	516720233
ATA-8	*Rugeria pomeroyi* DSS-3	56677770
ATA-9	*Sagittula stellata* E-37	126711082

A2 Production of Transaminases

A single microbial colony of *E. coli* BL21 (DE3) was inoculated into 3 mL Luria Bertani medium in a testing tube, containing 100 µg/mL ampicillin, and was grown overnight (about 18 h) at 30°C with shaking at 180 rpm. In a 300 mL shake flask,

30 mL of Terrific Broth containing 100 µg/mL ampicillin were inoculated with 1 % (v/v) of the overnight culture (0.3 mL), and incubated at 37°C with shaking at 180 rpm to an optical density at 600 nm (OD_{600}) of 0.5 to 0.7. The culture was cooled down to 20°C and isopropyl β D-thiogalactoside (IPTG) was added at a final concentration of 0.2 mM to induce the expression of the TA. Incubation continued overnight at 20°C with shaking, for about 16 h more. Cells were harvested via centrifugation (6000 *g*, 10 min, 4°C) and the supernatant was discarded. The cell pellet was resuspended in 6 mL pre-cooled (4°C) HEPES buffer (50 mM, pH 7.5), containing 0.1 mM pyridoxal-5-phosphate (PLP), and was treated with sonication (Bandelin Sonopuls HD2070; 2 times of 5 min with 5 min interval, 50 % pulse, 60 % power, kept always on ice) in order to lyse the cells. Cell debris was removed by centrifugation (6000 *g*, 30 min, 4°C). The lysate supernatant was filtered with 0.2 µm filters (Millipore) and was stored at +4°C, or at -20°C, with the addition of 30 % glycerol. In the case of a subsequent purification step, 0.3 M NaCl are included in the lysis buffer.

A3 Purification of Transaminases

Protein purification was performed using metal affinity chromatography, taking advantage of the C-terminal HisTag in all expressed TAs, with standard procedure for a person trained in the field. The crude lysate prepared as described in paragraph A2 is loaded to a HisTrap™ FastFlow 5mL column equipped on an Äkta protein chromatography system, after equilibration with the lysis buffer (HEPES buffer, 50 mM, 0.3 M NaCl, 0.1 mM PLP). Once the column was loaded with the TA, it was washed from the non-specifically bound proteins with application of 2 column volumes of 10 mM imidazole. The TA was eluted from the column by the application of 2 column volumes of 0.3 M imidazole. The purified TA fractions were pooled and subsequently desalted via the Äkta system, using a Sephadex™ G60 column equilibrated with a HEPES (50 mM, pH 7.5) buffer, containing 0.1 mM PLP, with a standard procedure known to the person trained in the field. The purified enzyme solutions are stored at +4°C, or at -20°C, with the addition of 30 % glycerol.

Date Reçue/Date Received 2022-07-08

B Biocatalysis

B1 Preparation of stock solutions of the compounds of interest

Due to the hydrophobicity of the substrates of interest (cf. Schemes 2 and 3), stock solutions were prepared in water-soluble organic solvents, such as 2-propanol or dimethylsulfoxide. In a standard procedure for the kinetic resolution experiments, 20 to 40 mM stock solutions of the ketones and amines of interest were prepared in 2-propanol. As amine 1 and 4 were prepared as HCl-salts, the solution was sonicated for 30 sec at room temperature (Bandeling Sonorex RK 512H) to facilitate the dispersion. After dispersion, the homogeneous solution was stable and no precipitation of the amine or the amine salt was observed.

B2 Activity measurement in kinetic resolution mode

The activity measurements were performed in a TECAN Infinite® 200 PRO reader, using the microtiter plate reader. For the direct photometric assay described by Schätzle et al. in Analytical Chemistry (2009; see also above), the enzymatic reaction takes place in CHES buffer (50 mM, pH 9.0) at 30°C, in a total volume of 200 µL. 1 mM of racemic amine (*rac*-amine) of interest and 2 mM of amine acceptor (preferably pyruvate or glyoxylate) are added to the buffer (final concentrations), which contains the TA of interest. Due to the preparation of the amine stock solution in organic solvent, the final concentration of the organic solvent (2-propanol or DMSO) is 5 % (v/v). The reaction is initiated by the addition of the second substrate (pyruvate). The concentration of the enzyme was varying between 2 and 800 µg/mL final concentration, depending on the specific activity towards the substrates in examination. The production of the ketone is monitored in the optimum wavelength for each compound, as determined with a method known to the person trained in the art. The wavelength used for each substrate, as well as the apparent extinction coefficients that were used to determine the specific activity of the TAs, as determined on the exact reaction conditions, are presented in Table 2.

Table 2: Optimum wavelength (nm) and apparent extinction coefficient ($M^{-1}cm^{-1}$) for the ketones of interest. Ketone 5a is not included, as it cannot be monitored by the direct photometric assay. Apparent coefficient was calculated simulating the reaction conditions, as some of the amines had a minor, but not detrimental absorbance in the monitored wavelength.

Substrate	Optimum wavelength	Apparent extinction coefficient
1a	265 nm	16562 $M^{-1}cm^{-1}$
2a	245 nm	7953 $M^{-1}cm^{-1}$
3a	245 nm	9646 $M^{-1}cm^{-1}$
4a	340 nm	5714 $M^{-1}cm^{-1}$
6a	245 nm	6115 $M^{-1}cm^{-1}$

For the compounds for which the direct photometric assay cannot be applied, such as substrate 5a/b and *iso*-propylamine, the test described by Weiß et al. in Analytical Chemistry (2014) was used. To use this test, a master mix in CHES buffer (pH 9.0, 50 mM) was prepared containing 2 mM amine 5a or 50 mM *iso*-propylamine (amine donor), 2.4 mM glyoxylate (amine acceptor), 0.12 mg/mL glycine oxidase from *Geobacillus kaustophilus*, 5 U/mL horseradish peroxidase, 3.9 mM vanillic acid and 1.2 mM 4-aminoantipyrine. The reaction was initiated with the addition of 20 µL of enzyme solution, normalized to a concentration of 1 mg/mL, to 130 µL of the master mix, and it was performed at 37ºC for 1 h. The enzymatic activity was determined by the increase of absorbance at 498 nm (ε = 4654 $M^{-1}cm^{-1}$) due to the formation of the quinone imine dye. As this test relies on the activity of three enzymes, the calculation of specific activities is not possible. Thus, the activity of the TAs is always presented as relative to the reference variant [activity (mutant) / activity (reference) * 100)].

EXAMPLE 1: Improvement of the TA specific activity for the kinetic resolution of *rac*-amines 1a, 3a and 4a by incorporation of the mutations

The specific activity of the presented mutants was determined for the kinetic resolution of *rac*-amines 1a, 3a, 4a and 6a according to the test described in section B2 of the General Procedures, with purified TAs. The positions were identified in the 3FCR scaffold and then transferred into the 3GJU scaffold, for confirmation of their significance in the acceptance of the amines of interest. The identity of SEQ ID NO: 1 and SEQ ID NO: 3 is 71.3 %.

Table 3: Specific activities (U/mg) for 3FCR and 3GJU mutants in kinetic resolution mode. n.a.: not active; n.d.: not determined.

TA \ Substrate	1a	3a	4a	6a
3FCR	n.a.	0.001	0.014	0.01
3FCR Y59W	0.002	0.50	0.27	0.50
3FCR T231A	n.a.	0.02	0.07	0.16
3FCR Y59W/T231A	0.005	0.92	0.08	0.83
3FCR Y59W/Y87F/Y152F/T231A/P423H	0.62	8.9	1.38	1.4
3GJU	n.a.	0.003	n.d.	0.003
3GJU Y59W	n.a.	0.11	n.d.	0.018
3GJU T231A	n.a.	0.01	n.d.	0.038
3GJU Y59W/T231A	0.003	0.66	0.11	0.344
3GJU Y59W/Y87F/Y152F/T231A/P423H	0.22	0.92	0.90	0.449

The superiority of the double mutant Y59W/T231A compared to the wild-type and the single mutants in 3FCR is proven, but also to an enzyme with only 71.3 % sequence identity (3GJU). More than that, the cumulative beneficial effect of the other positions is exemplified by the mutant Y59W/Y87F/Y152F/T231A/P423H.

More specifically, the most interesting intermediate mutants that were created on the scaffold of 3FCR had the specific activities presented in Table 4.

Table 4: Specific activities (U/mg) for 3FCR mutants in kinetic resolution mode.

TA \ Substrate	1a	3a	4a	6a
3FCR Y59W/T231A	0.005	0.92	0.08	0.83
3FCR Y59W/Y87F/T231A	0.32	3.3	0.51	2.3
3FCR Y59W/Y87F/Y152F/T231A	0.54	2.9	0.66	1.0
3FCR Y59W/Y87F/Y152F/T231A/P423H	0.62	8.9	1.38	1.4

In this table the gradual increase of the activity for substrates 1a, 3a and 4a with each mutation added is exemplified. The increase in the activity towards these bulky substrates does not necessarily goes hand-in-hand with the activity towards the standard substrate 6a.

The operational stability of the most interesting mutants was evaluated at 25°C. Purified enzymes were incubated in the storage buffer (HEPES buffer, 50 mM, pH 7.5, with the addition of 0.1 mM PLP) at the specified temperature, without shaking. At specific time intervals, aliquots of the incubated enzymes were withdrawn and the remaining activity was monitored with the direct test described in paragraph B2 of the General Procedures, using *rac*-amine 6a as amine donor.

The activity measured prior to the incubation for each mutant was defined as 100 %, respectively. The results are presented in Figure 1.

Mutations Y59W, Y87F, Y152F and T231A were incorporated into several putative transaminases that were identified by a BLAST search in NCBI. The amino acid sequence of 3FCR Y59W/Y87F/Y152F/T231A was used for the query in the non-redundant protein sequence database. No sequence was found in the first 100 results to have any of these four mutations; all sequences were conserved in these positions, namely having Tyr 59, Tyr 87, Tyr 152 and Thr 231. Sequences with identity as low as 69.6 % were selected (SEQ ID NO: 5-11) and the synthetic genes were ordered with the four mutations in the respective positions (SEQ ID NO: 32 and 34-38, respectively, for the amino acid sequence. All TAs were expressed solubly, apart from ATA-4. The specific activity of the transaminases was determined for the kinetic resolution of amines 1a, 3a, 4a and 6a according to the test described in paragraph B2 of the General Procedures, with purified TAs.

Table 5: Specific activities (U/mg) of the putative transaminases identified in the BLAST search, incorporating the Y59W/Y87F/Y152F/T231A mutations, in kinetic resolution mode.

TA \ Substrate	1a	3a	4a	6a
Mutant ATA-3	0.071	1.0	0.16	0.079
Mutant ATA-5	0.16	0.4	0.18	0.38
Mutant ATA-6	0.117	0.6	0.058	0.32
Mutant ATA-7	0.17	0.9	0.40	0.90
Mutant ATA-8	0.40	1.9	0.54	1.2
Mutant ATA-9	0.35	4.3	0.50	0.9

The motif found in 3FCR, and tested in 3GJU can be transferred to enzymes with sequence identity of at least 65 % and yield interesting activities for the substrates 1a, 3a and 4a.

EXAMPLE 2: Improvement of the TA specific activity for the kinetic resolution of *rac*-amines 1a, 2a, 3a, 4a and 6a by incorporation of the mutations

The specific activity of the presented mutants was determined for the kinetic resolution of *rac*-amines 1a, 2a, 3a, 4a and 6a according to the test described in paragraph B2 of the General Procedures, with purified TAs. The positions were identified in the 3FCR scaffold and then transferred into the 3GJU scaffold, for confirmation of their significance in the acceptance of the amines of interest. The identity of SEQ ID NO: 1 and SEQ ID NO: 3 is 71.3 %.

Table 6: Specific activities (U/mg) for 3FCR and 3GJU mutants in kinetic resolution mode. n.a.: not active; n.d.: not determined.

TA \ Substrate	1a	2a	3a	4a	6a
3FCR	n.a.	n.a.	0.001	0.014	0.01
3FCR Y59W/Y87L/T231A	0.23	0.009	0.4	0.02	0.1
3FCR Y59W/Y87V/T231A	0.17	0.005	0.1	0.01	0.1
3GJU	n.a.	n.a.	0.003	n.d.	0.003
3GJU Y59W/Y87L/T231A	0.09	0.004	0.22	0.050	0.008

Position 87 is significant for the acceptance of substrate 2a/b. An aliphatic hydrophobic residue like Leu or Val in necessary in this position to facilitate the acceptance. The effect can be transferred to homologeous proteins (like 3GJU, seq. identity: 71.3%).

EXAMPLE 3: Improvement of the TA specific activity for the kinetic resolution of *rac*-amine 5a by incorporation of the mutations

The specific activity of the presented mutants was determined for the kinetic resolution of *rac*-amine 5a according to the test described in paragraph B2 of the General Procedures, with purified TAs. The positions were identified in the 3FCR scaffold.

Table 7: Relative activity of 3FCR mutants in kinetic resolution of *rac*-amine 5a. The activity of 3FCR Y59W/T231A was used as reference (100 %). For comparison, the specific activity (U/mg) towards substrate 6a is provided.

TA \ Substrate	5a (%)	6a (U/mg)
3FCR Y59W/T231A	100 %	0.83
3FCR T231A	240 %	0.16
3FCR Y59W/T231G	900 %	2.4
3FCR Y59W/Y87F/T231A/P423H	540 %	4.5
3FCR Y59F/T231A	420 %	1.1
3FCR Y59F/Y152F/T231G	1470 %	0.71
3FCR Y59W/T231A/I234F	520 %	0.08
3FCR Y59W/T231A/I234M	460 %	0.90
3FCR Y59W/Y87F/Y152F/T231A/I234F	169 %	0.03
3FCR Y59W/Y87F/Y152F/T231A/I234M	247 %	0.26

The beneficial effect of Y59F and T231G substitution in 3FCR scaffold for the activity towards substrate 5a is proven. Their combination in the variant Y59F/Y152F/T231G yielded the best variant so far for substrate 5a relative to substrate 6a. More than that, the beneficial effect of the substitution of position 234 to Phe or Met could be shown.

EXAMPLE 4: Improvement of the TA specific activity for the use of *iso*-propylamine as amine donor

The specific activity of the presented mutants was determined for use of *iso*-propylamine as amine donor according to the test described in paragraph B2 of the General Procedures, with purified TAs. The positions were identified in the 3FCR scaffold.

Table 8: Relative activity of 3FCR mutants using *iso*-propylamine as amine donor. The activity of 3FCR Y59W/T231A was used as reference (100 %). For comparison, the specific activity (U/mg) towards substrate 6a is provided.

TA \ Substrate	*iso*-propylamine (%)	6a (U/mg)
3FCR Y59W/T231A	100 %	0.83
3FCR	145 %	0.01
3FCR T231A	280 %	0.16
3FCR Y59W/T231A/I234F	860 %	0.08
3FCR Y59W/T231A/I234M	475 %	0.90
3FCR Y59W/Y87F/Y152F/T231A	43 %	1.0
3FCR Y59W/Y87F/Y152F/T231A/I234F	208 %	0.03
3FCR Y59W/Y87F/Y152F/T231A/I234M	91 %	0.26

It can be concluded that the substitutions at position 234 have a beneficial effect of on the activity towards *iso*-propylamine.

EXAMPLE 5: Asymmetric synthesis of chiral amines 1a, 3a and 4a, using L-alanine as amine donor

A glass vial of 2 mL containing 0.5 mL of reaction mixture (HEPES buffer, 50 mM, pH 8.0) of 8 mM ketone (1, 3 or 4), 200 mM L-alanine, 20 % DMSO, 25 mM glucose, 5 mM NADH, 0.5 mg/mL glucose dehydrogenase GDH-105 (Codexis), 5 µL/mL L-lactic dehydrogenase (LDH) from bovine heart (L2625-50KU, Sigma), 1 mM PLP and the TA of interest, was incubated at 30°C in a thermoshaker (Eppendorf), with a shaking speed of 600 rpm. After 20 h the reaction is terminated by thermal denaturation of the enzymes at 90°C for 10 min. The reaction is cooled down to room temperature, and an equal amount of acetonitrile containing 0.1 % diethylamine is added to the reaction mixture. The mixture was centrifuged at 17000 *g* for 1 min to remove the precipitated protein and the supernatant was filtered into an inlet, for HPLC analysis (Chiralpak™ OD-RH column, 150*4.6 mm, 5 µm particle at 30°C; isocratic method with 50 % acetonitrile and 50 % H_2O containing 0.1 % diethylamine, at a flow of 0.5 mL/min). Conversion is defined as [total amine, mM / (total amine, mM + ketone, mM) * 100].

Table 9: Conversion (%) and enantiomeric ratio (er, %) in the asymmetric synthesis of chiral amines, using L-alanine as amine donor. Enzyme concentration was 1.8 mg/mL; n.d. not determined

Substr.	TA	*Conversion (%)*	er (S:R, %)
1b	3FCR Y59W/Y87F/Y152F/T231A	81 %	2:98 %
3b	3FCR Y59W/T231A	85 %	> 99.5:0.5 %
3b	3FCR Y59W/Y87F/Y152F/T231A	99 %	> 99.5:0.5 %
4b	3FCR Y59W/T231A	99 %	n.d.
4b	3FCR Y59W/Y87F/Y152F/T231A	100 %	n.d.

Asymmetric synthesis at a scale of 8 mM ketone (for production of chiral amines 1a, 3a and 4a) can be performed with desirable conversions and enantioselectivity, using L-alanine as amine donor in less than a day (20 h). Various variants catalyzed the reaction efficiently.

Date Reçue/Date Received 2022-01-06

EXAMPLE 6: Asymmetric synthesis of amine 5a, using alanine as amine donor

A glass vial of 2 mL containing 0.5 mL of reaction mixture (HEPES buffer, 50 mM, pH 8.0) of 8 mM ketone 5, 200 mM L-alanine, 20 % DMSO, 25 mM glucose, 5 mM NADH, 0.5 mg/mL glucose dehydrogenase GDH-105 (Codexis), 5 µL/mL L-lactic dehydrogenase (LDH) from bovine heart (L2625-50KU, Sigma), 1 mM PLP and the TA of interest, was incubated at 30°C in a thermoshaker (Eppendorf), with a shaking speed of 600 rpm. After 20 h the reaction is terminated by thermal denaturation of the enzymes at 90°C for 10 min. The reaction is cooled down to room temperature and an equal amount of acetonitrile containing 0.1 % diethylamine is added to the reaction mixture. The mixture was centrifuged at 17000 *g* for 1 min to remove the precipitated protein and the supernatant was filtered into an inlet, for HPLC analysis (Chiralpak OD-RH column, 150*4.6 mm, 5 µm particle at 30°C; isocratic method with 30 % acetonitrile and 70 % H_2O containing 0.1 % diethylamine, at a flow of 0.5 mL/min). Conversion is defined as [total amine, mM / (total amine, mM + ketone, mM) * 100].

Table 10: Conversion (%) of the asymmetric synthesis of amine 5a, using L-alanine as amine donor.

TA	**Enzyme concentration *(mg/mL)***	**Conversion *(%)***
3FCR Y59W/Y87F/Y152F/T231A/I234M	1.6	66 %
3FCR Y59W/Y87F/Y152F/T231A/I234F	0.2	40 %

Asymmetric synthesis at a scale of 8 mM ketone for substrate 5a/b can be performed with desirable conversions and enantioselectivity, using L-alanine as amine donor in less than a day (20 h). Various TA mutants catalyzed the reaction efficiently.

EXAMPLE 7: Asymmetric synthesis of chiral amine 1a, (*R*)-(4-chlorophenyl)-phenylmethaneamine, using alanine as amine donor

In 53.5 ml reaction buffer (HEPES 100 mM pH 8.0; 4 mM PLP; 400mM D,L-alanine) 4.5 g pyruvate reductase mix (5.4 mg GDH-102 [Codexis]; 21.6 mg LDH-101 [Codexis]; 126 mg NAD [Roche]; 4.32 g D-glucose) was dissolved. The purified mutant transaminase 3FCR Y59W/Y87F/Y152F/T231A was added subsequently as solution (22 ml, protein 2.6 mg/ml; see **A3**) and stirred for 5 minutes at 30°C. The reaction was started by the addition 100 mg (4-chlorophenyl)(phenyl)methanone dissolved in 20 ml DMSO. The reaction pH was manually adjusted to 8.0 (2N NaOH) and kept constant by the automated addition of 0.1 N NaOH. At the beginning the yellow reaction solution was slightly turbid. After 24 h and a conversion of 98 area% (IPC-HPLC) the reaction was acidified to pH 2.0 to precipitate the enzymes. 1.125 g filtration aid (Dicalite) was added and the mixture was stirred 20 minutes. Subsequently, the reaction mixture was filtrated through 20 g filter aid (Dicalite) bed and the filter cake was washed with 40 ml 0.1 N HCl. The pH of the combined, slightly yellow, clear aqueous solutions was adjusted to pH 10, changing the solution color to orange, for the amine extraction using twice 50 ml TBME. The combined organic phases were dried over $MgSO_4$, filtrated and evaporated under vacuum at 40°C yielding in 122 mg (122%) light yellow oil as *crude* product of the title compound **1a**.

HCl-salt formation: The crude product was dissolved in 5 ml TBME and under stirring 350 µl 2 M HCl was added using a syringe. The formed suspension was allowed to stir for 2 h at room temperature prior to its filtration (paper filter). The isolated HCl-salt was washed with TBME and dried 3 h under high vacuum at room temperature yielding in 95 mg (82%) white powder.

Chemical purity HPLC: 99.7 area% [210 nm; X-Bridge™ C8; 50*4.6 mm, 2.5 µm, flow 2 ml, 45°C, A: H_2O / ACN (95 / 5), 50 → 10% in 3 min, hold 0.5 min, B: ACN, 40 → 80% in 3 min, hold 0.5 min, C: 100 mmol ammonium acetate in H_2O / ACN (95 / 5), 10% isocratic]; chiral HPLC: 97.1% (R), 2.9% (S) [235 nm; Chrownpack CH-1(+), 150*3 mm, 5 µm, flow 0.4 ml, 40°C, A: 55% 3.6 g perchloric acid in H_2O,

Date Recue/Date Received 2022-07-08

B: 45 % ACN]; ^{1}H NMR (600 MHz, CH_3OD) δ ppm 7.20 - 7.49 (m, 9 H), 5.57 (s, 1 H); GC-MS: 217 $(M)^+$.

EXAMPLE 8: Kinetic resolution of amine 2a using pyruvate as amine acceptor for the preparation of (S)-2,2-dimethyl-1phenylpropan-1-amine

In 89.75 ml reaction buffer (TRIS 50 mM pH 8.5; 1 mM PLP) 129 mg sodium pyruvate and the purified solution of mutant transaminase 3FCR Y59W/Y87L/T231A (18.5 ml, protein 2.47 mg/ml; see **A3**) were added subsequently and stirred for 5 minutes at 30°C. The reaction was started by the addition 100 mg 2,2-dimethyl-1phenylpropan-1-amine dissolved in 1 ml DMSO. After 48 h and a conversion of roughly 55 area% (IPC-HPLC), the reaction was acidified to pH 2.0 to precipitate the enzymes and was stirred 20 minutes. Subsequently, the reaction mixture was filtrated through 25 g filter aid (Dicalite) bed and subsequently, the filter cake was washed with deionized water and 50ml TBME. After phase separation the aqueous phase was extracted with 50 ml TBME to remove the 2,2-dimethyl-1-phenyl-propan-1-one **2b**. The combined organic phases were dried over $MgSO_4$, filtrated and evaporated under vacuum at 40°C yielding in 25 mg (26.5%; chiral HPLC: 99 area%) 2,2-dimethyl-1-phenyl-propan-1-one **2b** as yellow vicious oil. The pH of the aqueous phase was adjusted to 12 using 2N NaOH and subsequently extracted twice with 50 ml TBME. The combined organic phases were dried over $MgSO_4$, filtrated and evaporated under vacuum at 40°C yielding in 38 mg (40%) (*S*)-2,2-dimethyl-1-phenyl-propan-1-amine **2a** as yellow oil.

Chiral HPLC: 98.1% (*S*), 1.9% (*R*) [220 nm; Chiracel OD-3R; 150*4.6 mm, 3 µm, flow 1.0 ml, 25°C, A: 50% ACN, B: 50% 6.3 g ammonium formate in 950 ml H_2O : 50 ml ACN]; ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.27 - 7.31 (m, 4 H), 7.24 (dt, *J*=6.0, 2.6 Hz, 1 H), 3.71 (s, 1 H), 0.91 (s, 9 H); GC-MS: 162 (M).

EXAMPLE 9: Asymmetric synthesis of chiral amine 3a, (*S*)-1.3-diphenyl propylamine, using alanine as amine donor

In 68 ml reaction buffer (HEPES 100 mM pH 8.0; 4 mM PLP; 400mM D,L-alanine) 4.5 g pyruvate reductase mix (5.4 mg GDH-102 [Codexis]; 21.6 mg LDH-101[Codexis]; 126 mg NAD [Roche]; 4.32 g D-glucose) was dissolved. The purified mutant transaminase 3FCR Y59W/Y87F/Y152F/T231A was added subsequently as solution (6.8 ml, protein 2.6 mg/ml; see **A3**) and stirred for 5 minutes at 30°C. The reaction was started by the addition 100 mg 1.3-diphenyl propan-1-one dissolved in 20 ml DMSO. The reaction pH was kept constant by the automated addition of 0.1 N NaOH. At the beginning the yellow reaction solution was slightly turbid. After 24 h and a conversion of 98 area% (IPC-HPLC), the reaction was acidified to pH 2.0 to precipitate the enzymes. 1.125 g filtration aid (Dicalite) was added and the mixture was stirred 20 minutes. Subsequently, the reaction mixture was filtrated through 20 g filter aid (Dicalite) bed and the filter cake was washed with 40 ml 0.1 N HCl. The pH of the combined, slightly yellow, clear aqueous solutions was adjusted to pH 10, changing its color to orange, for the amine extraction using twice 50 ml TBME. The combined organic phases were dried over $MgSO_4$, filtrated and evaporated under vacuum at 40°C yielding in 95 mg (95%) light yellow oil as *crude* product of the title compound **3a**.

HCl-salt formation: The crude product was dissolved in 5 ml TBME and under stirring 360 µl 2 M HCl was added using a syringe. The formed suspension was allowed to stir for 2 h at room temperature prior to its filtration (paper filter). The isolated HCl-salt was washed with TBME and dried 3 h under high vacuum at room temperature yielding in 84 mg (71%) white powder as HCl salt of the title compound **3a**.

Chemical purity HPLC: 99.0 area% [210 nm; X-Bridge C8; 50*4.6 mm, 2.5 µm, flow 2 ml, 45°C, A: H_2O / ACN (95 / 5), 60 → 10% in 3 min, hold 0.5 min, B: ACN, 30 → 80% in 3 min, hold 0.5 min, C: 100 mmol Ammonium acetate in H_2O / ACN (95 / 5), 10% isocratic]; chiral HPLC: 100% (*S*) [222 nm; Chiralpak IF 3; 150*4.6 mm, flow 1 ml, 40°C, A: 90% heptane with 0.1% diethylamine, B: 10 % ethanol]; ^1H NMR (600 MHz, CH_3OD) δ ppm 7.44 - 7.59 (m, 5 H), 7.28 - 7.33 (m, 2 H), 7.12

- 7.24 (m, 3 H), 4.24 (dd, *J*=9.6, 5.6 Hz, 1 H), 2.55 (br t, *J*=7.9 Hz, 2 H), 2.22 - 2.42 (m, 2 H); LC-MS: 212 $(M+H)^+$.

EXAMPLE 10: Asymmetric synthesis of chiral amine 4a, (S)-acenaphthen-1-ylamine, using alanine as amine donor

In 60.5 ml reaction buffer (HEPES 100 mM pH 8.0; 4 mM PLP; 400mM D,L-alanine) 4.5 g pyruvate reductase mix (5.4 mg GDH-102 [Codexis]; 21.6 mg LDH-101[Codexis]; 126 mg NAD [Roche]; 4.32 g D-glucose) was dissolved. The purified aminotransaminase 3FCR Y59W/Y87F/Y152F/T231A was added subsequently as solution (15 ml, protein 2.6 mg/ml; see **A3**) and stirred for 5 minutes at 30°C. The reaction was started by the addition 100 mg 2H-Acenaphthylen-1-one dissolved in 20 ml DMSO. The reaction pH was manually adjusted to 8.0 (2N NaOH) and kept constant by the automated addition of 0.1 N NaOH. At the beginning the yellow reaction solution was slightly turbid. To prevent potential oxidative aromatization the reaction was carried out under N_2 atmosphere and in the dark. After 46 h and a conversion of 98 area% (IPC-HPLC) the reaction was acidified to pH 2.0 to precipitate the enzymes. 5 g filtration aid (Dicalite) was added and the mixture was stirred 20 minutes. Subsequently, the reaction mixture was filtrated through 20 g filter aid (Dicalite) bed and the filter cake was washed with 40 ml 0.1 N HCl and with 50 ml TBME. The pH of the combined, slightly yellow, clear aqueous solutions was adjusted to pH 10, prior to the amine extraction using 50 ml TBME. The combined organic phases were dried over $MgSO_4$, filtrated and evaporated under vacuum at 40°C yielding in 112 mg (112%) orange-brown oil as *crude* product of the title compound **4a**.

HCl-salt formation: The crude product was dissolved in 5 ml TBME and under stirring 445 µl 2 M HCl was added using a syringe. The formed very fine suspension was allowed to stir for 1 h at room temperature prior to its centrifugation (5 min.; 4000 rpm). After decantation of TBME, the remaining solid was re-suspended in 10 ml TBME, vigorously mixed, centrifuged (5 min.; 4000 rpm) and once more decanted. The moist HCl-salt was evaporated under vacuum at 40°C and dried 3 h under high vacuum at room temperature yielding in 84 mg (69%) beige powder as HCl salt of the title compound **4a**.

Chemical and chiral purity HPLC: 81.5% (*S*) : 18.5% (*R*) [232 nm; Chiracel OJ-3; 150*4.6 mm, 3 µm; flow 2.5 ml, 30°C, A: 96% heptane with 0.1% diethylamine, B: 4 % ethanol]; ^{1}H NMR (600 MHz, CH_3OD) δ ppm : 7.90 (d, *J*=8.1 Hz, 1 H), 7.77 (d, *J*=8.3 Hz, 1 H), 7.54 - 7.70 (m, 3 H), 7.46 (d, *J*=6.9 Hz, 1 H), 5.32 (dd, *J*=8.1, 2.7 Hz, 1 H), 3.99 (dd, *J*=17.8, 8.1 Hz, 1 H), 3.41 (dt, *J*=17.8, 1.3 Hz, 1 H); LC-MS: 171.1 $(M+H)^+$.

EXAMPLE 11: Asymmetric synthesis of amine 5a, *exo*-3-amino-8-aza-bicyclo [3.2.1] oct-8-yl)-phenyl-methanone, using 2-propylamine as amine donor

Into 82 ml reaction buffer (HEPES 50 mM; 2 mM PLP; 0.2 M 2-propylamine hydrochloride; pH 7.5) the purified mutant transaminase 3FCR Y59W/Y87F/Y152F/T231A/I234M solution (in total 78 ml, protein 1.1 mg/ml; see **A3**) and 100 mg 8-Benzoyl-8-aza-bicyclo[3.2.1]octan-3-one **5b** dissolved in 20 ml DMSO were added under stirring at 30°C. The reaction was allowed to proceed for 9 d enabling a conversion of 75 area% (IPC-HPLC). After 9d the reaction was acidified to pH 2.0 to precipitate the enzymes and was stirred 15 minutes. Subsequently, the reaction mixture was filtrated through 25 g filter aid (Dicalite) bed and extracted twice with 50 ml dichloromethane to remove the 8-Benzoyl-8-aza-bicyclo[3.2.1]octan-3-one **5b**. The combined organic phases were dried over $MgSO_4$, filtrated and evaporated under vacuum at 40°C yielding in 37 mg (HPLC: 97 area%) 8-Benzoyl-8-aza-bicyclo[3.2.1]octan-3-one **5b** as slightly yellow oil. The pH of the aqueous phase was adjusted to 12 using 2N NaOH and subsequently extracted four times with 50 ml dichloromethane. The combined organic phases were dried over $MgSO_4$, filtrated, evaporated under vacuum at 40°C and dried 36 h under high vacuum at 60°C yielding in 60 mg (60%) as yellow oil of the title compound **5a**.

Chemical purity HPLC: 98.9 area% [210 nm; X-Bridge C8; 50*2.1 mm, 2.5 µm, flow ml, 40°C, A: 90 % 10 mmol Ammonium acetate in H_2O / ACN (95 / 5), B: ACN, 10%]; chiral SFC: 100% *exo* [210 nm; Chiralpak AD-3; 150*4.6 mm, 5 µm; flow 3 ml; left 40°C; right 42°C; A: 82% CO_2, B: 18 % methanol with 0.2% 2-

propylamine]; ^{1}H NMR (600 MHz, DMSO-d_6, 120°C) δ ppm 7.44 (s, 5 H), 4.29 (br s, 2 H), 3.16 (dt, *J*=11.1, 5.5 Hz, 1 H), 1.86-2.00 (m, 3 H), 1.79 (ddd, *J*=13.2, 5.1, 3.0 Hz, 3 H), 1.67-1.75 (m 3 H), 1.34-1.47(m, 2 H); LC-MS: 231 $(M+H)^+$.

SEQUENCES

Wild-type Sequences (plus tags):

3FCR wild-type (plus tag): amino acid (SEQ ID NO: 1 + SEQ ID NO: 56)

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DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI       180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG TGGIVPPPAG       240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS       300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN       360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR       420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG-(SHHHHHH)
        430         440         450
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3FCR wild-type plus tag: nucleic acid (SEQ ID NO: 2)

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3GJU wild-type (plus tag): amino acid (SEQ ID NO: 3 + SEQ ID NO: 56)

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SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV       180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL GTGGIVPPPA       240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG       300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF       360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR       420
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3GJU wild-type plus tag: nucleic acid (SEQ ID NO: 4)

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gcaatgccgc agggcgatat tctgggtttt gccccgccgc tgtgtctgac ccgtgaacag     1320
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caccaccatc accaccacta a                                               1401
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ATA-3: amino acid illustrated in Figure 2 (SEQ ID NO: 5)
ATA-4: amino acid illustrated in Figure 2 (SEQ ID NO: 6)
ATA-5: amino acid illustrated in Figure 2 (SEQ ID NO: 7)
ATA-6: amino acid illustrated in Figure 2 (SEQ ID NO: 8)
ATA-7: amino acid illustrated in Figure 2 (SEQ ID NO: 9)
ATA-8: amino acid illustrated in Figure 2 (SEQ ID NO: 10)
ATA-9: amino acid illustrated in Figure 2 (SEQ ID NO: 11)

Sequences of Double Mutants:

3FCR Y59W/T231A: amino acid (SEQ ID NO: 12)

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HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG AGGIVPPPAG      240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS      300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN      360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR      420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG                               457
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3FCR Y59W/T231G: amino acid (SEQ ID NO: 13)

```
MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGLWC       60
VNVGYGRQEI AEAIADQARE LAYYHSYVGH GTEASITLAK MILDRAPKNM SKVYFGLGGS      120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI      180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG GGGIVPPPAG      240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS      300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN      360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR      420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG                               457
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3FCR Y59F/T231A: amino acid (SEQ ID NO: 14)

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MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGLFC       60
VNVGYGRQEI AEAIADQARE LAYYHSYVGH GTEASITLAK MILDRAPKNM SKVYFGLGGS      120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI      180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG AGGIVPPPAG      240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS      300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN      360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR      420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG                               457
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3GJU Y59W/T231A: amino acid (SEQ ID NO: 15)

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MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGLW       60
CVNVGYGRQK IADAIATQAK NLAYYHAYVG HGTEASITLA KMIIDRAPKG MSRVYFGLSG      120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV      180
```

LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GGIVPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

3GJU Y59W/T231G: amino acid (SEQ ID NO: 16)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHAYVG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**G**GGIVPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

3GJU Y59F/T231A: amino acid (SEQ ID NO: 17)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**F** 60
CVNVGYGRQK IADAIATQAK NLAYYHAYVG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GGIVPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

Sequences of Triple Mutants:

3FCR Y59W/Y87F/T231A: amino acid (SEQ ID NO: 18)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHS**F**VGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **A**GGIVPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3FCR Y59W/Y87L/T231A: amino acid (SEQ ID NO: 19)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHS**L**VGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **A**GGIVPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3FCR Y59W/Y87V/T231A: amino acid (SEQ ID NO: 20)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHS**V**VGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **A**GGIVPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3FCR Y59W/Y87F/T231G: amino acid (SEQ ID NO: 21)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHS**F**VGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **G**GGIVPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420

AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3FCR Y59F/Y87F/T231G: amino acid (SEQ ID NO: 57)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**F**C 60
VNVGYGRQEI AEAIADQARE LAYYHS**F**VGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **G**GGIVPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3FCR Y59W/T231A/I234F: amino acid (SEQ ID NO: 22)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHSYVGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **A**GG**F**VPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3FCR Y59W/T231A/I234M: amino acid (SEQ ID NO: 23)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHSYVGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **A**GG**M**VPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3GJU Y59W/Y87F/T231A: amino acid (SEQ ID NO: 24)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHA**F**VG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GGIVPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

3GJU Y59W/Y87L/T231A: amino acid (SEQ ID NO: 25)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHA**L**VG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GGIVPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

3GJU Y59W/Y87V/T231A: amino acid (SEQ ID NO: 26)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHA**V**VG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GGIVPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

3GJU Y59W/Y87F/T231G: amino acid (SEQ ID NO: 27)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHA**F**VG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180

LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**G**GGIVPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

3GJU Y59W/T231A/I234F: amino acid (SEQ ID NO: 28)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHAYVG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GG**F**VPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

3GJU Y59W/T231A/I234M: amino acid (SEQ ID NO: 29)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHAYVG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GG**M**VPPPA 240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG 300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF 360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR 420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL 459

Sequences of 4-fold Mutants:

3FCR Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 30)
3GJU Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 31)
ATA-3 Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 32)
ATA-4 Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 33)
ATA-5 Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 34)
ATA-6 Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 35)
ATA-7 Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 36)
ATA-8 Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 37)
ATA-9 Y59W/Y87F/Y152F/T231A: amino acid as in Fig. 3 (SEQ ID NO: 38)

3FCR Y59W/Y87L/Y152F/T231A: amino acid (SEQ ID NO: 39)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHS**L**VGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR G**F**HGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **A**GGIVPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AMPQGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3FCR Y59W/Y87F/T231A/P423H: amino acid (SEQ ID NO: 40)

MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGL**W**C 60
VNVGYGRQEI AEAIADQARE LAYYHS**F**VGH GTEASITLAK MILDRAPKNM SKVYFGLGGS 120
DANETNVKLI WYYNNILGRP EKKKIISRWR GYHGSGLVTG SLTGLELFHK KFDLPVEQVI 180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG **A**GGIVPPPAG 240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS 300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN 360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR 420
AM**H**QGDILGF APPFCLTRAE ADQVVEGTLR AVKAVLG 457

3GJU Y59W/Y87L/Y152F/T231A: amino acid (SEQ ID NO: 41)

MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGL**W** 60
CVNVGYGRQK IADAIATQAK NLAYYHA**L**VG HGTEASITLA KMIIDRAPKG MSRVYFGLSG 120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RG**Y**HGSGVMT GSLTGLDLFH NAFDLPRAPV 180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL G**A**GGIVPPPA 240

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GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG   300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF   360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR   420
AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL                          459
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3GJU Y59W/Y87F/T231A/P423H: amino acid (SEQ ID NO: 42)

```
MLNQSNELNA WDRDHFFHPS THMGTHARGE SPTRIMAGGE GVTVWDNNGR KSIDAFAGLW    60
CVNVGYGRQK IADAIATQAK NLAYYHAFVG HGTEASITLA KMIIDRAPKG MSRVYFGLSG   120
SDANETNIKL IWYYNNVLGR PEKKKIISRW RGYHGSGVMT GSLTGLDLFH NAFDLPRAPV   180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL GAGGIVPPPA   240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG   300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF   360
RAELAKAVGG HKNVGEVRGD GMLAAVEFVA DKDDRVFFDA SQKIGPQVAT ALAASGVIGR   420
AMHQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL                          459
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Sequences of 5-fold Mutants:

3FCR Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 43)
3GJU Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 44)
ATA-3 Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 45)
ATA-4 Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 46)
ATA-5 Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 47)
ATA-6 Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 48)
ATA-7 Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 49)
ATA-8 Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 50)
ATA-9 Y59W/Y87F/Y152F/T231A/P423H: amino acid as in Fig. 4 (SEQ ID NO: 51)

3FCR Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 53)
3GJU Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 55)
ATA-3 Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 58)
ATA-4 Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 59)
ATA-5 Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 60)
ATA-6 Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 61)
ATA-7 Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 62)
ATA-8 Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 63)
ATA-9 Y59W/Y87F/Y152F/T231A/I234M: amino acid as in Fig. 5 (SEQ ID NO: 64)

3FCR Y59W/Y87F/Y152F/T231A/I234F: amino acid (SEQ ID NO: 52)

```
MLKNDQLDQW DRDNFFHPST HLAQHARGES ANRVIKTASG VFIEDRDGTK LLDAFAGLWC    60
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DANETNVKLI WYYNNILGRP EKKKIISRWR GFHGSGLVTG SLTGLELFHK KFDLPVEQVI   180
HTEAPYYFRR EDLNQTEEQF VAHCVAELEA LIEREGADTI AAFIGEPILG AGGFVPPPAG   240
YWEAIQTVLN KHDILLVADE VVTGFGRLGT MFGSDHYGLE PDIITIAKGL TSAYAPLSGS   300
IVSDKVWKVL EQGTDENGPI GHGWTYSAHP IGAAAGVANL KLLDELNLVS NAGEVGAYLN   360
ATMAEALSQH ANVGDVRGEG LLCAVEFVKD RDSRTFFDAA DKIGPQISAK LLEQDKIIAR   420
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GJU Y59W/Y87F/Y152F/T231A/I234F: amino acid (SEQ ID NO: 54)

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SDANETNIKL IWYYNNVLGR PEKKKIISRW RGFHGSGVMT GSLTGLDLFH NAFDLPRAPV   180
LHTEAPYYFR RTDRSMSEEQ FSQHCADKLE EMILAEGPET IAAFIGEPIL GAGGFVPPPA   240
GYWEKIQAVL KKYDVLLVAD EVVTGFGRLG TMFGSDHYGI KPDLITIAKG LTSAYAPLSG   300
VIVADRVWQV LVQGSDKLGS LGHGWTYSAH PICVAAGVAN LELIDEMDLV TNAGETGAYF   360
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AMPQGDILGF APPLCLTREQ ADIVVSKTAD AVKSVFANL                          459
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Claims

1. A mutant transaminase with increased transaminase activity relative to the wild-type transaminase having the amino acid sequence of SEQ ID NO: 1, wherein the mutant transaminase comprises an amino acid sequence that is at least 65 % identical to the amino acid sequence of SEQ ID NO: 1 and wherein the mutant transaminase has at least two amino acid substitutions relative to the wild-type transaminase, wherein the amino acid at the position corresponding to position 59 of SEQ ID NO: 1 is substituted with Trp or Phe and the amino acid at the position corresponding to position 231 of SEQ ID NO: 1 is substituted with Ala or Gly.

2. The mutant transaminase of claim 1,
   - wherein the amino acid at the position corresponding to position 87 of SEQ ID NO: 1 is substituted with the hydrophobic amino acid Leu or Val or Phe ; and/or
   - wherein the amino acid at the position corresponding to position 152 of SEQ ID NO: 1 is substituted with Phe and/or
   - wherein the amino acid at the position corresponding to position 234 of SEQ ID NO: 1 is substituted with Phe or Met , and/or
   - wherein the amino acid at the position corresponding to position 423 of SEQ ID NO: 1 is substituted with His .

3. The mutant transaminase of claim 1 or 2, wherein the transaminase has at least the mutations
   - Trp59 and Ala231, or
   - Trp59, Phe87 and Ala231; or
   - Trp59, Leu87 and Ala231; or
   - Trp59, Val87 and Ala231; or
   - Trp59, Phe87, Ala231 and His423; or
   - Trp59, Phe87, Phe152 and Ala231; or
   - Trp59, Leu87, Phe152 and Ala231; or
   - Trp59, Val87, Phe152 and Ala231; or
   - Trp59, Phe87, Phe152, Ala231 and His423, or
   - Trp59 and Gly231; or
   - Trp59, Phe87 and Gly231; or
   - Trp59, Leu87 and Gly231; or
   - Trp59, Val87 and Gly231; or

Date Reçue/Date Received 2022-07-08

- Trp59, Phe87, Phe152 and Gly231; or
- Trp59, Phe87, Phe152, Gly231 and His423; or
- Phe59, and Ala231; or
- Phe59, Phe87, and Gly231; or
- Trp59, Ala231 and Phe234; or
- Trp59, Ala231 and Met234; or
- Trp59, Phe87, Ala231 and Phe234; or
- Trp59, Leu87, Ala231 and Phe234; or
- Trp59, Val87, Ala231 and Phe234; or
- Trp59, Phe87, Phe152, Ala231 and Phe234; or
- Trp59, Phe87, Phe152, Ala231, Phe234 and His423, or
- Trp59, Gly231 and Phe234; or
- Trp59, Phe87, Gly231 and Phe234; or
- Trp59, Leu87, Gly231 and Phe234; or
- Trp59, Val87, Gly231 and Phe234; or
- Trp59, Phe87, Phe152, Gly231 and Phe234; or
- Trp59, Phe87, Phe152, Gly231, Phe234 and His423; or
- Phe59, Phe87, Gly231 and Phe234, or
- Trp59, Ala231 and Met234; or
- Trp59, Phe87, Ala231 and Met234; or
- Trp59, Leu87, Ala231 and Met234; or
- Trp59, Val87, Ala231 and Met234; or
- Trp59, Phe87, Phe152, Ala231 and Met234; or
- Trp59, Phe87, Phe152, Ala231, Met234 and His423, or
- Trp59, Gly231 and Met234; or
- Trp59, Phe87, Gly231 and Met234; or
- Trp59, Leu87, Gly231 and Met234; or
- Trp59, Val87, Gly231 and Met234; or
- Trp59, Phe87, Phe152, Gly231 and Met234; or
- Trp59, Phe87, Phe152, Gly231, Met234 and His423; or
- Phe59, Phe87, Gly231 and Met234.

4. The mutant transaminase according to any one of claims 1 to 3, wherein the transaminase has at least the mutations
   - Trp59, Phe87, Phe152 and Ala231, or
   - Trp59, Phe87, Phe152, Ala231 and Met234, or
   - Trp59, Phe87, Phe152, Ala231 and H423, or
   - Phe59, Phe87 and Gly231.

5. The mutant transaminase according to any one of claims 1 to 4, wherein the transaminase comprises or consists of the sequence selected from the group consisting of SEQ ID NOs: 30 to 38, 43 to 51, 53, 55 and 57-64.

6. The mutant transaminase according to any one of claims 1 to 5, wherein the mutant transaminase comprises an amino acid sequence that is at least 70 %, 75 %, 80 %, 85 %, 90 %, 95 %, 96 %, 97 %, 98 %, or 99 % identical to the amino acid sequence of SEQ ID NO: 1, at least 90 %, 95 %, 96 %, 97 %, 98 %, or 99 % identical to the amino acid sequence of any of SEQ ID NOs: 1, 3 or 5 to 11.

7. The mutant transaminase according to any one of claims 1 to 6, wherein the mutant transaminase has increased transaminase activity for transamination of at least one compound selected from the group consisting of 1-(4-chlorophenyl)-1-phenyl-1-aminomethane (1a), 2,2-dimethyl-1-phenyl-1-amino propane (2a), 1,3-diphenyl-1-aminopropane (3a), 1,2-dihydroacenaphthylen-1-amine (4a), and 3-amino-8-benzoyl-8-azabicyclo[3.2.1]octane (5a), 3(4-chlorophenyl)-phenyl-methanone (1b), 2,2-dimethyl-1-phenyl-propan-1-one (2b) 1,3-diphenylpropan-1-one (3b), 2H-acenaphthylen-1-one (4b), 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one (5b) and *iso*-propylamine.

8. The mutant transaminase according to any one of claims 1 to 7, wherein the mutant transaminase has an at least 2-fold increased transaminase activity relative to the corresponding wild type enzyme, for at least one compound selected from the group consisting of 1-(4-chlorophenyl)-1-phenyl-1-aminomethane (1a), 2,2-dimethyl-1-phenyl-1-amino propane (2a), 1,3-diphenyl-1-aminopropane (3a), 1,2-dihydroacenaphthylen-1-amine (4a), and 3-amino-8-benzoyl-8-azabicyclo[3.2.1]octane (5a), (4-chlorophenyl)-phenyl-methanone (1b), 2,2-dimethyl-1-phenyl-propan-1-one (2b) 1,3-diphenylpropan-1-one (3b), 2H-acenaphthylen-1-one (4b), 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one (5b) and *iso*-propylamine.

9. A fusion protein comprising the transaminase according to any one of claims 1 to 8.

10. A nucleic acid coding for the transaminase according to any one of claims 1 to 8 or the fusion protein of claim 9.

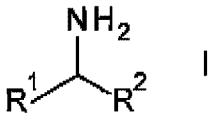
11. A host cell comprising the transaminase according to any one of claims 1 to 8, the fusion protein of claim 9, or the polynucleotide of claim 10.

12. A method of producing an amine comprising reacting an amine acceptor with the mutant transaminase according to any one of claims 1 to 8 or the fusion protein of claim 9 in the presence of an amine donor.

13. The method of claim 12 wherein an enantiomerically enriched chiral amine is produced and the method is either
    a) a kinetic resolution of a racemic amine in the presence of an amine acceptor and the mutant transaminase or the fusion protein; or
    b) an asymmetric transamination of a prochiral ketone in the presence of an amine donor and the mutant transaminase or the fusion protein,

    wherein the mutant transaminase or the fusion protein is stereoselective.

14. The method of claim 13, wherein in method a) the racemic amine has the formula

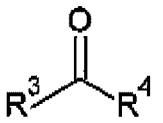


I

wherein
R^1 or R^2 independently of each other represent optionally substituted alkyl, aryl, carbocyclyl or heterocyclyl; or
R^1 and R^2 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring and wherein the optional substituents are selected from C_{1-12}-alkyl, C_{1-12}-alkoxy, aryl, aryloxy, halogen, hydroxyl or cyano.

15. The method of any one of claims 13 to 14, wherein in method a) the amine acceptor is selected from ketones and keto carboxylic acids.

16. The method of claim 13, wherein in method b) the prochiral ketone has the formula



II

wherein

R^3 or R^4 independently of each other represent optionally substituted alkyl, aryl, carbocyclyl or heterocyclyl or;

R^3 and R^4 together with the carbon atom they are attached to form an optionally substituted mono- or poly-cyclic carbocyclic or heterocyclic ring and wherein the optional substituents are selected from C_{1-12}-alkyl, C_{1-12}-alkoxy, aryl, aryloxy, halogen, hydroxyl or cyano.

17. The method of claim 13 or 16, wherein in method b) the amine donor is an achiral or chiral amine or amino acid.

18. Use of a mutant transaminase according to any one of claims 1 to 8 or of the fusion protein of claim 9 for the production of an enantiomerically enriched chiral amine by kinetic resolution or by asymmetric transamination.

Figures

Fig. 1

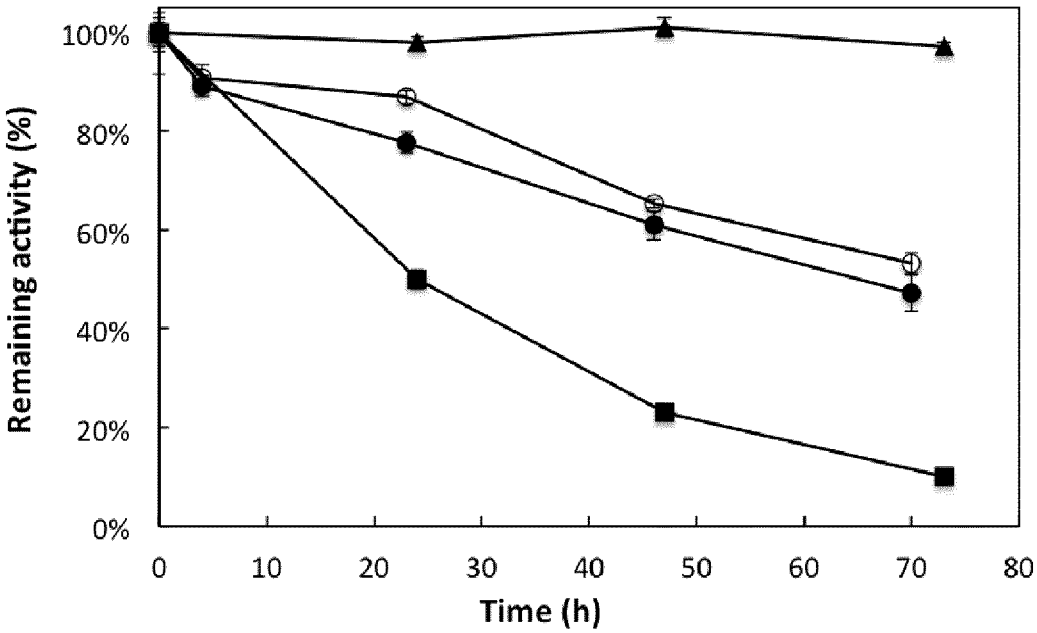


Fig. 2


SEQ ID NO: 1
SEQ ID NO: 3
SEQ ID NO: 5
SEQ ID NO: 6
SEQ ID NO: 7
SEQ ID NO: 8
SEQ ID NO: 9
SEQ ID NO: 10
SEQ ID NO: 11

Fig. 3

```
1   ML-----KNDQLDQWDRDNFFHPSTHLAQHARGESANRVIKTASGVFIEDRDGTKLLDAFAGLWCVNVGYGRQEIAEAIADQ  77
1   ML----nQSNELNAWDRDHFFHPSTHMGTHARGESPTRIMAGGEGVTVWDNNGRKSIDAFAGLWCVNVGYGRQKIADAIATQ  78
1   ML-----KNDPLEQWDRDHFLHPSTHLAEFARGNVAHRIVSGGEGSHIVDRNGTRLLDGFAGLWCVNVGYGRREIADAIAKQ  77
1   ML-----TNDQLSQWDQDHFFHPSTALGAHARGEAPGMVVQTAEGCHITDRNGNRMLDAFAGLWCVNIGYGRQEVAEAIAAQ  77
1   MLdhpapASNAFDSWDRDHFFHPSTHMGQHARGETPNRIITGAEGVYIVDREGRRSLDAFGGLWCVNVGYGRSKITDAIAEQ  82
1   ML-----RNDQLAEWDRENFFHASTHLAAHARGDTPTRIITGGEGVYIQDRDGAKILDGFAGLWCVNVGYGRREITDAIAAQ  77
1   ML-----TNDQLDRFDRENFFHPSTHLAQHARGESPSRIVKTAKGVFIEDRDGNKLLDGFGGLWCVNVGYGQESIIEAIAEQ  77
1   ML-----KNDQLDQWDRENFFHPSTHLAQHARGDSANRVIKTASGVFIEDRDGNKLLDAFAGLWCVNVGYGRQEIADAIADQ  77
1   ML-----TNDQLSQWDRENFFHPSTHLAQHARGETATRVITTGQGCHIEDRDGTKLLDAFAGLWCVNVGYGRTEIADAIAAQ  77

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79    AKNLAYYHAFVGHGTEASITLAKMIIDRAPKGMSRVYFGLSGSDANETNIKLIWYYNNVLGRPEKKKIISRWRGFHGSGV  158
78    ARELSYYHSFVGHGTEASVTLAHMILERAPANMSKVFFGLGGSDANETNIKLIWYMNNILGRPGKKKIISRWRGFHGSGL  157
78    ARELAYYHSFMGNGTEASITLAKMVTERAPEGMNRVYFGQGGSDANETNIKLVWYYNNILGRPEKKKIVSRWRGFHGSGL  157
83    ASKLAYYHAFAGHGSEPSIRLARMVIERAPAGMSRVFFGLSGSDANETNIKLVWYINNVLGRPQKKKIISRWRGFHGSGV  162
78    VSELSYYHAFAGHGTEASVTLAKMVLDRAPDNMSKVYFGLGGSDANETNIKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157
78    AKELAYYHAFAGHGTEASINLAKMVIDRAPDHMSKVYFGLSGSDANETNIKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157
78    ARELAYYHSFVGHGTEASITLAKMILDRAPANMSKVYFGLGGSDANETNVKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157
78    AKELAYYHAFVGHGTEASITLSKMILDRAPAHMSKVYFGLSGSDANETNIKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157

158   VTGSLTGLELFHKKFDLPVEQVIHTEAPYYFRREDLNQTEEQFVAHCVAELEALIEREGADTIAAFIGEPILGAGGIVPP  237
159   MTGSLTGLDLFHNAFDLPRAPVLHTEAPYYFRRTDRSMSEEQFSQHCADKLEEMILAEGPETIAAFIGEPILGAGGIVPP  238
158   MSGSLTGLPLFHKAFDLPLAPILHTEAPYYYRRPNADMSEEAFSAWCASELEAMIQREGPDTIAAFWAEPVLGAGGIVPP  237
158   MSGSLTGLSLFHRKFDLPLDKVLHTTAPYYFQRENVAQSEQEFTAQCVADLEELIAREGADTIAAFIAEPVIGAGGIVPP  237
163   MTGSLTGLAGFHKLFDLPRAPILHTEAPYYFRREDRSMSEEQFSQHCADRLEEMILTEGADTIAAFIGEPVLGAGGIVPP  242
158   MTGSLTGLGLFHAKFDLPMDGVLHTEAPHYLHRADRNQTEEQFSAYCAAKLEEMILAEGPDTIAAFIGEPILGAGGIVPP  237
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238   PAGYWEAIQAVLRKHDILLIADEVVTGFGRLGTMFGSDHYGIEADIITIAKGLTSAYAPLSGSIISDKVWKVLEQGTDEN  317
238   PKGYWEAIQPILEKHDILLVADEVVTGFGRLGTMFGSDHYGLKPDLITIAKGLTSAYAPLSGSIVGDKMWKVLEQGTDEN  317

318   GPIGHGWTYSAHPIGAAAGVANLKLLDELNLVSNAGEVGAYLNATMAEALSQHANVGDVRGEGLLCAVEFVKDRDSRT     395
319   GSLGHGWTYSAHPICVAAGVANLELIDEMDLVTNAGETGAYFRAELAKAVGGHKNVGEVRGDGMLAAVEFVADKDDRV     396
318   GAIGHGWTYSAHPIGAAAGVANLKLIDELGLIDNAAEVGAHLRAGMRDALGEHPNVGDIRGEGMLCAVELVSDRESKE     395
318   GPLGHGWTYSAHPIGAAAGVANLTLLDTLGLVDNAADVGPYLTAQMRAAMQDHAHVGDIRGVGMLTAVELVADRDKGSgvga 399
323   GPIGHGWTYSSHPLCTAAGVANLELVDELDLVTNARETGAYFNAALKDALSGHRHVGEVRGEGLLAAVELVRDRDDRT     400
318   GAFGHGWTYSAHPIGAAAGVANLKLIDDLGLIANASETGAYLKSALQAALGDHPNVAEIRGEGMLAAVEFCADRDDLK     395
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396   FFDAADKIGPQISAKLLEQDkIIARAMPQGDILGFAPPFCLTRAEADQVVEGTLRAVKAV---LG     457   SEQ ID NO: 30
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396   GFDPSRKVTVNAVAHLMENG-VIGRAMPHSETIGFAPPFCLTRDEADEIVAKTAAAVKAV---LG     456   SEQ ID NO: 32
400   GFDPAAKIVPQISAAMAKRG-VIARAMPQADIVGFSPPLCLTRAEADTIVSVTAEAVAEV---LG     460   SEQ ID NO: 33
401   FFEASEKVGPRVAAAMLERG-VIARAMPQGDILGFAPPLCLTREEADIVVDATRGAVEAVcatLG     464   SEQ ID NO: 34
396   QFDTSATIGPRLAAELLTRG-VIGRAMPQSDTIGFAPPLCITRAEVDQIVAAMKGAVDAV---LPa    457   SEQ ID NO: 35
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396   FFDAADKIGPQISAKLLEQDkVIARAMPQGDILGFAPPFCLSRAEADQVVDATLRAVRTV---LG     457   SEQ ID NO: 37
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Fig. 4

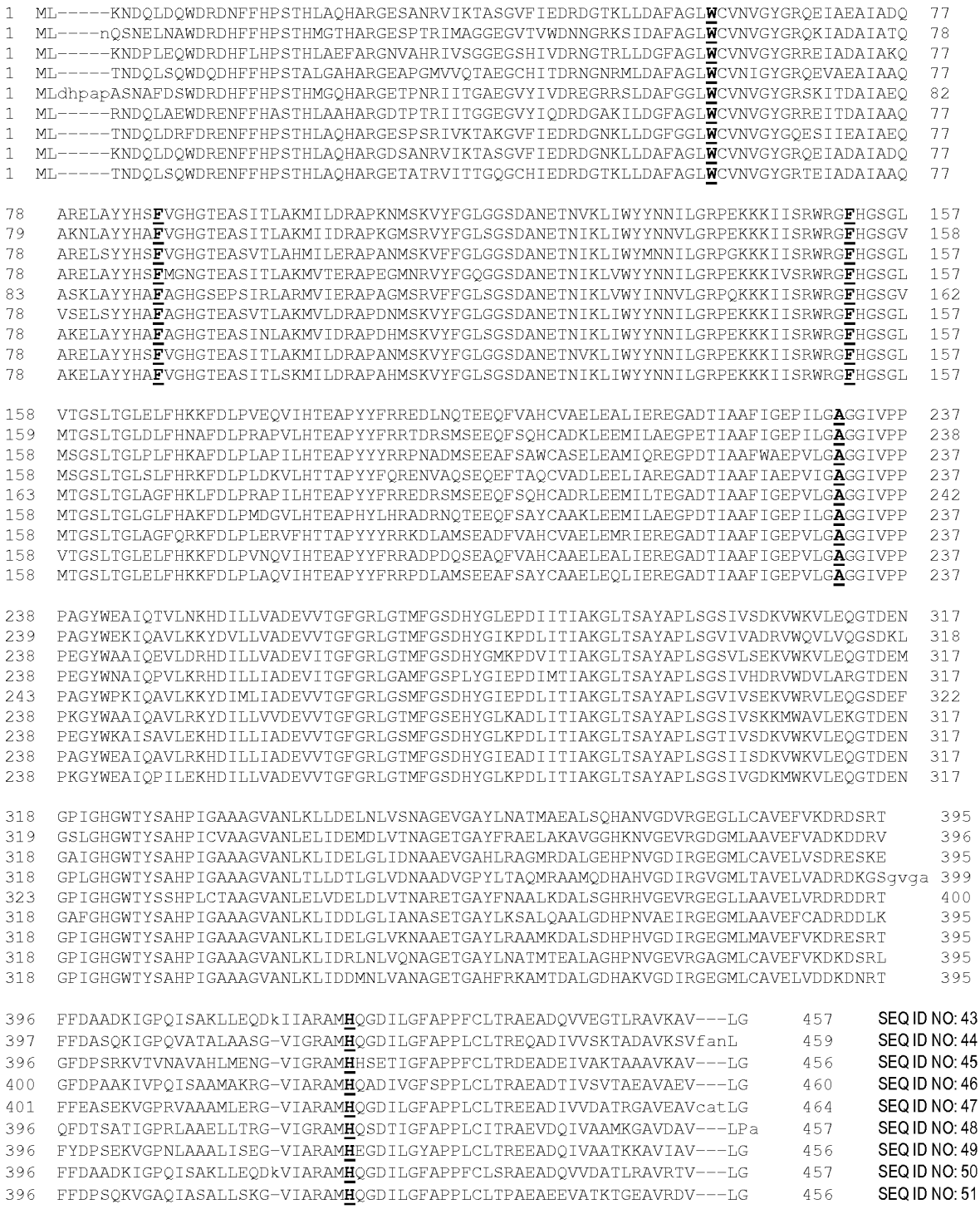
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Fig. 5

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78    ARELAYYHSFMGNGTEASITLAKMVTERAPEGMNRVYFGQGGSDANETNIKLVWYYNNILGRPEKKKIVSRWRGFHGSGL  157
83    ASKLAYYHAFAGHGSEPSIRLARMVIERAPAGMSRVFFGLSGSDANETNIKLVWYINNVLGRPQKKKIISRWRGFHGSGV  162
78    VSELSYYHAFAGHGTEASVTLAKMVLDRAPDNMSKVYFGLGGSDANETNIKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157
78    AKELAYYHAFAGHGTEASINLAKMVIDRAPDHMSKVYFGLSGSDANETNIKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157
78    ARELAYYHSFVGHGTEASITLAKMILDRAPANMSKVYFGLGGSDANETNVKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157
78    AKELAYYHAFVGHGTEASITLSKMILDRAPAHMSKVYFGLSGSDANETNIKLIWYYNNILGRPEKKKIISRWRGFHGSGL  157

158   VTGSLTGLELFHKKFDLPVEQVIHTEAPYYFRREDLNQTEEQFVAHCVAELEALIEREGADTIAAFIGEPILGAGGMVPP  237
159   MTGSLTGLDLFHNAFDLPRAPVLHTEAPYYFRRTDRSMSEEQFSQHCADKLEEMILAEGPETIAAFIGEPILGAGGMVPP  238
158   MSGSLTGLPLFHKAFDLPLAPILHTEAPYYYRRPNADMSEEAFSAWCASELEAMIQREGPDTIAAFWAEPVLGAGGMVPP  237
158   MSGSLTGLSLFHRKFDLPLDKVLHTTAPYYFQRENVAQSEQEFTAQCVADLEELIAREGADTIAAFIAEPVIGAGGMVPP  237
163   MTGSLTGLAGFHKLFDLPRAPILHTEAPYYFRREDRSMSEEQFSQHCADRLEEMILTEGADTIAAFIGEPVLGAGGMVPP  242
158   MTGSLTGLGLFHAKFDLPMDGVLHTEAPHYLHRADRNQTEEQFSAYCAAKLEEMILAEGPDTIAAFIGEPILGAGGMVPP  237
158   MTGSLTGLAGFQRKFDLPLERVFHTTAPYYYRRKDLAMSEADFVAHCVAELEMRIEREGADTIAAFIGEPVLGAGGMVPP  237
158   VTGSLTGLELFHKKFDLPVNQVIHTEAPYYFRRADPDQSEAQFVAHCAAELEALIEREGADTIAAFIGEPVLGAGGMVPP  237
158   MTGSLTGLELFHKKFDLPLAQVIHTEAPYYFRRPDLAMSEEAFSAYCAAELEQLIEREGADTIAAFIGEPVLGAGGMVPP  237

238   PAGYWEAIQTVLNKHDILLVADEVVTGFGRLGTMFGSDHYGLEPDIITIAKGLTSAYAPLSGSIVSDKVWKVLEQGTDEN  317
239   PAGYWEKIQAVLKKYDVLLVADEVVTGFGRLGTMFGSDHYGIKPDLITIAKGLTSAYAPLSGVIVADRVWQVLVQGSDKL  318
238   PEGYWAAIQEVLDRHDILLVADEVITGFGRLGTMFGSDHYGMKPDVITIAKGLTSAYAPLSGSVLSEKVWKVLEQGTDEM  317
238   PEGYWNAIQPVLKRHDILLIADEVITGFGRLGAMFGSPLYGIEPDIMTIAKGLTSAYAPLSGSIVHDRVWDVLARGTDEN  317
243   PAGYWPKIQAVLKKYDIMLIADEVVTGFGRLGSMFGSDHYGIEPDLITIAKGLTSAYAPLSGVIVSEKVWRVLEQGSDEF  322
238   PKGYWAAIQAVLRKYDILLVVDEVVTGFGRLGTMFGSEHYGLKADLITIAKGLTSAYAPLSGSIVSKKMWAVLEKGTDEN  317
238   PEGYWKAISAVLEKHDILLIADEVVTGFGRLGSMFGSDHYGLKPDLITIAKGLTSAYAPLSGTIVSDKVWKVLEQGTDEN  317
238   PAGYWEAIQAVLRKHDILLIADEVVTGFGRLGTMFGSDHYGIEADIITIAKGLTSAYAPLSGSIISDKVWKVLEQGTDEN  317
238   PKGYWEAIQPILEKHDILLVADEVVTGFGRLGTMFGSDHYGLKPDLITIAKGLTSAYAPLSGSIVGDKMWKVLEQGTDEN  317

318   GPIGHGWTYSAHPIGAAAGVANLKLLDELNLVSNAGEVGAYLNATMAEALSQHANVGDVRGEGLLCAVEFVKDRDSRT     395
319   GSLGHGWTYSAHPICVAAGVANLELIDEMDLVTNAGETGAYFRAELAKAVGGHKNVGEVRGDGMLAAVEFVADKDDRV     396
318   GAIGHGWTYSAHPIGAAAGVANLKLIDELGLIDNAAEVGAHLRAGMRDALGEHPNVGDIRGEGMLCAVELVSDRESKE     395
318   GPLGHGWTYSAHPIGAAAGVANLTLLDTLGLVDNAADVGPYLTAQMRAAMQDHAHVGDIRGVGMLTAVELVADRDKGSgvga 399
323   GPIGHGWTYSSHPLCTAAGVANLELVDELDLVTNARETGAYFNAALKDALSGHRHVGEVRGEGLLAAVELVRDRDDRT     400
318   GAFGHGWTYSAHPIGAAAGVANLKLIDDLGLIANASETGAYLKSALQAALGDHPNVAEIRGEGMLAAVEFCADRDDLK     395
318   GPIGHGWTYSAHPIGAAAGVANLKLIDELGLVKNAAETGAYLRAAMKDALSDHPHVGDIRGEGMLMAVEFVKDRESRT     395
318   GPIGHGWTYSAHPIGAAAGVANLKLIDRLNLVQNAGETGAYLNATMTEALAGHPNVGEVRGAGMLCAVEFVKDKDSRL     395
318   GPIGHGWTYSAHPIGAAAGVANLKLIDDMNLVANAGETGAHFRKAMTDALGDHAKVGDIRGEGMLCAVELVDDKDNRT     395

396   FFDAADKIGPQISAKLLEQDkIIARAMPQGDILGFAPPFCLTRAEADQVVEGTLRAVKAV---LG     457   SEQ ID NO: 53
397   FFDASQKIGPQVATALAASG-VIGRAMPQGDILGFAPPLCLTREQADIVVSKTADAVKSVfanL     459   SEQ ID NO: 55
396   GFDPSRKVTVNAVAHLMENG-VIGRAMPHSETIGFAPPFCLTRDEADEIVAKTAAAVKAV---LG     456   SEQ ID NO: 58
400   GFDPAAKIVPQISAAMAKRG-VIARAMPQADIVGFSPPLCLTRAEADTIVSVTAEAVAEV---LG     460   SEQ ID NO: 59
401   FFEASEKVGPRVAAAMLERG-VIARAMPQGDILGFAPPLCLTREEADIVVDATRGAVEAVcatLG     464   SEQ ID NO: 60
396   QFDTSATIGPRLAAELLTRG-VIGRAMPQSDTIGFAPPLCITRAEVDQIVAAMKGAVDAV---LPa    457   SEQ ID NO: 61
396   FYDPSEKVGPNLAAALISEG-VIARAMPEGDILGYAPPLCLTREEADQIVAATKKAVIAV---LG     456   SEQ ID NO: 62
396   FFDAADKIGPQISAKLLEQDkVIARAMPQGDILGFAPPFCLSRAEADQVVDATLRAVRTV---LG     457   SEQ ID NO: 63
396   FFDPSQKVGAQIASALLSKG-VIARAMPQGDILGFAPPLCLTPAEAEEVATKTGEAVRDV---LG     456   SEQ ID NO: 64
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