

Enzymatic method for the production of L-glufosinate *P*-alkyl esters

The present invention relates in a first aspect to an enzymatic method for the production of an L-glufosinate *P*-alkyl ester. This method is characterized by a step (c) in which an L-glufosinate *P*-alkyl ester carbamoylate is reacted to give the corresponding L-glufosinate *P*-alkyl ester. This step (c) is catalyzed by a carbamoylase, preferably by an L-enantioselective carbamoylase. The L-glufosinate *P*-alkyl ester carbamoylate employed in step (c) may be obtained by reaction from the corresponding L-glufosinate hydantoin *P*-alkyl ester. This optional, antecedent step (b) is catalyzed by a hydantoinase, preferably an L-enantioselective hydantoinase. Even more preferred, the L-glufosinate hydantoin *P*-alkyl ester employed in step (b) may be obtained by reaction from the corresponding D-glufosinate hydantoin *P*-alkyl ester. This optional, antecedent step (a) is preferably catalyzed by a hydantoin racemase.

In a second aspect, the present invention relates to an enzymatic method for enantioselective production of an L-glufosinate *P*-alkyl ester from a mixture **M_{IIIA}** of L- and D-glufosinate *P*-alkyl ester hydantoins. In the method according to the second aspect of the invention, the mixture **M_{IIIA}** is provided in a step (i-A) and employed in steps (ii) and (iii). Steps (ii) and (iii) correspond to steps (b) and (c) described above, respectively, wherein in step (c), an L-carbamoylase is employed.

In an optional, preferred step (i-B) of the method according to the second aspect of the invention, corresponding to preferred step (a) described above, a hydantoin racemase is preferably used to convert at least a part of the D-glufosinate *P*-alkyl ester hydantoin in the mixture **M_{IIIA}** into its corresponding L-enantiomer, giving a mixture **M_{IIIB}**, which is then employed in steps (ii) and (iii). The L-glufosinate *P*-alkyl ester obtained in the methods according to the first or second aspect of the invention may be saponified to give L-glufosinate.

1. Background of the Invention

Organic phosphorous compounds, i.e. chemical agents comprising a carbon-phosphor bond, are widely applied as herbicides in the area of plant protection. Agents such as the herbicides glyphosate (Roundup®, Touchdown®) and glufosinate (Basta®, Liberty®) as well as the growth regulator glyphosine (Polaris®) are used for this purpose (as described for example by G. Hörlein, Rev. Environ. Contam. Toxicol. **1994**, 138, 73 - 145).

The esters of *P*-methyl phosphinic acid (for example, *P*-methyl phosphinic acid butyl ester; "MPBE"; CAS-No: 6172-80-1) have a key role as synthetic building blocks in the synthesis of the non-selective herbicide glufosinate. These esters are accessible via two fundamental synthetic pathways (summarized in Figures 3 a and 3 b, page 130, of the article of K. Haack, Chem. Unserer Zeit **2003**, 37, 128 - 138):

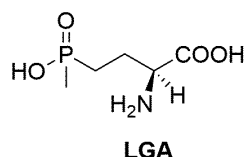
- a. Reacting diethyl chlorophosphite [$\text{ClP}(\text{OC}_2\text{H}_5)_2$] with CH_3MgCl provides methyl diethoxy phosphine [$\text{H}_3\text{CP}(\text{OC}_2\text{H}_5)_2$; "DEMP"; CAS-No. 15715-41-0], which is partially hydrolyzed to give the corresponding methylphosphinic acid ethyl ester (MPEE; CAS-Nr: 16391-07-4).
- b. Alternatively, methane can be reacted with phosphor trichloride at 500 °C to give methyl dichloro phosphane H_3CPCl_2 . The latter can then be solvolized in alcohols to give the corresponding methyl phosphinic acid esters.

The esters of *P*-methyl phosphinic acid add to carbon-carbon double bonds regioselectively. This property is used in the synthesis of glufosinate for the formation of the second phosphor-carbon bond. For example, $\text{H}_3\text{CPH}(\text{O})\text{OR}$ ($\text{R} = \text{Alkyl}$) reacts with 1-cyano allyl acetate in an addition reaction to provide an intermediate. Subsequent exchange of the acetate substituent with ammonia and hydrolysis of the cyano group and the ester group of the phosphinic acid moiety give glufosinate.

Acrylic acid ester is a cheaper alternative starting material. It can react with the ester of *P*-methyl phosphinic acid to 3-[alkoxy(methyl)phosphinyl]propionic acid alkyl ester. Claisen reaction of this diester with diethyl oxalate, hydrolysis and decarboxylation provide the corresponding α -keto acid, which can be reductively aminated to give glufosinate.

These and further synthetic routes towards glufosinate are also described in the art, e.g. in WO 1999/009039 A1, EP 0 508 296 A1.

A general disadvantage of all synthetic routes to glufosinate is that the obtained glufosinate is a racemic mixture. However, as there is no herbicidal activity of the D-enantiomer, L-glufosinate [hereinafter "LGA"; CAS-Nr. 35597-44-5; other names "(*S*)-glufosinate", "(*-*)-glufosinate"] is the enantiomer of economical interest.



For example, CN 111662325 A discloses a synthetic pathway in which L-homoserine is reacted to the respective hydantoin, followed by addition of methane phosphor dichloride, which results in a methane phosphane which is disubstituted with L-homoserine hydantoin. After a final Arbuzov reaction and hydrolysis, LGA is obtained. Although the disclosure describes a high enantiomeric excess (= "ee"), this ee could not be reproduced by the inventors of the present invention. A reason could be that during this synthesis pathway, strong acidic (HCl for saponification of the hydantoin phosphor bond) and alkaline conditions (NaOH, 100 °C for ring opening) are applied. Such conditions usually lead to racemization, as described by M. Bovarnick & H.T. Clarke, Journal of the American Chemical Society **1938**, 60, 2426 – 2430, by R. A. Lazarus, J. Org. Chem. **1990**, 55, 4755 – 4757, and by A. S. Bommarius, M. Kottenhahn, H. Klenk, K. Drauz: "A direct route from

hydantoins to D-amino acids employing a resting cell biocatalyst with D-hydantoinase and D-carbamoylase activity" on page 164 and 167 in "Microbial Reagents in Organic Synthesis" Series C: Mathematical and Physical Sciences - Vol. 381, S. Servi (Ed.), 1992, Springer Science+Business Media, B.V., Dordrecht.

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For enantioselective syntheses of LGA, chemical and enzymatic pathways are described in the art.

WO 2020/145513 A1 and WO 2020/145514 A1 describe a chemical route to LGA. In this route, an L-homoserine derivative such as O-acetyl L-homoserine or O-succinyl L-homoserine is used as starting material and L-glufosinate is obtained by a sequence of reactions including lactonization and halogenation.

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WO 2020/145627 A1 describes a similar route, wherein during halogenation, a bromo derivative is obtained.

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The route disclosed by CN 106083922 A resembles these synthetic pathways but starts off from L-methionine.

CN 108516991 A describes another synthetic pathway to LGA, starting from the azeotropic dehydration of L-homoserine to give L-3,6-bis(2-haloethyl)-2,5-diketopiperazine, followed by introduction of a methylphosphonate diester group, and hydrolysis.

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WO 2017/151573 A1 discloses a two-step enzymatic synthesis of LGA from D-glufosinate. In the first step, D-glufosinate is oxidatively deaminated to give 2-oxo-4-[hydroxy(methyl)phosphinoyl]-butyric acid ("PPO"), followed by the specific amination of PPO to LGA as the second step. The first step is carried out by catalysis of a D-amino acid oxidase, the second step is catalyzed by a transaminase.

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WO 2020/051188 A1 discloses a similar method of converting racemic glufosinate to the L-enantiomer. In addition, it discloses a step in which the α -ketoacid or ketone byproduct formed during amination of PPO with an amine donor is converted by ketoglutarate decarboxylase to further shift the equilibrium to LGA.

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WO 2019/018406 A1 discloses a method of purifying LGA from a mixture comprising LGA and glutamate. Glutamate is converted to pyroglutamate enzymatically by glutaminy-peptidyl cyclotransferase, and LGA is then purified from the resulting mixture with an ion-exchange resin.

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WO 2013/072486 A1 disclose hydantoinase mutants which have a greater activity towards D-amino acids.

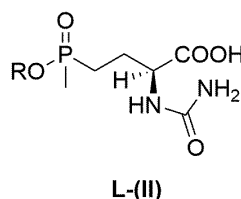
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WO 00/58449 A1 disclose hydantoinase mutants which have a greater activity towards L-amino acids.

The object of the present invention is to provide a further enzymatic process for producing
 5 glufosinate or glufosinate *P*-alkyl esters. This process should provide products with a high excess of the L-enantiomer over the D-enantiomer. Moreover, this process should allow to use new substrates which heretofore were not used in the enzymatic synthesis of glufosinate or glufosinate esters. Moreover, there is a need in the art for an enantioselective method for the production of
 10 L-glufosinate or L-glufosinate esters from starting materials comprising D- and L-enantiomers, such as racemic mixtures, in a minimal amount of synthetic steps.

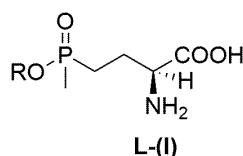
2. Short Summary of the Invention

Namely, it was surprisingly found that the desired L-glufosinate *P*-alkyl esters can be produced by
 15 an enzymatically catalyzed reaction from the corresponding carbamoylates of formula **L-(II)**, wherein R is an alkyl group or an aryl group:



20 Such carbamoylates were not used in the enzymatically catalyzed production of L-glufosinate or its *P*-alkyl esters before. This finding was even more surprising, as it turned out that the presence of an alkyl group or aryl group for R in formula **L-(II)** is mandatory, as there is no corresponding reaction of compounds according to formula **L-(II)** in which R = H.

25 α.1) The present invention hence solves the problems mentioned above by providing in a first aspect a method for the production of a glufosinate *P*-alkyl ester according to formula **L-(I)**, comprising a step (c) in which a compound according to the above-mentioned formula **L-(II)** is reacted to give a compound according to formula **L-(I)**:



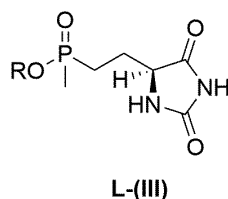
30 wherein the reaction according step (c) is catalyzed by a carbamoylase **E₁**, preferably by an L-enantioselective carbamoylase **E₁** ("L-enantioselective carbamoylase" = "L-carbamoylase") and wherein R is an alkyl group or aryl group, in particular an alkyl group or phenyl or benzyl, more

preferably an alkyl group, more preferably an alkyl group with 1 to 10, even more preferably with 1 to 6, even more preferably with 1 to 4 carbon atoms, even more preferably R = ethyl or *n*-butyl, most preferably R = *n*-butyl.

- 5 The L-carbamoylase **E₁** is preferably categorized in the EC class 3.5.1.87.

The polypeptide sequence of the L-carbamoylase **E₁** is preferably selected from SEQ ID NO: 1 and variants thereof, SEQ ID NO: 2 and variants thereof, SEQ ID NO: 3 and variants thereof, SEQ ID NO: 4 and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 6 and variants thereof, SEQ ID NO: 7 and variants thereof, SEQ ID NO: 8 and variants thereof, SEQ ID NO: 9 and variants thereof.

- α.2) In a preferred embodiment of the method according to the first aspect of the invention, the compound according to formula **L-(II)** that is employed in step (c) is obtained by a step (b) in which a compound according to formula **L-(III)** is reacted to give the compound according to formula **L-(II)**:



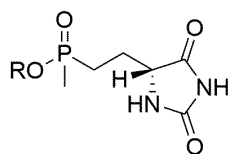
- wherein the reaction according to step (b) is catalyzed by a hydantoinase **E₂**, preferably an L-enantioselective hydantoinase **E₂** ("L-enantioselective hydantoinase" = "L-hydantoinase"), and wherein R in formula **L-(III)** has the same meaning as described for **L-(I)** above.

The hydantoinase **E₂**, in particular the L-hydantoinase **E₂**, is preferably categorized in the EC class 3.5.2.2.

- 25 The polypeptide sequence of the hydantoinase **E₂**, in particular the L-hydantoinase **E₂**, is preferably selected from SEQ ID NO: 10 and variants thereof, SEQ ID NO: 11 and variants thereof, SEQ ID NO: 12 and variants thereof, SEQ ID NO: 13 and variants thereof, SEQ ID NO: 14 and variants thereof, SEQ ID NO: 15 and variants thereof, SEQ ID NO: 16 and variants thereof.

- α.3) In an even more preferred embodiment of the method according to the first aspect of the invention, the compound according to formula **L-(III)** that is employed in step (b) is obtained by a step (a) in which a compound according to formula **D-(III)** is reacted to give the compound according to formula **L-(III)**:

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D-(III)

wherein the reaction according to step (a) is preferably catalyzed by a hydantoin racemase E_3 , and wherein R in formula D-(III) has the same meaning as described for L-(III) above [which is the same meaning of R as described for L-(I) above].

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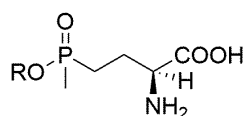
The hydantoin racemase E_3 is preferably categorized in the EC class 5.1.99.5.

The polypeptide sequence of the hydantoin racemase E_3 in step (a) is preferably selected from SEQ ID NO: 17 and variants thereof, SEQ ID NO: 18 and variants thereof, SEQ ID NO: 19 and variants thereof, SEQ ID NO: 20 and variants thereof, SEQ ID NO: 21 and variants thereof, SEQ ID NO: 22 and variants thereof, SEQ ID NO: 23 and variants thereof, SEQ ID NO: 24 and variants thereof, SEQ ID NO: 25 and variants thereof, SEQ ID NO: 26 and variants thereof.

It was further surprisingly found that the above-mentioned finding can be advantageously used for a method for enantioselective production of an L-glufosinate *P*-alkyl ester from mixtures of enantiomers such as racemic mixtures.

β.1) Hence, the present invention relates in a second aspect to a method for the production of an L-glufosinate *P*-alkyl ester according to formula L-(I):

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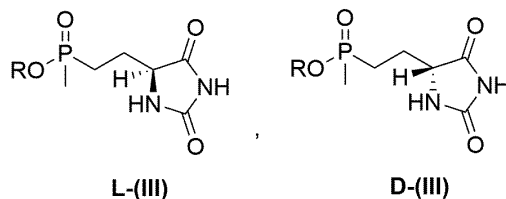


L-(I)

wherein R is an alkyl group or aryl group, in particular an alkyl group or phenyl or benzyl, more preferably an alkyl group, more preferably an alkyl group with 1 to 10, even more preferably with 1 to 6, even more preferably with 1 to 4 carbon atoms, even more preferably R = ethyl or *n*-butyl, most preferably R = *n*-butyl.

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In this method, a mixture M_{IIIa} comprising both enantiomers L-(III) and D-(III):

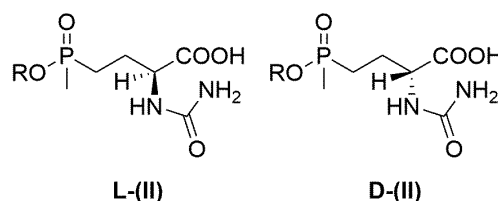


L-(III)

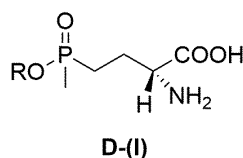
D-(III)

wherein R has the same meaning as described for **L-(I)** above, is provided [step (i)-A].

- β.2) In a step (ii), the mixture **M_{III A}** is subjected to a reaction with a hydantoinase **E₂**, which is more preferably as defined under item α.2), giving a composition **M_{II}** comprising **L-(II)** and optionally its enantiomer **D-(II)**, wherein **L-(II)** and **D-(II)** have the following formulae and wherein R in formulae **L-(II)** and **D-(II)** has the same meaning as described for **L-(I)** above:



- β.3) Then, in a further step (iii), **M_{II}** is subjected to a reaction, which is catalyzed by an L-carbamoylase **E₁**, which is preferably as defined under item α.1), giving a composition **M_I** comprising **L-(I)** and optionally its enantiomer **D-(I)**, wherein **D-(I)** has the following formula and wherein R in formula **D-(I)** has the same meaning as described for **L-(I)** above:



- The method according to the second aspect of the invention is enantioselective, because **M_I** comprises either only **L-(I)** or, in case **M_I** comprises both enantiomers **L-(I)** and **D-(I)**, the molar ratio of **L-(I)** to **D-(I)** in **M_I** is greater than the molar ratio of **L-(III)** to **D-(III)** in **M_{III A}**.

- β.4) Furthermore, it was surprisingly found that the method for enantioselective production according to the second aspect of the invention can be further improved. Namely, in an optional step (i-B) which is carried out after step (i-A) [and which may be carried out before or concomitantly with steps (ii) and (iii), preferably concomitantly with steps (ii) and (iii)], at least a part of the compounds **D-(III)** comprised by the mixture **M_{III A}** are reacted, preferably enzymatically catalyzed by a hydantoin racemase **E₃**, into **L-(III)**, which is the enantiomer of **D-(III)**, giving a composition **M_{III B}** comprising **L-(III)** and optionally its enantiomer **D-(III)**.

- In case the reaction according to step (i-B) is enzymatically catalyzed, it is preferably catalyzed by a hydantoin racemase **E₃**.

The hydantoin racemase **E₃** is preferably as defined under item α.3).

3. Brief Description of the Drawings

- Figure 1 shows the pOM21c plasmid map.
- 5 Figure 2 shows the pOM22c plasmid map.
- Figure 3 shows the pOM17c {Prha} plasmid map.
- Figure 4 shows the pOM17c {Prha}{amaB_Gst} plasmid map.
- Figure 5 shows the pOM17c {Prha}{atc_Ps} plasmid map.
- Figure 6 shows the pOM17c {Prha}{hyuC_Pau} plasmid map.
- 10 Figure 7 shows the pOM17c {Prha}{hyuC_Asp (co_Ec)} plasmid map.

4. Detailed Description of the Invention

4.1 Definitions

- 15 Any of the enzymes used according to any aspect of the present invention, may be an isolated enzyme. In particular, the enzymes used according to any aspect of the present invention may be used in an active state and in the presence of all cofactors, substrates, auxiliary and/or activating polypeptides or factors essential for its activity. Such factors may be metal ions such as Mn^{2+} or
- 20 Co^{2+} .

In particular, an enzyme according to the present application may be a carbamoylase **E₁**, a hydantoinase **E₂**, or a hydantoin racemase **E₃**.

- 25 “Enzymatically catalyzed” means that the respective reaction is catalyzed by an enzyme, which may be a carbamoylase **E₁**, a hydantoinase **E₂**, or a hydantoin racemase **E₃**.

- The enzyme used according to any aspect of the present invention may be recombinant. The term “recombinant” as used herein, refers to a molecule or is encoded by such a molecule, particularly a
- 30 polypeptide or nucleic acid that, as such, does not occur naturally but is the result of genetic engineering or refers to a cell that comprises a recombinant molecule. For example, a nucleic acid molecule is recombinant if it comprises a promoter functionally linked to a sequence encoding a catalytically active polypeptide and the promoter has been engineered such that the catalytically active polypeptide is overexpressed relative to the level of the polypeptide in the corresponding
- 35 wild type cell that comprises the original unaltered nucleic acid molecule.

- A “polypeptide” (one or more peptides) is a chain of chemical building blocks called amino acids that are linked together by chemical bonds called peptide bonds. A protein or polypeptide, including an enzyme, may be “native” or “wild-type”, meaning that it occurs in nature or has the amino acid
- 40 sequence of a native protein, respectively. These terms are sometimes used interchangeably. A polypeptide may or may not be glycosylated.

The term “overexpressed”, as used herein, means that the respective polypeptide encoded or expressed is expressed at a level higher or at higher activity than would normally be found in the cell under identical conditions in the absence of genetic modifications carried out to increase the expression, for example in the respective wild type cell.

4.2 Methods to obtain enzymes

The enzymes that can be used in the method according to the present invention can be synthesized by methods that are known to the skilled person.

One approach, which is a preferred approach according to the invention, is to express the enzyme(s) in microorganism(s) such as *Escherichia coli* (= “*E. coli*”), *Saccharomyces cerevisiae*, *Pichia pastoris*, and others, and to add the whole cells to the reactions as whole cell biocatalysts. Another approach is to express the enzyme(s), lyse the microorganisms, and add the cell lysate. Yet another approach is to purify, or partially purify, the enzyme(s) from a lysate and add pure or partially pure enzyme(s) to the reaction. If multiple enzymes are required for a reaction, the enzymes can be expressed in one or several microorganisms, including expressing all enzymes within a single microorganism.

For example, the skilled person can obtain the enzymes according to the invention by expression, in particular, overexpression, [hereinafter, “expression, in particular overexpression” is abbreviated as (over)expression”, and “express, in particular overexpress” is abbreviated as (over)express”] of these enzymes in a cell and subsequent isolation thereof, e.g. as described in DE 100 31 999 A1. Episomal plasmids, for example, are employed for increasing the expression of the respective genes. In such plasmids, the nucleic acid molecule to be (over)expressed or encoding the polypeptide or enzyme to be (over)expressed may be placed under the control of a strong inducible promoter such as the *lac* promoter, located upstream of the gene. A promoter is a DNA sequence consisting of about 40 to 50 base pairs which constitutes the binding site for an RNA polymerase holoenzyme and the transcriptional start point (M. Pátek, J. Holátko, T. Busche, J. Kalinowski, J. Nešvera, *Microbial Biotechnology* **2013**, *6*, 103-117), whereby the strength of expression of the controlled polynucleotide or gene can be influenced. A “functional linkage” is obtained by the sequential arrangement of a promoter with a gene, which leads to a transcription of the gene.

Suitable strong promoters or methods of producing such promoters for increasing expression are known from the literature (e.g. S. Lisser & H. Margalit, *Nucleic Acid Research* **1993**, *21*, 1507-1516; M. Pátek and J. Nesvera in H. Yukawa and M Inui (eds.), *Corynebacterium glutamicum*, *Microbiology Monographs* 23, Springer Verlag Berlin Heidelberg 2013, 51-88; B. J. Eikmanns, E. Kleinertz, W. Liebl, H. Sahm, *Gene* **1991**, *102*, 93-98). For instance, native promoters may be optimized by altering the promoter sequence in the direction of known consensus sequences with

respect to increasing the expression of the genes functionally linked to these promoters (M. Pátek, B.J. Eikmanns, J. Pátek, H. Sahm, *Microbiology* **1996**, 142, 1297-1309; M. Pátek, J. Holátko, T. Busche, J. Kalinowski, J. Nešvera, *Microbial Biotechnology* **2013**, 6, 103-117).

- 5 Constitutive promoters are also suitable for the (over)expression, in which the gene encoding the enzyme activity is expressed continuously under the control of the promoter such as, for example, the glucose dependent *deo* promoter. Chemically induced promoters are also suitable, such as *tac*, *lac*, *rha* or *trp*. The most widespread system for the induction of promoters is the *lac* operon of *E. coli*. In this case, either lactose or isopropyl β -D-thiogalactopyranoside (IPTG) is used as
- 10 inducer. Also, systems using arabinose (e.g. the pBAD system) or rhamnose (e.g. *E. coli* KRX) are common as inducers. A system for physical induction is, for example, the temperature-induced cold shock promoter system based on the *E. coli cspA* promoter from Takara or Lambda PL and also osmotically inducible promoters, for example, *osmB* (e.g. WO 95/25785 A1).
- 15 Suitable plasmids or vectors are in principle all embodiments available for this purpose to the person skilled in the art. The state of the art describes standard plasmids that may be used for this purpose, for example the pET system of vectors exemplified by pET-3a or pET-26b(+) (commercially available from Novagen). Further plasmids and vectors can be taken, for example, pOM21 described in WO 2004/111227 A2, pOM22 described in WO 00/058449 A1 or pOM18
- 20 described in WO 2013/072486 A1 or from the brochures of the companies Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids and vectors can be found in: Glover, D.M. (1985) DNA cloning: a practical approach, Vol. I-III, IRL Press Ltd., Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) (1988) Vectors: a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V. (1990) Systems for heterologous
- 25 gene expression, *Methods Enzymol.* 185, 3-7; Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York. Of these plasmids, pOM21 and pOM22 are preferred.

- The plasmid vector, which contains the gene to be amplified, is then converted to the desired
- 30 strain, e.g. by conjugation or transformation. The method of conjugation is described, for example, by A. Schäfer, J. Kalinowski, A. Pühler, *Applied and Environmental Microbiology* **1994**, 60, 756 - 759. Methods for transformation are described, for example, in G. Thierbach, A. Schwarzer, A. Pühler, *Applied Microbiology and Biotechnology* **1988**, 29, 356 - 362, L.K. Dunican & E. Shivan, *Bio/Technology* **1989**, 7, 1067-1070 and A. Tauch, O. Kirchner, L. Wehmeier,
- 35 J. Kalinowski, A. Pühler, *FEMS Microbiology Letters* **1994**, 123, 343-347. After homologous recombination by means of a "cross-over" event, the resulting strain contains at least two copies of the gene concerned.

- The desired enzyme can be isolated by disrupting cells which contain the desired activity in a
- 40 manner known to the person skilled in the art, for example with the aid of a ball mill, a French press

or of an ultrasonic disintegrator and subsequently separating off cells, cell debris and disruption aids, such as, for example, glass beads, by centrifugation for 10 minutes at 13,000 rpm and 4 °C. Using the resulting cell-free crude extract, enzyme assays with subsequent LC-ESI-MS detection of the products can then be carried out. Alternatively, the enzyme can be enriched in the manner

5 known to the person skilled in the art by chromatographic methods (such as nickel-nitrilotriacetic acid affinity chromatography, streptavidin affinity chromatography, gel filtration chromatography or ion-exchange chromatography) or else purified to homogeneity. Quantification of the enzyme can be performed by methods known to the person skilled in the art, for example by determination of the concentration of the respective polypeptide of the enzyme (e.g. carbamoylase, hydantoinase

10 and racemase) in the obtained solution by SDS page and analysis of the respective bands via the software GelQuant® (BiochemLabSolutions).

Moreover, whether or not a nucleic acid or polypeptide is (over)expressed, may be determined by way of quantitative PCR reaction in the case of a nucleic acid molecule, SDS polyacrylamide

15 electrophoreses, Western blotting or comparative activity assays in the case of a polypeptide. Genetic modifications may be directed to transcriptional, translational, and/or post-translational modifications that result in a change of enzyme activity and/or selectivity under selected and/or identified culture conditions.

20 4.3 Variants

In the context of the present invention, the term “variant” with respect to polypeptide sequences refers to a polypeptide sequence with a degree of identity to the reference sequence (“sequence identity”) of at least 60 %, preferably at least 70 %, more preferably at least 71 %, more preferably

25 at least 72 %, more preferably at least 73 %, more preferably at least 74 %, more preferably at least 75 %, more preferably at least 76 %, more preferably at least 77 %, more preferably at least 78 %, more preferably at least 79 %, more preferably at least 80 %, more preferably at least 81 %, more preferably at least 82 %, more preferably at least 83 %, more preferably at least 84 %, more preferably at least 85 %, more preferably at least 86 %, more preferably at least 87 %, more

30 preferably at least 88 %, more preferably at least 89 %, more preferably at least 90 %, more preferably at least 91 %, more preferably at least 92 %, more preferably at least 93 %, more preferably at least 94 %, more preferably at least 95 %, more preferably at least 96 %, more preferably at least 97 %, more preferably at least 98 %, more preferably at least 99 %, more preferably at least 99.9 %. In still further particular embodiments, the degree of identity is at least

35 98.0 %, more preferably at least 98.2 %, more preferably at least 98.4 %, more preferably at least 98.6 %, more preferably at least 98.8 %, more preferably at least 99.0 %, more preferably at least 99.1 %, more preferably at least 99.2 %, more preferably at least 99.3 %, more preferably at least 99.4 %, more preferably at least 99.5 %, more preferably at least 99.6 %, more preferably at least 99.7 %, more preferably at least 99.8 %, or at least more preferably at least 99.9 %.

It goes without saying that a "variant" of a certain polypeptide sequence is not identical to the polypeptide sequence.

Such variants may be prepared by introducing deletions, insertions, substitutions, or combinations thereof, in particular in amino acid sequences, as well as fusions comprising such macromolecules or variants thereof.

Modifications of amino acid residues of a given polypeptide sequence which lead to no significant modifications of the properties and function of the given polypeptide are known to those skilled in the art. Thus for example many amino acids can often be exchanged for one another without problems; examples of such suitable amino acid substitutions are: Ala by Ser; Arg by Lys; Asn by Gln or His; Asp by Glu; Cys by Ser; Gln by Asn; Glu by Asp; Gly by Pro; His by Asn or Gln; Ile by Leu or Val; Leu by Met or Val; Lys by Arg or Gln or Glu; Met by Leu or Ile; Phe by Met or Leu or Tyr; Ser by Thr; Thr by Ser; Trp by Tyr; Tyr by Trp or Phe; Val by Ile or Leu. It is also known that modifications, particularly at the N- or C-terminus of a polypeptide in the form of for example amino acid insertions or deletions, often exert no significant influence on the function of the polypeptide.

In line with this, preferable variants according to the invention of any of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, 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, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, respectively, have a polypeptide sequence that comprises the complete polypeptide sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, 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, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, respectively, or at least the amino acids of the respective sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, 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, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 that are essential for the function, for example the catalytic activity of a protein, or the fold or structure of the protein. The other amino acids may be deleted, substituted or replaced by insertions or essential amino acids are replaced in a conservative manner to the effect that the activity of the enzyme, in particular the L-carbamoylase (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9), hydantoinase (SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14,

SEQ ID NO: 15, SEQ ID NO: 16), hydantoin racemase (SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26), is preserved.

3.4 Sequence Identity

5

The person skilled in the art is aware that various computer programs are available for the calculation of similarity or identity between two nucleotide or polypeptide sequences.

Preferred methods for determining the sequence identity initially generate the greatest alignment between the sequences to be compared. Computer programs for determining the sequence identity include, but are not limited to, the GCG program package including

- GAP [J. Deveroy *et al.*, Nucleic Acid Research **1984**, 12, page 387, Genetics Computer Group University of Wisconsin, Medicine (WI)], and
- BLASTP, BLASTN and FASTA (S. Altschul *et al.*, Journal of Molecular Biology **1990**, 215, 403 - 410). The BLAST program can be obtained from the National Center for Biotechnology Information (NCBI) and from other sources (BLAST Handbook, S. Altschul *et al.*, NCBI NLM NIH Bethesda ND 22894; S. Altschul *et al.*, above).

For instance, the percentage identity between two polypeptide sequences can be determined by the algorithm developed by S. B. Needleman & C. D. Wunsch, J. Mol. Biol. **1970**, 48, 443 - 453, which has been integrated into the GAP program in the GCG software package, using either a BLOSUM62 matrix or a PAM250 matrix, a gap weight of 16, 14, 12, 10, 8, 6 or 4 and a length weight of 1, 2, 3, 4, 5 or 6. The person skilled in the art will recognize that the use of different parameters will lead to slightly different results, but that the percentage identity between two polypeptide overall will not be significantly different. The BLOSUM62 matrix is typically used applying the default settings (gap weight: 12, length weight: 1).

In the context of the present invention, a sequence identity of 60% according to the above algorithm means 60% homology. The same applies to higher sequence identities.

Most preferably, the degree of identity between sequences is determined in the context of the present invention by the programme "Needle" using the substitution matrix BLOSUM62, the gap opening penalty of 10, and the gap extension penalty of 0.5. The Needle program implements the global alignment algorithm described in S. B. Needleman & C. D. Wunsch, J. Mol. Biol. **1970**, 48, 443-453. The substitution matrix used according to the present invention is BLOSUM62, gap opening penalty is 10, and gap extension penalty is 0.5. The preferred version used in the context of this invention is the one presented by F. Madeira, Y.M. Park, J. Lee, N. Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A.R.N. Tivey, S.C. Potter, R.D. Finn, Nucleic Acids Research **2019**, 47, W636-W641, Web Server issue (preferred version accessible online on June 16, 2021 via https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

40

In a particular embodiment, the percentage of identity of an amino acid sequence of a polypeptide with, or to, a reference polypeptide sequence is determined by

- i) aligning the two amino acid sequences using the Needle program, with the BLOSUM62 substitution matrix, a gap opening penalty of 10, and a gap extension penalty of 0.5;
- 5 ii) counting the number of exact matches in the alignment;
- iii) dividing the number of exact matches by the length of the longest of the two amino acid sequences, and
- iv) converting the result of the division of iii) into percentage.

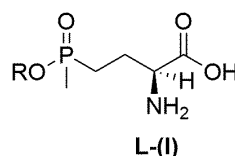
10 4.5 First aspect: Method for production of a glufosinate alkyl ester

4.5.1 Step (c)

4.5.1.1 Enzymatic catalysis of step (c)

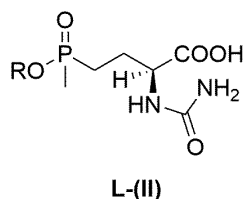
15

The present invention relates in a first aspect to a method for the production of an L-glufosinate *P*-alkyl ester according to formula **L-(I)**:



The method according to the first aspect of the invention comprises a step (c).

- 20 In step (c), a compound according to formula **L-(II)** is reacted to give a compound according to formula **L-(I)**. Formula **L-(II)** has the following structure:



The reaction according step (c) is enzymatically catalyzed, namely it is catalyzed by a carbamoylase **E₁**.

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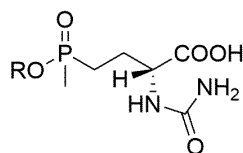
In formulae **L-(II)** and **L-(I)**, R is an alkyl group or an aryl group. In particular, R is selected from the group consisting of alkyl group, phenyl group, benzyl group. Preferably, R is an alkyl group, more preferably an alkyl group with 1 to 10, even more preferably with 1 to 6, even more preferably with 1 to 4 carbon atoms. Even more preferably R = ethyl or *n*-butyl, most preferably R = *n*-butyl.

30

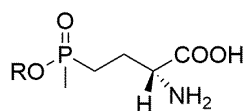
4.5.1.2 Enantioselective and enantiospecific catalysis of step (c)

Step (c) of the method according to the first aspect of the present invention is preferably L-enantioselective, even more preferably L-enantiospecific.

- In such a preferred embodiment, **L-(II)** is in particular employed in step (c) as a mixture **M_{II}** comprising, besides **L-(II)**, the enantiomer of **L-(II)**, which is **D-(II)**, wherein **D-(II)** has the following structure, wherein R in **D-(II)** has the same meaning as defined for **L-(II)** and wherein R in **L-(II)** and **D-(II)** is the same:

**D-(II)**

- For such mixtures **M_{II}**, a parallel reaction according to step (c)* may be observed. Namely, in the reaction according to step (c)*, **D-(II)**, i.e. the enantiomer of **L-(II)**, in mixture **M_{II}** is reacted to give a compound according to formula **D-(I)**:

**D-(I)**

- In case that step (c) is "L-enantioselective", this means that in case a mixture **M_{II}** is employed in step (c), then there is either no reaction according to step (c)* or, in case there is a reaction according to step (c)*, then the rate of reaction according to step (c)* is lesser than the rate of the reaction according to step (c).

- Step (c) is "L-enantiospecific", if the rate of reaction according to step (c)* is essentially zero, i.e. there is no reaction according to step (c)*.

20

In a preferred embodiment, the mixture **M_{II}** is a racemic mixture of enantiomer **L-(II)** and enantiomer **D-(II)**, meaning that the molar ratio of enantiomer **L-(II)** to enantiomer **D-(II)** is essentially 1 : 1.

- In other preferred embodiments, the molar ratio of enantiomer **L-(II)** to enantiomer **D-(II)** in mixture **M_{II}** is in the range of from 3 : 2 to 1 : 99, more preferably in the range of from 1.01 : 1 to 1 : 99, more preferably in the range of from 1 : 1 to 1 : 99, more preferably in the range of from 1 : 1.01 to 1 : 99, more preferably in the range of from 1 : 1.01 to 1 : 9, more preferably in the range of from 1 : 1.01 to 1 : 8, more preferably in the range of from 1 : 1.01 to 1 : 3.

30

Alternatively, enantiomer **D-(II)** is comprised in an excess to **L-(II)** in mixture **M_{II}**, meaning that, while **L-(II)** is present in the mixture **M_{II}**, the molar ratio of enantiomer **L-(II)** to enantiomer **D-(II)** in

mixture M_{II} is $< 1 : 1$, preferably $< 0.9 : 1$, more preferably $< 0.75 : 1$, more preferably $< 0.5 : 1$, more preferably $< 0.2 : 1$, more preferably $< 0.1 : 1$, more preferably $< 0.01 : 1$.

Step (c) is in particular L-enantioselective, if it is catalyzed by an L-carbamoylase E_1 , which may be determined by the skilled person as set forth under 4.5.4.3.

In case step (c) is L-enantioselective, the reaction according to step (c) proceeds preferably at a reaction rate that is at least 2 times greater, preferably at least 10 times greater, more preferably at least 100 times greater, even more preferably at least 10^3 times greater, even more preferably at least 10^4 times greater, even more preferably at least 10^5 times greater than the reaction rate at which step (c)* proceeds.

To quantify the factor at which the reaction rate of step (c) proceeds compared to the reaction rate of step (c)*, the following test may be carried out:

(1) An equimolar mixture [molar ratio of the two enantiomers **L-(II)** and **D-(II)** is $1 : 1$] is subjected to the respective reactions conditions and the development of the two products **L-(I)** and **D-(I)** is monitored over time (e.g. by LC-MS as set forth under item 5.4).

(2) When $n_{LH0} = 10$ mol-% of the initially employed **L-(II)** has reacted to the product **L-(I)**, the amount of **D-(I)** that was formed by reaction from **D-(II)** [in mol-% relative to the initially employed **D-(II)**] is measured ($= n_{DH0}$).

(3) The ratio of $n_{LH0} / n_{DH0} = 10 / n_{DH0}$ gives the factor at which the reaction rate of step (c) proceeds compared to the reaction rate of step (c)*.

→ If $n_{LH0} / n_{DH0} > 1$, the reaction rate of step (c) is greater than the reaction rate of step (c)*.

→ If $n_{LH0} / n_{DH0} < 1$, the reaction rate of step (c) is lesser than the reaction rate of step (c)*.

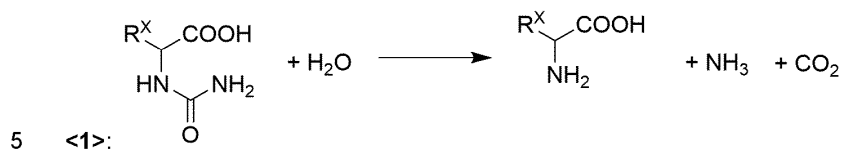
→ If $n_{LH0} / n_{DH0} = 1$, the reaction rates of steps (c) and (c)* are the same.

4.5.2 Carbamoylases

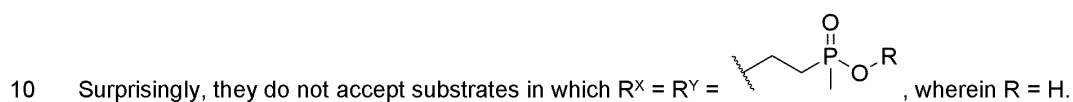
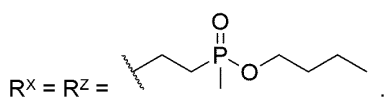
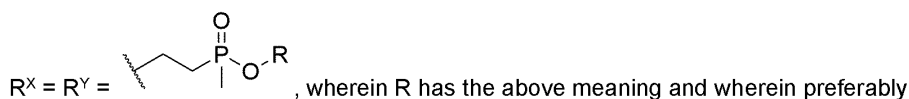
The reaction according to step (c) of the method according to the first aspect of the invention is catalyzed by a carbamoylase E_1 .

Namely, it was surprisingly found that carbamoylases accept compounds of formula **L-(II)** as substrates and convert them to products **L-(I)**, and hence can be used to catalyze the reaction according to step (c). This finding is of high scientific and economic value, as it opens new synthetic routes based on new starting materials for the production of L-glufosinate *P*-alkyl esters and L-glufosinate. Even more surprisingly, it was found that L-glufosinate carbamoylate, i.e. the compound according to **L-(II)**, in which $R = H$, does not undergo reaction by carbamoylases to give L-glufosinate.

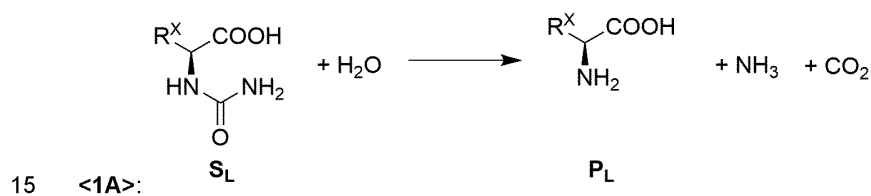
In nature, carbamoylases generally catalyze the following reaction <1>, wherein R^X may be an organic residue, e.g. a side chain of one of the naturally occurring amino acids.



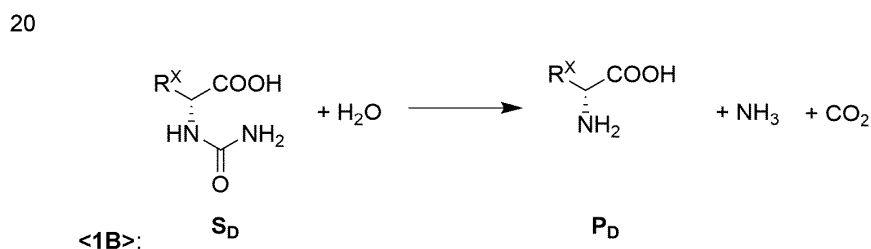
It was now surprisingly found that carbamoylases also accept substrates in which



In the context of the present invention, a “carbamoylase E_1 ” is a carbamoylase that catalyzes the following reaction <1A> of a carbamoyl substrate S_L to the respective amino acid product P_L , wherein $R^X = R^Y$ and preferably $R^X = R^Z$.



In particular, the carbamoylase E_1 is a “L-carbamoylase”, i.e. it has a greater catalytic activity for reaction <1A> than for reaction <1B>, wherein the substrate S_D in the reaction <1B> is the enantiomer of the substrate S_L in the reaction <1A>:



As an L-carbamoylase has a higher catalytic activity for reaction <1A> than for reaction <1B>, it is "L-enantioselective". An L-carbamoylase that has no catalytic activity for reaction <1B> and thus only has catalytic activity for reaction <1A> is "L-enantiospecific".

- 5 A "D-carbamoylase" is defined as a carbamoylase which is "D-enantioselective", i.e. it has a higher catalytic activity for reaction <1B> than for reaction <1A>. A D-carbamoylase that does not catalyze reaction <1A> and thus only has catalytic activity for reaction <1B> is "D-enantiospecific".

- 10 A carbamoylase which has the same catalytic activity for reaction <1B> as for reaction <1A>, is referred to as "non-enantioselective carbamoylase".

For determination whether a carbamoylase may be denoted as "L-carbamoylase", "D-carbamoylase" or "non-enantioselective carbamoylase" in the context of the present invention, the procedure according to **Assay B** (item 4.5.4) may preferably be used.

15

- The carbamoylase **E₁**, in particular the L-carbamoylase **E₁**, that may be used in step (c) of the method according to the first aspect of the invention may originate from *Achromobacter* sp., in particular *Achromobacter xylosoxidans*; *Agrobacterium* sp., in particular *Agrobacterium tumefaciens*; *Arthrobacter* sp., in particular *Arthrobacter crystallopoietes*, *Arthrobacter aurescens*,
 20 *Arthrobacter* sp. BT801; *Bacillus* sp., in particular *Bacillus fordii*; *Blastobacter* sp.; *Bradyrhizobium* sp., in particular *Bradyrhizobium japonicum*; *Brevibacillus* sp., in particular *Brevibacillus reuszeri*; *Comamonas* sp.; *Ensifer* sp., in particular *Ensifer adhaerens*; *Flavobacterium* sp. *Geobacillus* sp., in particular *Geobacillus kaustophilus*, *Geobacillus stearothermophilus*; *Microbacterium* sp., in particular *Microbacterium liquefaciens* strain AJ3912; *Paenarthrobacter* sp., in particular
 25 *Paenarthrobacter aurescens*; *Pasteurella* sp.; *Pseudomonas* sp.; *Ralstonia* sp., in particular *Ralstonia pickettii*; *Sinorhizobium* sp., in particular *Sinorhizobium meliloti*.

- An L-carbamoylase **E₁** suitable for the method according to the present invention may be the enzyme HyuC, which originates from *Arthrobacter*. Other enzymes are AmaB, AtcC, Inc,
 30 SinmeB_2280.

WO 01/23582 A1 discloses an example of an enzyme having carbamoylase activity according to the invention.

- 35 The carbamoylase **E₁** that may be used in step (c) of the method according to the present invention may be an L-carbamoylase categorized in the EC class EC 3.5.1.87.

L-carbamoylase enzymes are for example described by J. Ogawa, H. Miyake, S. Shimizu, *Appl. Microbiol. Biotechnol.* **1995** 43, 1039 – 1043 and in WO 01/23582 A1.

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- A L-carbamoylase E₁ that may preferably be used in step (c) according to the first aspect of the invention may originate from *Arthrobacter* sp., in particular *Arthrobacter crystallopoietes*, *Arthrobacter aurescens*, *Arthrobacter* sp. BT801, *Arthrobacter aurescens* DSM 3747; *Bacillus* sp., in particular *Bacillus fordii*; *Geobacillus* sp., in particular *Geobacillus stearothermophilus*,
 5 *Geobacillus kaustophilus*; *Microbacterium* sp., in particular *Microbacterium liquefaciens* strain AJ3912; *Paenarthrobacter* sp., in particular *Paenarthrobacter aurescens*; *Pseudomonas* sp., in particular *Pseudomonas* sp. QR-101; *Sinorhizobium* sp., in particular *Sinorhizobium meliloti*. Even more preferably, the L-carbamoylase E₁ that may preferably be used in step (c) according to the first aspect of the invention may originate from *Arthrobacter* sp., in particular *Arthrobacter*
 10 *crystallopoietes*, *Arthrobacter aurescens*, *Arthrobacter* sp. BT801, *Arthrobacter aurescens* DSM 3747, most preferably from *Arthrobacter aurescens* DSM 3747.

- The respective sequences can be derived from databases such as the Braunschweig Enzyme Database (BRENDA, Germany, available under www.brenda-enzymes.org/index.php), the National
 15 Center for Biochemical Information (NCBI, available under <https://www.ncbi.nlm.nih.gov/>) or the Kyoto Encyclopedia of Genes and Genomes (KEGG, Japan, available under www.genome.jp/kegg/).

- The following table 1 gives preferred examples for polypeptide sequences of L-carbamoylases E₁ that may be preferably used in step (c) of the method according to the first aspect of the invention.
 20 The genes encoding the respective L-carbamoylase E₁ and the respective accession code are indicated as far as known.

Table 1

L-Carbamoylases (EC 3.5.1.87)			
Strain	Gene name	GenBank/UniProt accession	SEQ ID NO: of the polypeptide
<i>Arthrobacter aurescens</i> DSM 3747	<i>hyuC</i>		SEQ ID NO: 1
<i>Geobacillus stearothermophilus</i>	<i>amaB</i>	Q53389	SEQ ID NO: 2
<i>Pseudomonas</i> sp. QR-101	<i>atcC</i>	H9B8T5	SEQ ID NO: 3
<i>Geobacillus kaustophilus</i>	<i>Inc</i>	Q8GQG5	SEQ ID NO: 4
<i>Paenarthrobacter aurescens</i>	<i>hyuC</i>	Q9F464	SEQ ID NO: 5
<i>Sinorhizobium meliloti</i>	<i>SinmeB_2280</i>	A0A0E0UEY4	SEQ ID NO: 6
<i>Bacillus fordii</i> strain MH602	not assigned	ABL14248	SEQ ID NO: 7
<i>Arthrobacter</i> sp. BT801	<i>hyuC</i>	AAL55413	SEQ ID NO: 8
<i>Microbacterium liquefaciens</i> strain AJ3912	not assigned		SEQ ID NO: 9

In a preferred embodiment of the method according to the first aspect of the present invention, the reaction according to step (c) is catalyzed by an L-carbamoylases **E₁**, wherein the polypeptide sequence of **E₁** is selected from the group consisting of SEQ ID NO: 1 and variants thereof, SEQ ID NO: 2 and variants thereof, SEQ ID NO: 3 and variants thereof, SEQ ID NO: 4 and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 6 and variants thereof, SEQ ID NO: 7 and variants thereof, SEQ ID NO: 8 and variants thereof, SEQ ID NO: 9 and variants thereof, preferably SEQ ID NO: 1 and variants thereof.

4.5.3 Assays **A_L** and **A_D** for determining carbamoylase activity

The skilled person is aware of carbamoylases, in particular L-carbamoylases, that may be used in step (c) of the method according to the first aspect of the invention.

In particular, **Assay A_L**, described in the following, may be used to determine carbamoylase and L-carbamoylase activity of a given enzyme **E_x** and may advantageously be used according to the invention to determine carbamoylase and L-carbamoylase activity in variants of SEQ ID NO: 1, variants of SEQ ID NO: 2, variants of SEQ ID NO: 3, variants of SEQ ID NO: 4, variants of SEQ ID NO: 5, variants of SEQ ID NO: 6, variants of SEQ ID NO: 7, variants of SEQ ID NO: 8, variants of SEQ ID NO: 9.

For comparative reasons, **Assay A_D** may be used to determine D-carbamoylase activity of a given enzyme **E_x**.

For the purpose of **Assay A_L** and **Assay A_D**, the molar mass of the enzyme **E_x** to be tested is calculated as the molar mass of the polypeptide sequence of **E_x**.

4.5.3.1 Assay **A_L**:

To 0.9 ml of an aqueous reaction solution (phosphate buffer, pH 7.2, 10 mM MnCl₂), containing 50 mM of an *n*-butyl *P*-ester of carbamoyl glufosinate according to formula **L-(II)**, wherein R = *n*-butyl, are added 400 nmol of **E_x** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂). The resulting solution is incubated at 25 °C, and the pH is held at pH 7.2 by addition of 0.5 M NaOH. After 300 minutes, the reaction is stopped by addition of 2 M HCl to achieve a pH of 2.5, and the molar amount of the respective LGA *P*-(*n*-butyl) ester according to formula **L-(I)**, wherein R = *n*-butyl, is determined. **L-(I)**, i.e. wherein R = *n*-butyl, may be detected by the LC-MS method as described in the example section (item 5.4) for detection of LGA.

4.5.3.2 Assay **A_D**:

40

To 0.9 ml of an aqueous reaction solution (phosphate buffer, pH 7.2, 10 mM MnCl₂), containing 50 mM of an *n*-butyl *P*-ester of carbamoyl glufosinate according to formula **D-(II)**, wherein R = *n*-butyl, are added 400 nmol of **E_x** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂). The resulting solution is incubated at 25 °C, and the pH is held at pH 7.2 by addition of

5 0.5 M NaOH. After 300 minutes, the reaction is stopped by addition of 2 M HCl to achieve a pH of 2.5, and the molar amount of the respective D-glufosinate *P*-(*n*-butyl) ester **D-(I)**, wherein R = *n*-butyl, is determined. **D-(I)**, wherein R = *n*-butyl, may be detected by the LC-MS method described in the example section (item 5.4) for detection of LGA.

10 **4.5.4 Assay B for identifying carbamoylases, L-carbamoylases, D-carbamoylases, L- and D-enantiospecificity**

The carbamoylase **E₁** according to the invention is preferably an L-carbamoylase, more preferably L-enantiospecific.

15

Whether a given enzyme **E_x** may be considered a carbamoylase **E₁**, in particular an L-carbamoylase, may be determined in the context of the present invention by the following **Assay B**.

20 **4.5.4.1 Assay B:**

B-1. Firstly, **Assay A_L** as set forth under item 4.5.3.1 is conducted, and the obtained molar amount of the compound of the formula **L-(I)**, wherein R = *n*-butyl, is determined according to **Assay A_L**.

25 B-2. Secondly, **Assay A_D** as set forth under item 4.5.3.2 is conducted, and the obtained molar amount of the compound of the formula **D-(I)**, wherein R = *n*-butyl, is determined according to **Assay A_D**.

B-3. Then, step B-1 is repeated, except that instead of the addition of 400 nmol **E_x** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂), 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂) without **E_x** is added.

30

B-4. Then, step B-2 is repeated, except that instead of the addition of 400 nmol **E_x** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂), 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂) without **E_x** is added.

35

4.5.4.2 Carbamoylase activity

If the molar amount of the compound of the formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, is greater than the molar amount of the compound of the formula **L-(I)**, wherein

40

R = *n*-butyl, that is determined in step B-3, then E_x is deemed to have carbamoylase activity, and hence may be considered a carbamoylase E_1 in the context of the invention.

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4.5.4.3 L-Carbamoylase activity

4.5.4.3.1 L-Carbamoylases

- 10 (i) If the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, is greater than the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-3, and
- (ii) if the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2, is greater than or the same as the molar amount of the compound of formula **D-(I)**,
- 15 wherein R = *n*-butyl, that is determined in step B-4, and
- (iii) if, in addition, the molar amount of the compound of formula of **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, is greater than the molar amount of the compound of formula of **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2,
- 20 → then E_x is deemed to have L-carbamoylase activity, and hence may be considered an L-carbamoylase in the context of the invention. In this case, E_x is deemed to be “L-enantioselective” in the context of the invention.

25 For the sake of clarity it is pointed out that condition (iii) is automatically fulfilled in those cases in which the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2, is the same as the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-4.

- 30 (i) If the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, is greater than the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-3, and
- (ii) if the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2 is the same as the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-4,
- 35 → then E_x is deemed to have L-carbamoylase activity, and hence may be considered to be an L-carbamoylase in the context of the invention. In this case, E_x is not only “L-enantioselective”, but also “L-enantiospecific” in the context of the invention.

For L-carbamoylases E_x that are not L-enantiospecific, the L-enantioselectivity may then be quantified by dividing the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, by the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2, and then multiplying the obtained value by 100, giving the L-enantioselectivity of E_x in %.

4.5.4.3.2 D-carbamoylases

- (i) If the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, is greater than or the same as the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-3, and
- (ii) if the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2, is greater than the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-4, and
- (iii) if, in addition, the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2 is greater than the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1,
→ then E_x is deemed to have D-carbamoylase activity, and hence may be considered a D-carbamoylase. In this case, E_x is "D-enantioselective" in the context of the invention.

For the sake of clarity it is pointed out that condition (iii) is automatically fulfilled in those cases in which the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1 is the same as the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-3.

- (i) If the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, is the same as the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-3, and
- (ii) if the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2, is greater than the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-4,
→ then E_x is deemed to have D-carbamoylase activity, and hence may be considered to be a D-carbamoylase. In this case, E_x is not only "D-enantioselective", but also "D-enantiospecific" in the context of the invention.

For D-carbamoylases E_x , that are not D-enantiospecific, the D-enantioselectivity may then be quantified by dividing the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2 by the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1 and then multiplying the obtained value by 100, giving the D-enantioselectivity of E_x in %.

4.5.4.3.3 Non-enantioselective carbamoylases

- (i) If the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1, is greater than the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-3, and
- (ii) if the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2 is greater than the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-4, and
- (iii) if, in addition, the molar amount of the compound of formula **D-(I)**, wherein R = *n*-butyl, that is determined in step B-2 is the same as the molar amount of the compound of formula **L-(I)**, wherein R = *n*-butyl, that is determined in step B-1,
 → then **E_x** is deemed to be a non-enantioselective carbamoylase in the context of the invention. In this case, **E_x** is "non-enantioselective".

4.5.5 Assay C for identifying preferred carbamoylase variants of SEQ ID NOs: 1 - 9

4.5.5.1 L- and D-enantioselective carbamoylase variants

An enzyme, the polypeptide sequence of which is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9 has carbamoylase, and L-carbamoylase activity.

- In a preferred embodiment of the method according to the first aspect of the invention, the polypeptide sequence of the L-carbamoylase **E₁** is selected from the group consisting of SEQ ID NO: 1 and variants thereof, SEQ ID NO: 2 and variants thereof, SEQ ID NO: 3 and variants thereof, SEQ ID NO: 4, and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 6 and variants thereof, SEQ ID NO: 7, and variants thereof, SEQ ID NO: 8 and variants thereof, SEQ ID NO: 9 and variants thereof.

- In an even more preferred embodiment of the method according to the first aspect of the invention, the polypeptide sequence of the L-carbamoylase **E₁** is selected from the group consisting of SEQ ID NO: 1 and variants thereof, SEQ ID NO: 2 and variants thereof, SEQ ID NO: 3 and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 8 and variants thereof, more preferably SEQ ID NO: 1 and variants thereof.

The term "variant" is defined under item 4.3.

In the context of the invention, an enzyme **E₁**, the polypeptide sequence of which is a variant of one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9 has carbamoylase activity, preferably L-carbamoylase activity, more preferably is L-enantiospecific.

5

Whether a given enzyme **E_x**, the polypeptide sequence of which is a variant of one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, has carbamoylase activity, L carbamoylase activity and/or is L-enantiospecific may be determined as set forth under items 4.5.4.2 and 4.5.4.3.1, respectively.

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The carbamoylase activity of a given L-carbamoylase **E_{1V}**, the polypeptide sequence of which is a variant of one of one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, relative to the carbamoylase activity of an L-carbamoylase **E_{1S}**, wherein the polypeptide sequence of **E_{1S}** is selected from one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, may be quantified in the context of the present invention by the following **Assay C**:

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4.5.5.2 Assay C

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C-1 **Assay A_L** as set forth under item 4.5.3.1 is conducted, wherein **E_{1S}** is the enzyme to be tested. The obtained molar amount of the compound according to formula **L-(I)**, wherein R = *n*-butyl, is determined according to **Assay A_L**.

C-2 Step C-1 is repeated, except that, instead of **E_{1S}**, **E_{1V}** is used as the enzyme to be tested.

25

C-3. Then, the molar amount of the compound according to formula **L-(I)**, wherein R = *n*-butyl, that is determined in step C-2, is divided by the molar amount of the compound according to formula **L-(I)**, wherein R = *n*-butyl, that is determined in step C-1, and the obtained ratio is multiplied by 100, giving the carbamoylase activity of L-carbamoylase **E_{1V}**, relative to the carbamoylase activity of the L-carbamoylase **E_{1S}**, in %.

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4.5.6 Preferred carbamoylase variants of SEQ ID NOs: 1 - 9

In the context of the invention, L-carbamoylases **E₁**, the polypeptide sequence of which is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, are generally denoted as "**E_{1S}**".

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L-carbamoylases **E₁**, the polypeptide sequence of which is selected from variants of a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, are generally denoted as "**E_{1V}**".

40

In a preferred embodiment of the method according to the first aspect of the present invention, the reaction in step (c) is catalyzed by an L-carbamoylase **E₁**, and the polypeptide sequence of the L-carbamoylase **E₁** is selected from the group consisting of SEQ ID NO: 1 and variants thereof,
5 SEQ ID NO: 2 and variants thereof, SEQ ID NO: 3 and variants thereof, SEQ ID NO: 4 and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 6 and variants thereof, SEQ ID NO: 7 and variants thereof, SEQ ID NO: 8 and variants thereof, SEQ ID NO: 9 and variants thereof.

More preferably, the reaction in step (c) is catalyzed by an L-carbamoylase **E₁**, the reaction in step
10 (c) is catalyzed by an L-carbamoylase **E₁**, and the polypeptide sequence of the L-carbamoylase **E₁** is selected from the group consisting of SEQ ID NO: 1 and variants of SEQ ID NO: 1, SEQ ID NO: 2 and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 8 and variants thereof.

15 More preferably, the reaction in step (c) is catalyzed by an L-carbamoylase **E₁**, and the polypeptide sequence of the L-carbamoylase **E₁** is selected from the group consisting of SEQ ID NO: 1 and variants of SEQ ID NO: 1.

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4.5.6.1 Preferred variants of SEQ ID NO: 1

According to the invention, the polypeptide sequence of the L-carbamoylase **E₁** may also be a variant of SEQ ID NO: 1.

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The L-carbamoylase **E₁**, the polypeptide sequence of which is SEQ ID NO: 1, is denoted as "**E_{101S}**". L-carbamoylases **E₁**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 1, are generally denoted as "**E_{101V}**".

30 A variant of the polypeptide sequence of SEQ ID NO: 1 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably
35 ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 1.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 1 is not identical to SEQ ID NO: 1.

According to the invention, an L-carbamoylase **E_{101V}** has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E_{101V}** preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E_{101S}**, wherein the carbamoylase activity of **E_{101V}**, relative to the carbamoylase activity of **E_{101S}**, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E_{101V}** has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E_{101S}**, wherein the carbamoylase activity of **E_{101V}**, relative to the carbamoylase activity of **E_{101S}**, is determined by **Assay C** described under item 4.5.5.2.

4.5.6.2 Preferred variants of SEQ ID NO: 2

According to the invention, the polypeptide sequence of the L-carbamoylase **E₁** may also be a variant of SEQ ID NO: 2.

The L-carbamoylase **E₁**, the polypeptide sequence of which is SEQ ID NO: 2, is denoted as "**E_{102S}**". L-carbamoylases **E₁**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 2, are generally denoted as "**E_{102V}**".

A variant of the polypeptide sequence of SEQ ID NO: 2 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 2.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 2 is not identical to SEQ ID NO: 2.

According to the invention, an L-carbamoylase **E**_{102v} has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E**_{102v} preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E**_{102s}, wherein the carbamoylase activity of **E**_{102v}, relative to the carbamoylase activity of **E**_{102s}, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E**_{102v} has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E**_{102s}, wherein the carbamoylase activity of **E**_{102v}, relative to the carbamoylase activity of **E**_{102s}, is determined by **Assay C** described under item 4.5.5.2.

4.5.6.3 Preferred variants of SEQ ID NO: 3

According to the invention, the polypeptide sequence of the L-carbamoylase **E**₁ may also be a variant of SEQ ID NO: 3.

The L-carbamoylase **E**₁, the polypeptide sequence of which is SEQ ID NO: 3, is denoted as "**E**_{103s}". L-carbamoylases **E**₁, the polypeptide sequence of which is selected from variants of SEQ ID NO: 3, are generally denoted as "**E**_{103v}".

A variant of the polypeptide sequence of SEQ ID NO: 3 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 3.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 3 is not identical to SEQ ID NO: 3.

According to the invention, an L-carbamoylase **E**_{103V} has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E**_{103V} preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E**_{103S}, wherein the carbamoylase activity of **E**_{103V}, relative to the carbamoylase activity of **E**_{103S}, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E**_{103V} has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E**_{103S}, wherein the carbamoylase activity of **E**_{103V}, relative to the carbamoylase activity of **E**_{103S}, is determined by **Assay C** described under item 4.5.5.2.

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4.5.6.4 Preferred variants of SEQ ID NO: 4

According to the invention, the polypeptide sequence of the L-carbamoylase **E**₁ may also be a variant of SEQ ID NO: 4.

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The L-carbamoylase **E**₁, the polypeptide sequence of which is SEQ ID NO: 4, is denoted as "**E**_{104S}". L-carbamoylases **E**₁, the polypeptide sequence of which is selected from variants of SEQ ID NO: 4, are generally denoted as "**E**_{104V}".

30

A variant of the polypeptide sequence of SEQ ID NO: 4 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 4.

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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 4 is not identical to SEQ ID NO: 4.

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According to the invention, an L-carbamoylase **E**_{104V} has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E**_{104V} preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E**_{104S}, wherein the carbamoylase activity of **E**_{104V}, relative to the carbamoylase activity of **E**_{104S}, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E**_{104V} has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E**_{104S}, wherein the carbamoylase activity of **E**_{104V}, relative to the carbamoylase activity of **E**_{104S}, is determined by **Assay C** described under item 4.5.5.2.

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4.5.6.5 Preferred variants of SEQ ID NO: 5

According to the invention, the polypeptide sequence of the L-carbamoylase **E**₁ may also be a variant of SEQ ID NO: 5.

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The L-carbamoylase **E**₁, the polypeptide sequence of which is SEQ ID NO: 5, is denoted as "**E**_{105S}". L-carbamoylases **E**₁, the polypeptide sequence of which is selected from variants of SEQ ID NO: 5, are generally denoted as "**E**_{105V}".

30

A variant of the polypeptide sequence of SEQ ID NO: 5 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 5.

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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 5 is not identical to SEQ ID NO: 5.

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According to the invention, an L-carbamoylase **E**_{105V} has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E**_{105V} preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E**_{105S}, wherein the carbamoylase activity of **E**_{105V}, relative to the carbamoylase activity of **E**_{105S}, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E**_{105V} has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E**_{105S}, wherein the carbamoylase activity of **E**_{105V}, relative to the carbamoylase activity of **E**_{105S}, is determined by **Assay C** described under item 4.5.5.2.

4.5.6.6 Preferred variants of SEQ ID NO: 6

According to the invention, the polypeptide sequence of the L-carbamoylase **E**₁ may also be a variant of SEQ ID NO: 6.

The L-carbamoylase **E**₁, the polypeptide sequence of which is SEQ ID NO: 6, is denoted as "**E**_{106S}". L-carbamoylases **E**₁, the polypeptide sequence of which is selected from variants of SEQ ID NO: 6, are generally denoted as "**E**_{106V}".

A variant of the polypeptide sequence of SEQ ID NO: 6 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 6.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 6 is not identical to SEQ ID NO: 6.

According to the invention, an L-carbamoylase **E_{106V}** has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E_{106V}** preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E_{106S}**, wherein the carbamoylase activity of **E_{106V}**, relative to the carbamoylase activity of **E_{106S}**, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E_{106V}** has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E_{106S}**, wherein the carbamoylase activity of **E_{106V}**, relative to the carbamoylase activity of **E_{106S}**, is determined by **Assay C** described under item 4.5.5.2.

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4.5.6.7 Preferred variants of SEQ ID NO: 7

According to the invention, the polypeptide sequence of the L-carbamoylase **E₁** may also be a variant of SEQ ID NO: 7.

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The L-carbamoylase **E₁**, the polypeptide sequence of which is SEQ ID NO: 7, is denoted as "**E_{107S}**". L-carbamoylases **E₁**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 7, are generally denoted as "**E_{107V}**".

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A variant of the polypeptide sequence of SEQ ID NO: 7 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 7.

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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 7 is not identical to SEQ ID NO: 7.

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According to the invention, an L-carbamoylase **E**_{107V} has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E**_{107V} preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E**_{107S}, wherein the carbamoylase activity of **E**_{107V}, relative to the carbamoylase activity of **E**_{107S}, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E**_{107V} has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E**_{107S}, wherein the carbamoylase activity of **E**_{107V}, relative to the carbamoylase activity of **E**_{107S}, is determined by **Assay C** described under item 4.5.5.2.

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4.5.6.8 Preferred variants of SEQ ID NO: 8

According to the invention, the polypeptide sequence of the L-carbamoylase **E**₁ may also be a variant of SEQ ID NO: 8.

The L-carbamoylase **E**₁, the polypeptide sequence of which is SEQ ID NO: 8, is denoted as "**E**_{108S}". L-carbamoylases **E**₁, the polypeptide sequence of which is selected from variants of SEQ ID NO: 8, are generally denoted as "**E**_{108V}".

A variant of the polypeptide sequence of SEQ ID NO: 8 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 8.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 8 is not identical to SEQ ID NO: 8.

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According to the invention, an L-carbamoylase **E_{108V}** has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E_{108V}** preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E_{108S}**, wherein the carbamoylase activity of **E_{108V}**, relative to the carbamoylase activity of **E_{108S}**, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E_{108V}** has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E_{108S}**, wherein the carbamoylase activity of **E_{108V}**, relative to the carbamoylase activity of **E_{108S}**, is determined by **Assay C** described under item 4.5.5.2.

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4.5.6.9 Preferred variants of SEQ ID NO: 9

According to the invention, the polypeptide sequence of the L-carbamoylase **E₁** may also be a variant of SEQ ID NO: 9.

The L-carbamoylase **E₁**, the polypeptide sequence of which is SEQ ID NO: 9, is denoted as "**E_{109S}**". L-carbamoylases **E₁**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 9, are generally denoted as "**E_{109V}**".

A variant of the polypeptide sequence of SEQ ID NO: 9 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 9.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 9 is not identical to SEQ ID NO: 9.

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According to the invention, an L-carbamoylase **E_{109V}** has carbamoylase activity and L-carbamoylase activity, determined as described under items 4.5.4.2 and 4.5.4.3.1.

According to the invention, an L-carbamoylase **E_{109V}** preferably has carbamoylase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the carbamoylase activity of the L-carbamoylase **E_{109S}**, wherein the carbamoylase activity of **E_{109V}**, relative to the carbamoylase activity of **E_{109S}**, is determined by **Assay C** described under item 4.5.5.2.

It is even more preferable according to the invention, that an L-carbamoylase **E_{109V}** has carbamoylase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the carbamoylase activity of the L-carbamoylase **E_{109S}**, wherein the carbamoylase activity of **E_{109V}**, relative to the carbamoylase activity of **E_{109S}**, is determined by **Assay C** described under item 4.5.5.2.

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4.5.7 Preferred method conditions in step (c)

The reaction in step (c) of the method according to the first aspect of the present invention may be carried out under conditions known to the skilled person.

The reaction medium is preferably aqueous, more preferably an aqueous buffer.

Exemplary buffers commonly used in biotransformation reactions and advantageously used herein include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2-aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-morpholinepropanesulfonic acid ("MOPSO"), choline chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine, 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"),

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3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").

In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be added to the reaction.

5

The buffer preferably contains metal salts, more preferably metal salts such as halogenides of metals, preferably halogenides of monovalent or bivalent or trivalent metals, preferably chlorides of monovalent or bivalent metals, preferably CoCl_2 or MnCl_2 , preferably CoCl_2 .

- 10 The concentration of these metal salts in the reaction medium is preferably in the range from $1 \mu\text{M}$ to 1 M, more preferably 1 mM to 100 mM, even more preferably 1 to 10 mM.

Preferably, step (c) of the method according to the first aspect of the invention is carried out in a phosphate buffer.

15

The pH of the reaction medium in step (c) of the method according to the first aspect of the invention is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8, more preferably 7.2 to 7.5, most preferably 7.5.

- 20 Preferably, step (c) of the method according to the first aspect of the invention is carried out at a temperature in the range of from 20°C to 70°C , more preferably in the range of from 30°C to 55°C , most preferably 50°C .

- 25 Preferably, the total concentration of all carbamoylases E_1 in the reaction solution in step (c) is in the range of from $1 \mu\text{M}$ to 10 mM, preferably $10 \mu\text{M}$ to 1 mM, more preferably 0.1 mM to 0.5 mM, most preferably 0.4 mM.

- 30 In alternative preferred embodiments, the total concentration of all carbamoylases E_1 in the reaction solution in step (c) is in the range of from $1 \mu\text{g/l}$ to 10 g/l, preferably 0.1 mg/l to 5 g/l, more preferably 1 mg/l to 1 g/l, more preferably 5 mg/l to 500 mg/l.

- 35 Preferably, the initial concentration of all the compounds according to formula **L-(II)** in the reaction medium in step (c) is in the range of from $1 \mu\text{M}$ to 1 M, preferably of from $10 \mu\text{M}$ to 0.5 M, more preferably of from 0.1 mM to 0.1 M, more preferably of from 1 mM to 10 mM, most preferably 1.25 mM.

If compounds according to formula **D-(II)** are present in the reaction medium in step (c), the initial concentration of all the compounds according to formula **D-(II)** in the reaction medium is preferably from 1 % to 100 % the concentration of all the compounds according to formula **L-(II)**, more

preferably 10 % to 100 % the concentration, even more preferably 50 to 100 %, even more preferably 100 % the concentration of all the compounds according to formula **L-(II)**.

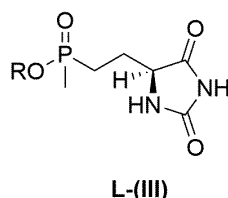
- “Initial concentration of all the compounds according to formula **L-(II)/ D-(II)**” refers to the concentration of the respective compound **L-(II)** or **D-(II)** respectively, in the reaction medium when the respective compounds are employed in step (c).

4.5.8 Step (b)

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4.5.8.1 Enzymatic catalysis of step (b)

- In a preferred embodiment of the method according to the first aspect of the invention, the compound according to formula **L-(II)** is obtained by a step (b) in which a compound according to formula **L-(III)** is reacted to give a compound according to formula **L-(II)**:



- Step (b) gives the starting material for step (c), and R in **L-(III)** has the same meaning as described for **L-(I)**.

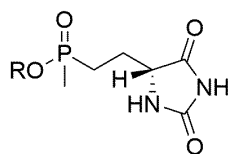
The reaction according step (b) is enzymatically catalyzed, namely it is catalyzed by a hydantoinase **E₂**.

- 25 **4.5.8.2 Enantioselective and enantiospecific catalysis of step (b)**

Step (b) of the method according to the first aspect of the present invention is preferably L-enantioselective, even more preferably L-enantiospecific.

- 30 In such a preferred embodiment, **L-(III)** is in particular employed in step (b) as a mixture **M_{III}** comprising, besides **L-(III)**, the enantiomer of **L-(III)**, which is **D-(III)**, wherein **D-(III)** has the following structure, wherein R in **L-(III)** and **D-(III)** is the same:

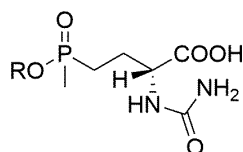
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D-(III)

For such mixtures **M_{III}**, a parallel reaction according to step (b)* may be observed. Namely, in the reaction according to step (b)*, **D-(III)**, i.e. the enantiomer of **L-(III)**, in mixture **M_{III}** is reacted to give a compound according to formula **D-(II)**:

5



D-(II)

In case that step (b) is "L-enantioselective", this means that in case a mixture **M_{III}** is employed in step (b), then there is either no reaction according to step (b)* or, in case there is a reaction according to step (b)*, then the rate of reaction according to step (b)* is lesser than the rate of the reaction according to step (b).

Step (b) is "L-enantiospecific", if the rate of reaction according to step (b)* is essentially zero, i.e. there is no reaction according to step (b)*.

15

In a preferred embodiment, the mixture **M_{III}** is a racemic mixture of enantiomer **L-(III)** and enantiomer **D-(III)**, meaning that the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** is essentially 1 : 1.

In other preferred embodiments, the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** in mixture **M_{III}** is in the range of from 3 : 2 to 1 : 99, more preferably in the range of from 1.01 : 1 to 1 : 99, more preferably in the range of from 1 : 1 to 1 : 99, more preferably in the range of from 1 : 1.01 to 1 : 99, more preferably in the range of from 1 : 1.01 to 1 : 9, more preferably in the range of from 1 : 1.01 to 1 : 8, more preferably in the range of from 1 : 1.01 to 1 : 3.

25

Alternatively, enantiomer **D-(III)** is comprised in an excess to **L-(III)** in mixture **M_{III}**, meaning that, while **L-(III)** is present in the mixture **M_{III}**, the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** in mixture **M_{III}** is < 1 : 1, preferably < 0.9 : 1, more preferably < 0.75 : 1, more preferably < 0.5 : 1, more preferably < 0.2 : 1, more preferably < 0.1 : 1, more preferably < 0.01 : 1.

30

Step (b) is in particular L-enantioselective, if it is preferably catalyzed by an L-hydantoinase **E₂**, which may be determined by the skilled person as set forth under 4.5.10.3.

In case step (b) is L-enantioselective, the reaction according to step (b) proceeds preferably at a reaction rate that is at least 2 times greater, preferably at least 10 times greater, more preferably at least 100 times greater, even more preferably at least 10³ times greater, even more preferably at least 10⁴ times greater, even more preferably at least 10⁵ times greater than the reaction rate at which step (b)* proceeds.

To quantify the factor at which the reaction rate of step (b) proceeds compared to the reaction rate of step (b)*, the following test may be carried out:

(1) An equimolar mixture [molar ratio of the two enantiomers **L-(III)** and **D-(III)** is 1 : 1] is subjected to the respective reactions conditions and the development of the two products **L-(II)** and **D-(II)** is monitored over time (e.g. by LC-MS as set forth under item 5.4).

(2) When $n_{LII10} = 10$ mol-% of the initially employed **L-(III)** has reacted to the product **L-(II)**, the molar amount of **D-(II)** that was formed by reaction from **D-(III)** [in mol-% relative to the initially employed **D-(III)**] is measured (= n_{DII10}).

(3) The ratio of $n_{LII10} / n_{DII10} = 10 / n_{DII10}$ gives the factor by which the reaction rate of step (b) is higher than the reaction rate of step (b)*.

→ If $n_{LII10} / n_{DII10} > 1$, the reaction rate of step (b) is greater than the reaction rate of step (b)*.

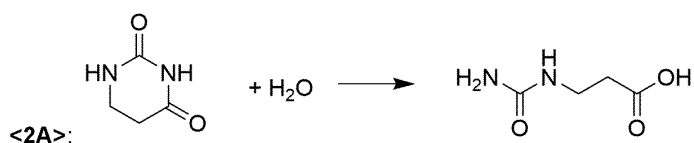
→ If $n_{LII10} / n_{DII10} < 1$, the reaction rate of step (b) is lesser than the reaction rate of step (b)*.

→ If $n_{LII10} / n_{DII10} = 1$, the reaction rates of steps (b) and (b)* are the same.

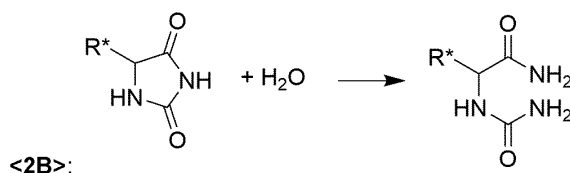
The reaction according to step (b) of the preferred embodiment of the first aspect of the invention is catalyzed by a hydantoinase ("dihydropyrimidinase") **E₂**.

Namely, it was surprisingly found that hydantoinases accept compounds of formula **L-(III)** as substrates and convert them to products according to formulae **L-(II)**, and hence catalyze the reaction according to step (b). This finding is of high scientific and economic value, as it further broadens the scope of synthetic routes based on new starting materials for the production of L-glufosinate *P*-alkyl esters and L-glufosinate. Even more surprisingly, it is suggested that L-glufosinate hydantoin, i.e. the compound according to formula **L-(III)**, in which R = H, does not undergo reaction by hydantoinases to give the respective LGA carbamoylate.

In nature, hydantoinases ("dihydropyrimidinases") generally catalyze the reaction of 5,6-dihydrouracil to produce ureidopropionate (see the following reaction **<2A>**):

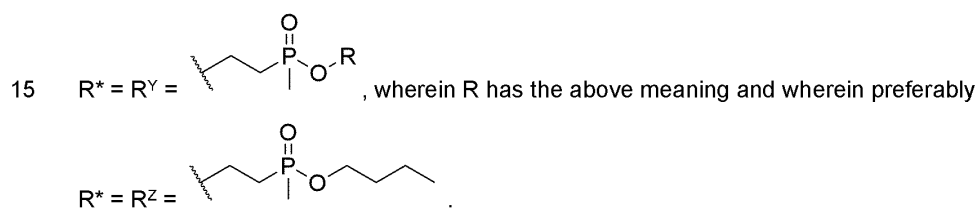


They also catalyze the ring opening of monosubstituted hydantoins to give the respective carbamoyl amino acid according to the following reaction <2B>, wherein R* may be an organic residue, e.g. a side chain of one of the naturally occurring amino acids.



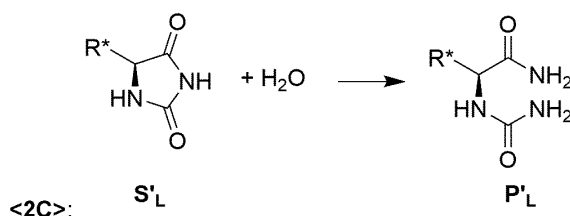
Chapter 1.3 (pages 7 to 11) of the dissertation “Untersuchungen zur Substratspezifität und Enantioselektivität mikrobieller Hydantoinasen/ Investigations of substrate specificity and enantioselectivity of microbial hydantoinases” by T. Waniek, University of Stuttgart, **2000** (available under: <https://elib.uni-stuttgart.de/bitstream/11682/1511/1/Diss.pdf>) gives an overview over hydantoinases.

It was now surprisingly found that hydantoinases also accept substrates in which

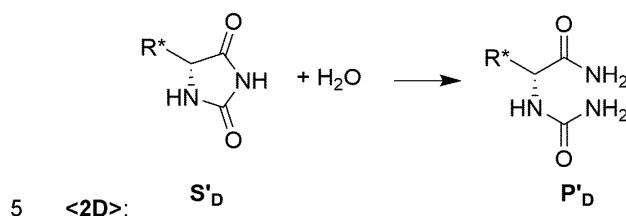


Surprisingly, they supposedly do not accept substrates in which $R^* = R^Y =$, wherein R = H.

20 In the context of the present invention, a “hydantoinase **E₂**” is a hydantoinase that catalyzes the following reaction <2C> of a carbamoyl substrate **S'_L** to the respective amino acid product **P'_L**, wherein $R^* = R^Y$ and preferably $R^* = R^Z$:



In particular, the hydantoinase E_2 is a "L-hydantoinase", i.e. it has a greater catalytic activity for reaction <2C> than for reaction <2D>, wherein the substrate S'_D in the reaction <2D> is the enantiomer of the substrate S_L in the reaction <2C>:



As an L-hydantoinase has a higher catalytic activity for reaction <2C> than for reaction <2D>, it is "L-enantioselective". An L-hydantoinase that has no catalytic activity for reaction <2D> and thus only has catalytic activity for reaction <2C> is "L-enantiospecific".

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A "D-hydantoinase" is defined as a hydantoinase which is "D-enantioselective", i.e. it has a higher catalytic activity for reaction <2D> than for reaction <2C>. A D-carbamoylase that does not catalyze reaction <2C> and thus only has catalytic activity for reaction <2D> is "D-enantiospecific".

15 A hydantoinase which has the same catalytic activity for reaction <2C> as for reaction <2D>, is referred to as "non enantioselective hydantoinase".

For determination whether a hydantoinase may be denoted as "L-hydantoinase", "D-hydantoinase" or "non-enantioselective hydantoinase" in the context of the present invention, the procedure
20 according to **Assay E** (item 4.5.10) may preferably be used.

The hydantoinase E_2 , in particular the L-hydantoinase E_2 , that may be used in step (b) of the preferred embodiment of the method according to the first aspect of the invention may originate from *Arthrobacter sp.*, in particular *Arthrobacter crystallopoietes*, *Arthrobacter aurescens*,
25 *Arthrobacter sp.* BT801; *Alcaligenes sp.*, in particular *Alcaligenes faecalis* subsp. *faecalis*; *Bacillus sp.*, in particular *Bacillus fordii*; *Microbacterium sp.*, in particular *Microbacterium liquefaciens* strain AJ3912; *Pseudomonas sp.*, in particular *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*.

A hydantoinase E_2 , in particular an L-hydantoinase E_2 suitable for the method according to the
30 present invention may be the enzyme HyuH, which originates from *Arthrobacter*. Another enzyme may be Dht.

Even more preferably, the hydantoinase E_2 , in particular the L-hydantoinase E_2 , that may preferably be used in step (b) according to the first aspect of the invention may originate from
35 *Arthrobacter sp.*, in particular *Arthrobacter crystallopoietes*, *Arthrobacter aurescens*, *Arthrobacter*

sp. BT801, *Arthrobacter aureescens* DSM 9771, most preferably from *Arthrobacter aureescens* DSM 9771.

- 5 A hydantoinase suitable for the method according to the present invention is described e.g. in WO 01/23582 A1 and by J.M. Clemente-Jiménez, S. Martínez-Rodríguez, F. Rodríguez-Vico, F.J.L. Heras-Vázquez, Recent Pat. Biotechnology **2008**, 2, 35 – 46; G. Latacz, E. Pekala, K. Kiec-Kononowicz, Biotechnologia **2006**, 2, 189 – 205.

- 10 Further suitable hydantoinases are described by K. Yokozeki, H. Yoshiteru, K. Kubota, Agric. Biol. Chem. **1987**, **51**, 737 - 746.

The hydantoinase **E₂** that may be used in preferred step (b) of the method according to the present invention may be a hydantoinase categorized in the EC class EC 3.5.2.2.

- 15 The following table 2 gives preferred examples for polypeptide sequences of hydantoinases **E₂** that may be preferably used in step (b) of the preferred embodiment of the method according to the first aspect of the invention. The genes encoding the respective hydantoinase **E₂** and the respective accession code are indicated as far as known.

Table 2

Hydantoinases (EC 3.5.2.2)			
Strain	Gene name	GenBank/UniProt accession	SEQ ID NO: of the polypeptide
<i>Arthrobacter aurescens</i> DSM 9771	<i>hyuH</i>		SEQ ID NO: 10
<i>Pseudomonas fluorescens</i>	not assigned	S5MPT0	SEQ ID NO: 11
<i>Pseudomonas aeruginosa</i>	<i>dht</i>	Q9I676	SEQ ID NO: 12
<i>Bacillus fordii</i> MH602	not assigned	ABL14245	SEQ ID NO: 13
<i>Arthrobacter</i> sp. BT801	<i>hyuH</i>	AAL55412	SEQ ID NO: 14
<i>Alcaligenes faecalis</i> subsp. <i>faecalis</i>	not assigned		SEQ ID NO: 15
<i>Microbacterium liquefaciens</i> strain AJ3912	not assigned		SEQ ID NO: 16

- 5 In a preferred embodiment of the method according to the first aspect of the present invention, the reaction according to step (b) is catalyzed by an hydantoinase **E₂**, wherein the polypeptide sequence of **E₂** is selected from the group consisting of SEQ ID NO: 10 and variants thereof, SEQ ID NO: 11 and variants thereof, SEQ ID NO: 12 and variants thereof, SEQ ID NO: 13 and variants thereof, SEQ ID NO: 14 and variants thereof, SEQ ID NO: 15 and variants thereof,
- 10 SEQ ID NO: 16 and variants thereof, preferably SEQ ID NO: 10 and variants thereof.

4.5.9 Assays **D_L** and **D_D** for determining hydantoinase activity

- The skilled person is aware of hydantoinases, in particular L-hydantoinases, that may be used in
- 15 step (b) of the preferred method according to the first aspect of the invention.

- In particular, **Assay D_L**, described in the following, may be used to determine hydantoinase and L-hydantoinase activity of a given enzyme **E_Y** and may advantageously be used according to the invention to determine hydantoinase and L-hydantoinase activity in variants of SEQ ID NO: 10,
- 20 variants of SEQ ID NO: 11, variants of SEQ ID NO: 12, variants of SEQ ID NO: 13, variants of SEQ ID NO: 14, variants of SEQ ID NO: 15, variants of SEQ ID NO: 16.

- For comparative reasons, **Assay D_D** may be used to determine D-hydantoinase activity of a given enzyme **E_Y**.
- 25

For the purpose of **Assay D_L** and **Assay D_D**, the molar mass of the enzyme **E_Y** to be tested is calculated as the molar mass of the polypeptide sequence of **E_Y**.

4.5.9.1 Assay D_L:

To 0.9 ml of an aqueous reaction solution (phosphate buffer, pH 7.2, 10 mM MnCl₂), containing
 5 50 mM of an *n*-butyl *P*-ester of hydantoin glufosinate of the formula **L-(III)**, wherein R = *n*-butyl, are
 added 400 nmol of **E_V** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂). The resulting
 solution is incubated at 25 °C, and the pH is held at pH 7.2 by addition of 1 M NaOH. After 300
 minutes, the reaction is stopped by addition of 2 M HCl to achieve a pH of 2.5, and the molar
 amount of the *n*-butyl *P*-ester of carbamoyl glufosinate according to formula **L-(II)**, wherein
 10 R = *n*-butyl, is determined. **L-(II)**, wherein R = *n*-butyl, may be detected by the LC-MS method as
 described in the example section (item 5.4) for detection of LGA.

4.5.9.2 Assay D_D:

15 To 0.9 ml of an aqueous reaction solution (phosphate buffer, pH 7.2, 10 mM MnCl₂), containing
 50 mM of an *n*-butyl *P*-ester of hydantoin glufosinate of the formula **D-(III)**, wherein R = *n*-butyl, are
 added 400 nmol of **E_V** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂). The resulting
 solution is incubated at 25 °C, and the pH is held at pH 7.2 by addition of 1 M NaOH. After 300
 20 minutes, the reaction is stopped by addition of 2 M HCl to achieve a pH of 2.5, and the molar
 amount of the *n*-butyl *P*-ester of carbamoyl glufosinate according to formula **D-(II)**, wherein R =
n-butyl, is determined. **D-(II)**, wherein R = *n*-butyl, may be detected by the LC-MS method as
 described in the example section (item 5.4) for detection of LGA.

25 4.5.10 Assay E for identifying hydantoinases, L-hydantoinases, D-hydantoinases, L- and D-enantiospecificity

The hydantoinase **E₂** according to the invention is preferably an L-hydantoinase, more preferably
 L-enantiospecific.

30 Whether a given enzyme **E_V** may be considered a hydantoinase **E₂**, in particular an
 L-hydantoinase, may be determined in the context of the present invention by the following
Assay E:

35 4.5.10.1 Assay E:

E-1. Firstly, **Assay D_L** as set forth under item 4.5.9.1 is conducted, and the obtained molar amount
 of the compound of the formula **L-(II)**, wherein R = *n*-butyl, is determined according to **Assay D_L**.

E-2 Secondly, **Assay D_D** as set forth under item 4.5.9.2 is conducted, and the obtained molar amount of the compound of the formula **D-(II)**, wherein R = *n*-butyl, is determined according to **Assay D_D**.

- 5 E-3. Then, step E-1 is repeated, except that instead of the addition of 400 nmol **E_Y** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂), 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂) without **E_Y** is added.

- 10 E-4 Then, step E-2 is repeated, except that instead of the addition of 400 nmol **E_Y** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂), 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂) without **E_Y** is added.

4.5.10.2 Hydantoinase activity:

- 15 If the molar amount of the compound of the formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1, is greater than the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-3, then **E_Y** is deemed to have hydantoinase activity, and hence may be considered a hydantoinase **E₂** in the context of the invention.

20 4.5.10.3 L-Hydantoinase activity

4.5.10.3.1 L-hydantoinases

- (i) If the molar amount of the compound of the formula **L-(II)**, wherein R = *n*-butyl, that is
 25 determined in step E-1, is greater than the molar amount of the compound of the formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-3, and
 (ii) if the molar amount of the compound of the formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2, is greater than or the same as the molar amount of the compound of the formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-4, and
 30 (iii) if, in addition, the molar amount of the compound of the formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1 is greater than the molar amount of the compound of the formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2,

- then **E_Y** is deemed to have L-hydantoinase activity, and hence may be considered an
 35 L-hydantoinase in the context of the invention. In this case, **E_Y** is deemed to be "L-enantioselective" in the context of the invention.

- For the sake of clarity it is pointed out that condition (iii) is automatically fulfilled in those cases in which the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined
 40 in step E-2 is the same as the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-4.

- (i) If the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1, is greater than the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-3, and
- 5 (ii) if the molar amount of the compound of the formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2, is the same as the molar amount of the compound of the formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-4, and

→ then **E_Y** is deemed to have L-hydantoinase activity, and hence may be considered a

10 L-hydantoinase in the context of the invention. In this case, **E_Y** is deemed to be not only "L-enantioselective", but also "L-enantiospecific" in the context of the invention.

For L-hydantoinases **E_Y** that are not L-enantiospecific, the L-enantioselectivity may then be quantified by dividing the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in E-1, by the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in E-2, and then multiplying the obtained value by 100, giving the

15 L-enantioselectivity of **E_Y** in %.

4.5.10.3.2 D-hydantoinases

- 20 (i) If the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1, is greater than or the same as the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-3, and
- (ii) if the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in
- 25 step E-2 is greater than the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-4, and
- (iii) if, in addition, the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2, is greater than the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1,

30 → then **E_Y** is deemed to have D-hydantoinase activity, and hence may be considered a D-hydantoinase. In this case, **E_Y** is "D-enantioselective" in the context of the invention.

For the sake of clarity it is pointed out that condition (iii) is automatically fulfilled in those cases in

35 which the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1 is the same as the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-3.

- (i) If the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1, is the same as the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-3, and
- (ii) if the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2, is the greater than the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-4,

→ then **E_Y** is deemed to have D-hydantoinase activity, and hence may be considered a D-hydantoinase in the context of the invention. In this case, **E_Y** is deemed to be not only “D-enantioselective”, but also “D-enantiospecific” in the context of the invention.

For D-hydantoinases **E_Y** that are not D-enantiospecific, the D-enantioselectivity may then be quantified by dividing the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2, by the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1 and then multiplying the obtained value by 100, giving the D-enantioselectivity of **E_Y** in %.

4.5.10.3.3 Non-enantioselective hydantoinases

- (i) If the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1, is greater than the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-3, and
- (ii) if the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2, is greater than the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-4, and
- (iii) if, in addition, the molar amount of the compound of formula **D-(II)**, wherein R = *n*-butyl, that is determined in step E-2 is the same as the molar amount of the compound of formula **L-(II)**, wherein R = *n*-butyl, that is determined in step E-1,
- then **E_Y** is deemed to be a non-enantioselective hydantoinase in the context of the invention. In this case, **E_Y** is “non-enantioselective”.

4.5.11 Assay F for identifying preferred hydantoinases variants of SEQ ID NOs: 10 - 16

4.5.11.1 L- and D-enantioselective hydantoinase variants

An enzyme, the polypeptide sequence of which is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16 has hydantoinase, in particular L-hydantoinase activity.

In a preferred embodiment of the method according to the first aspect of the invention, the polypeptide sequence of the hydantoinase, in particular the L-hydantoinase **E₂** is selected from the group consisting SEQ ID NO: 10 and variants thereof, SEQ ID NO: 11 and variants thereof, SEQ ID NO: 12 and variants thereof, SEQ ID NO: 13 and variants thereof, SEQ ID NO: 14, and variants thereof, SEQ ID NO: 15 and variants thereof, SEQ ID NO: 16 and variants thereof, more preferably SEQ ID NO: 10 and variants thereof.

The term variant is defined under item 4.3.

10 In the context of the invention, an enzyme, the polypeptide sequence of which is a variant of one of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, has hydantoinase activity, preferably L-hydantoinase activity, more preferably is L-enantiospecific.

15 Whether a given enzyme **E_Y**, the polypeptide sequence of which is a variant of one of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, has hydantoinase activity, L-hydantoinase activity and is L-enantiospecific may be determined as set forth under items 4.5.10.2 and 4.5.10.3.1, respectively.

20 The hydantoinase activity of a given hydantoinase **E_{2v}**, the polypeptide sequence of which is a variant of one of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, relative to the hydantoinase activity of an hydantoinase **E_{2s}**, wherein the polypeptide sequence of **E_{2s}** is selected from SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, may be determined in
25 the context of the present invention by the following **Assay F**:

4.5.11.2 **Assay F**

F-1 **Assay D_L** as set forth under item 4.5.9.1 is conducted, wherein **E_{2s}** is the enzyme to be tested.

30 The obtained molar amount of the compound according to formula **L-(II)**, wherein R = *n*-butyl, is determined according to **Assay D_L**.

F-2 Step F-1 is repeated, except that, instead of **E_{2s}**, **E_{2v}** is used as the enzyme to be tested.

F-3. Then, the molar amount of the compound according to formula **L-(II)**, wherein R = *n*-butyl, that is determined in step F-2, is divided by the molar amount of the compound according to formula
35 **L-(II)**, wherein R = *n*-butyl, that is determined in step F-1, and the obtained ratio is multiplied by 100, giving the hydantoinase activity of hydantoinase **E_{2v}** relative to the hydantoinase activity of the hydantoinase **E_{2s}**, in %.

4.5.12 Preferred hydantoinase variants of SEQ ID NOs: 10 - 16

In the context of the invention, hydantoinases **E₂**, the polypeptide sequence of which is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11; SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, are generally denoted as "**E_{2s}**".

- Hydantoinases **E₂**, the polypeptide sequence of which is selected from variants of a sequence
 5 selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11; SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, are generally denoted as "**E_{2v}**".

- In a preferred embodiment of the method according to the first aspect of the present invention, the reaction in step (b) is catalyzed by a hydantoinase **E₂**, preferably an L-hydantoinase **E₂**, and the
 10 polypeptide sequence of the hydantoinase **E₂**, preferably the L-hydantoinase **E₂**, is selected from the group consisting of SEQ ID NO: 10 and variants thereof, SEQ ID NO: 11 and variants thereof, SEQ ID NO: 12 and variants thereof, SEQ ID NO: 13 and variants thereof, SEQ ID NO: 14 and variants thereof, SEQ ID NO: 15 and variants thereof, SEQ ID NO: 16 and variants thereof.
 More preferably, the reaction in step (b) is catalyzed by a hydantoinase **E₂**, preferably an
 15 L-hydantoinase **E₂**, and the polypeptide sequence of the hydantoinase **E₂**, preferably the L-hydantoinase **E₂**, is selected from the group consisting of SEQ ID NO: 10 and variants of SEQ ID NO: 10.

4.5.12.1 Preferred variants of SEQ ID NO: 10 20

According to the invention, the polypeptide sequence of the hydantoinase **E₂**, preferably the polypeptide sequence of the L-hydantoinase **E₂**, may also be a variant of SEQ ID NO: 10.

- The hydantoinase **E₂**, the polypeptide sequence of which is SEQ ID NO: 10, is denoted as "**E_{210s}**".
 25 The hydantoinase **E₂**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 10, are generally denoted as "**E_{210v}**".

- A variant of the polypeptide sequence of SEQ ID NO: 10 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably
 30 ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 10.

- 35 The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 10 is not identical to SEQ ID NO: 10.

According to the invention, a hydantoinase **E_{210v}** has hydantoinase activity and preferably L-hydantoinase activity, determined as described under items 4.5.10.2 and 4.5.10.3.1.

According to the invention, a hydantoinase **E_{210V}** preferably has hydantoinase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{210S}**, wherein the hydantoinase activity of **E_{210V}**, relative to the hydantoinase activity of **E_{210S}** is determined by **Assay F** described under item 4.5.11.2.

It is even more preferably according to the invention, that a hydantoinase **E_{210V}** has hydantoinase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoinase activity of the hydantoinase **E_{210S}**, wherein the hydantoinase activity of **E_{210V}**, relative to the hydantoinase activity **E_{210S}** is determined by **Assay F** described under item 4.5.11.2.

4.5.12.2 Preferred variants of SEQ ID NO: 11

According to the invention, the polypeptide sequence of the hydantoinase **E₂**, preferably the polypeptide sequence of the L-hydantoinase **E₂**, may also be a variant of SEQ ID NO: 11.

The hydantoinase **E₂**, the polypeptide sequence of which is SEQ ID NO: 11, is denoted as "**E_{211S}**". The hydantoinase **E₂**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 11, are generally denoted as "**E_{211V}**".

A variant of the polypeptide sequence of SEQ ID NO: 11 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 11.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 11 is not identical to SEQ ID NO: 11.

According to the invention, a hydantoinase **E_{211V}** has hydantoinase activity and preferably L-hydantoinase activity, determined as described under items 4.5.10.2 and 4.5.10.3.1.

According to the invention, a hydantoinase **E_{211V}** preferably has hydantoinase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{211S}**, wherein the hydantoinase activity of **E_{211V}**, relative to the hydantoinase activity of **E_{211S}** is determined by **Assay F** described under item 4.5.11.2.

It is even more preferably according to the invention, that a hydantoinase **E_{211V}** has hydantoinase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoinase activity of the hydantoinase **E_{211S}**, wherein the hydantoinase activity of **E_{211V}**, relative to the hydantoinase activity **E_{211S}** is determined by **Assay F** described under item 4.5.11.2.

4.5.12.3 Preferred variants of SEQ ID NO: 12

According to the invention, the polypeptide sequence of the hydantoinase **E₂**, preferably the polypeptide sequence of the L-hydantoinase **E₂**, may also be a variant of SEQ ID NO: 12.

The hydantoinase **E₂**, the polypeptide sequence of which is SEQ ID NO: 12, is denoted as "**E_{212S}**". The hydantoinase **E₂**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 12, are generally denoted as "**E_{212V}**".

A variant of the polypeptide sequence of SEQ ID NO: 12 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 12.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 12 is not identical to SEQ ID NO: 12.

According to the invention, a hydantoinase **E_{212V}** has hydantoinase activity and preferably L-hydantoinase activity, determined as described under items 4.5.10.2 and 4.5.10.3.1.

According to the invention, a hydantoinase **E_{212V}** preferably has hydantoinase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{212S}**, wherein the hydantoinase activity of **E_{212V}**, relative to the hydantoinase activity of **E_{212S}** is determined by **Assay F** described under item 4.5.11.2.

It is even more preferably according to the invention, that a hydantoinase **E_{212V}** has hydantoinase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoinase activity of the hydantoinase **E_{212S}**, wherein the hydantoinase activity of **E_{212V}**, relative to the hydantoinase activity **E_{212S}** is determined by **Assay F** described under item 4.5.11.2.

4.5.12.4 Preferred variants of SEQ ID NO: 13

According to the invention, the polypeptide sequence of the hydantoinase **E₂**, preferably the polypeptide sequence of the L-hydantoinase **E₂**, may also be a variant of SEQ ID NO: 13.

The hydantoinase **E₂**, the polypeptide sequence of which is SEQ ID NO: 13, is denoted as "**E_{213S}**". The hydantoinase **E₂**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 13, are generally denoted as "**E_{213V}**".

A variant of the polypeptide sequence of SEQ ID NO: 13 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 13.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 13 is not identical to SEQ ID NO: 13.

According to the invention, a hydantoinase **E_{213V}** has hydantoinase activity and preferably L-hydantoinase activity, determined as described under items 4.5.10.2 and 4.5.10.3.1.

According to the invention, a hydantoinase **E_{213V}** preferably has hydantoinase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{213S}**, wherein the hydantoinase activity of **E_{213V}**, relative to the hydantoinase activity of **E_{213S}** is determined by **Assay F** described under item 4.5.11.2.

It is even more preferably according to the invention, that a hydantoinase **E_{213V}** has hydantoinase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoinase activity of the hydantoinase **E_{213S}**, wherein the hydantoinase activity of **E_{213V}**, relative to the hydantoinase activity **E_{213S}** is determined by **Assay F** described under item 4.5.11.2.

4.5.12.5 Preferred variants of SEQ ID NO: 14

According to the invention, the polypeptide sequence of the hydantoinase **E₂**, preferably the polypeptide sequence of the L-hydantoinase **E₂**, may also be a variant of SEQ ID NO: 14.

The hydantoinase **E₂**, the polypeptide sequence of which is SEQ ID NO: 14, is denoted as "**E_{214S}**". The hydantoinase **E₂**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 14, are generally denoted as "**E_{214V}**".

A variant of the polypeptide sequence of SEQ ID NO: 14 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 14.

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 14 is not identical to SEQ ID NO: 14.

According to the invention, a hydantoinase **E_{214V}** has hydantoinase activity and preferably L-hydantoinase activity, determined as described under items 4.5.10.2 and 4.5.10.3.1.

According to the invention, a hydantoinase **E_{214V}** preferably has hydantoinase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{213S}**, wherein the hydantoinase activity of **E_{214V}**, relative to the hydantoinase activity of **E_{213S}** is determined by **Assay F** described under item 4.5.11.2.

more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{214S}**, wherein the hydantoinase activity of **E_{214V}**, relative to the hydantoinase activity of **E_{214S}** is determined by **Assay F** described under item 4.5.11.2.

It is even more preferably according to the invention, that a hydantoinase **E_{214V}** has hydantoinase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoinase activity of the hydantoinase **E_{214S}**, wherein the hydantoinase activity of **E_{214V}**, relative to the hydantoinase activity **E_{214S}** is determined by **Assay F** described under item 4.5.11.2.

15

4.5.12.6 Preferred variants of SEQ ID NO: 15

According to the invention, the polypeptide sequence of the hydantoinase **E₂**, preferably the polypeptide sequence of the L-hydantoinase **E₂**, may also be a variant of SEQ ID NO: 15.

20

The hydantoinase **E₂**, the polypeptide sequence of which is SEQ ID NO: 15, is denoted as "**E_{215S}**". The hydantoinase **E₂**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 15, are generally denoted as "**E_{215V}**".

A variant of the polypeptide sequence of SEQ ID NO: 15 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 15.

30

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 15 is not identical to SEQ ID NO: 15.

According to the invention, a hydantoinase **E_{215V}** has hydantoinase activity and preferably L-hydantoinase activity, determined as described under items 4.5.10.2 and 4.5.10.3.1.

According to the invention, a hydantoinase **E_{215V}** preferably has hydantoinase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{214S}**, wherein the hydantoinase activity of **E_{215V}**, relative to the hydantoinase activity of **E_{214S}** is determined by **Assay F** described under item 4.5.11.2.

40

more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{215S}**, wherein the hydantoinase activity of **E_{215V}**, relative to the hydantoinase activity of **E_{215S}** is determined by **Assay F** described under item 4.5.11.2.

5

It is even more preferably according to the invention, that a hydantoinase **E_{215V}** has hydantoinase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %
 10 % , more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoinase activity of the hydantoinase **E_{215S}**, wherein the hydantoinase activity of **E_{215V}**, relative to the hydantoinase activity **E_{215S}** is determined by **Assay F** described under item 4.5.11.2.

15 4.5.12.7 Preferred variants of SEQ ID NO: 16

According to the invention, the polypeptide sequence of the hydantoinase **E₂**, preferably the polypeptide sequence of the L-hydantoinase **E₂**, may also be a variant of SEQ ID NO: 16.

20

The hydantoinase **E₂**, the polypeptide sequence of which is SEQ ID NO: 16, is denoted as "**E_{216S}**". The hydantoinase **E₂**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 16, are generally denoted as "**E_{216V}**".

25 A variant of the polypeptide sequence of SEQ ID NO: 16 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 16.
 30

The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 16 is not identical to SEQ ID NO: 16.

35 According to the invention, a hydantoinase **E_{216V}** has hydantoinase activity and preferably L-hydantoinase activity, determined as described under items 4.5.10.2 and 4.5.10.3.1.

According to the invention, a hydantoinase **E_{216V}** preferably has hydantoinase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{215S}**, wherein the hydantoinase activity of **E_{216V}**, relative to the hydantoinase activity of **E_{215S}** is determined by **Assay F** described under item 4.5.11.2.
 40

more preferably of at least 99 %, more preferably of at least 100 % the hydantoinase activity of the hydantoinase **E_{216S}**, wherein the hydantoinase activity of **E_{216V}**, relative to the hydantoinase activity of **E_{216S}** is determined by **Assay F** described under item 4.5.11.2.

- 5 It is even more preferably according to the invention, that a hydantoinase **E_{216V}** has hydantoinase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more
10 preferably 100 % the hydantoinase activity of the hydantoinase **E_{216S}**, wherein the hydantoinase activity of **E_{216V}**, relative to the hydantoinase activity **E_{216S}** is determined by **Assay F** described under item 4.5.11.2.

4.5.13 Preferred method conditions in step (b)

- 15 The reaction in step (b) of the method according to the first aspect of the present invention may be carried out under conditions known to the skilled person.

The reaction medium is preferably aqueous, more preferably an aqueous buffer.

- 20 Exemplary buffers commonly used in biotransformation reactions and advantageously used herein include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2- aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-
25 morpholinepropanesulfonic acid ("MOPSO"), cholamine chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)- 2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2- yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-
1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine,
30 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2- Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"),
3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycineamide, bicine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").
35 In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be added to the reaction.

- The buffer preferably contains metal salts, more preferably metal salts such as halogenides of metals, preferably halogenides of monovalent or bivalent or trivalent metals, preferably chlorides of
40 monovalent or bivalent metals, preferably CoCl₂ or MnCl₂, preferably CoCl₂.

The concentration of these metal salts in the reaction medium is preferably in the range from 1 μ M to 1 M, more preferably 1 mM to 100 mM, even more preferably 1 to 10 mM.

- 5 Preferably, step (b) of the method according to the first aspect of the invention is carried out in a phosphate buffer.

- The pH of the reaction medium in step (b) of the method according to the first aspect of the invention is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8,
10 more preferably 7.2 to 7.5, most preferably 7.5.

- Preferably, step (b) of the method according to the first aspect of the invention is carried out at a temperature in the range of from 20 °C to 70 °C, more preferably in the range of from 30 °C to 55 °C, most preferably 50 °C.

- 15 Preferably, the total concentration of all hydantoinases **E₂** in the reaction solution in step (b) is in the range of from 1 μ M to 10 mM, preferably 10 μ M to 1 mM, more preferably 0.1 mM to 0.5 mM, most preferably 0.4 mM.

- 20 In alternative preferred embodiments, the total concentration of all hydantoinases **E₂** in the reaction solution in step (b) is in the range of from 1 μ g/l to 10 g/l, preferably 0.1 mg/l to 5 g/l, more preferably 1 mg/l to 1 g/l, more preferably 5 mg/l to 500 mg/l.

- Preferably, the initial concentration of all the compounds according to formula **L-(III)** in the reaction medium in step (b) is in the range of from 1 μ M to 1 M, preferably of from 10 μ M to 0.5 M, more preferably of from 0.1 mM to 0.1 M, more preferably of from 1 mM to 10 mM, most preferably 1.25 mM.

- 30 If compounds according to formula **D-(III)** are present in the reaction medium in step (b), the initial concentration of all the compounds according to formula **D-(III)** in the reaction medium is preferably from 1 % to 100 % the concentration of all the compounds according to formula **L-(III)**, more preferably 10 % to 100 % the concentration, even more preferably 50 to 100 %, even more preferably 100 % the concentration of all the compounds according to formula **L-(III)**.

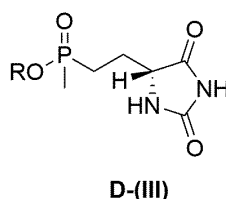
- 35 Preferably, step (b) is carried out in the same reaction medium in which step (c) is carried out. In this case, preferably, the initial concentration of all the compounds according to formula **L-(III)** in the reaction medium in step (b) is in the range of from 1 μ M to 1 M, preferably of from 10 μ M to 0.5 M, more preferably of from 0.1 mM to 0.1 M, more preferably of from 1 mM to 10 mM, most preferably 1.25 mM.

"Initial concentration of all the compounds according to formula **L-(III)**/ **D-(III)**" refers to the concentration of the respective compound **L-(III)** or **D-(III)**, respectively, in the reaction medium when the respective compounds are employed in step (b).

5 4.5.14 Step (a)

In a preferred embodiment of the method according to the first aspect of the invention, the compound according to formula **L-(III)** is obtained by a step (a) in which a compound according to formula **D-(III)** is reacted to give a compound according to formula **L-(III)**:

10



Step (a) gives the starting material for step (b), and R in **D-(III)** has the same meaning as described for **L-(III)** [or **L-(I)**].

15

The reaction according to step (a) may be carried out enzymatically or non-enzymatically, preferably enzymatically. More preferably, the reaction according to step (a) is catalyzed by a hydantoin racemase **E₃**.

20 In preferred step (a), the compound according to formula **D-(III)** is employed in step (a) as a mixture **M_{III}** of **D-(III)** with its enantiomer **L-(III)**.

In a preferred embodiment, the mixture **M_{III}** is a racemic mixture of enantiomer **L-(III)** and enantiomer **D-(III)**, meaning that the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** is essentially 1 : 1.

25

In other preferred embodiments, the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** in mixture **M_{III}** is in the range of from 3 : 2 to 1 : 99, more preferably in the range of from 1.01 : 1 to 1 : 99, more preferably in the range of from 1 : 1 to 1 : 99, more preferably in the range of from 1 : 1.01 to 1 : 99, more preferably in the range of from 1 : 1.01 to 1 : 9, more preferably in the range of from 1 : 1.01 to 1 : 8, more preferably in the range of from 1 : 1.01 to 1 : 3.

30

Alternatively, enantiomer **D-(III)** is comprised in an excess to **L-(III)** in mixture **M_{III}**, meaning that, while **L-(III)** is present in the mixture **M_{III}**, the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** in mixture **M_{III}** is < 1 : 1, preferably < 0.9 : 1, more preferably < 0.75 : 1, more preferably < 0.5 : 1, more preferably < 0.2 : 1, more preferably < 0.1 : 1, more preferably < 0.01 : 1.

35

4.5.14.1 Step (a) without enzymatic catalysis

Step (a) may be carried out non-enzymatically, i.e. without the use of an enzyme. The reaction of compounds according to step **D-(III)** to compounds according to **L-(III)** proceeds in alkaline solution, as known to the skilled person and as described by Slomka *et al.*, M. Bovarnick & H.T. Clarke, Journal of the American Chemical Society **1938**, *60*, 2426 – 2430, by R. A. Lazarus, J. Org. Chem. **1990**, *55*, 4755 – 4757, and by A. S. Bommarius, M. Kottenhahn, H. Klenk, K. Drauz: "A direct route from hydantoins to D-amino acids employing a resting cell biocatalyst with D-hydantoinase and D-carbamoylase activity" on page 164 and 167 in "Microbial Reagents in Organic Synthesis" Series C: Mathematical and Physical Sciences - Vol. 381, S. Servi (Ed.), 1992, Springer Science+Business Media, B.V., Dordrecht.

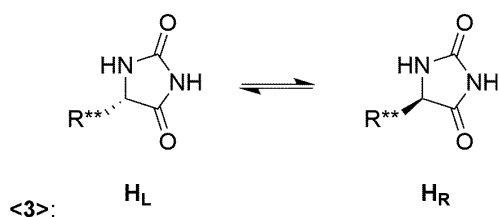
Therefore, if step (a) is carried out non-enzymatically, the conditions that are preferably applied in the reaction medium in which non-enzymatic step (a) is carried out are preferably those that are described for the preferred conditions for step (b) (item 4.5.13), except that the pH is ≥ 8 , preferably in the range of 8 to 12, more preferably 8 to 11, more preferably 8 to 10, even more preferably 8 to 9. As in these embodiments, the preferred conditions in step (a) and (b) with respect to the pH ranges are different, it is preferable that the reaction media in steps (a) and (b) are different.

4.5.14.2 Enzymatic conversion of **D-(III)** into **L-(III)**

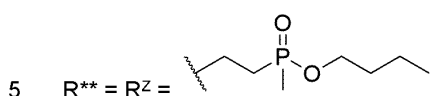
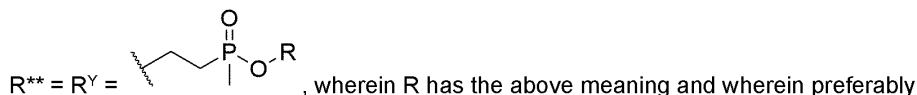
Step (a) is preferably carried out enzymatically, i.e. the reaction according to step (a) is preferably catalyzed by a hydantoin racemase **E₃**. Namely, it was surprisingly found that hydantoin racemases accept compounds of formula **D-(III)** as substrates and convert them to products according to formulae **L-(III)**, and hence catalyze the reaction according to step (a), while there is no catalysis of the corresponding compound according to formula **D-(III)**, in which R = H.

This finding is of high scientific and economic value, as it further expands the scope of new starting materials for the production of L-glufosinate alkyl esters and L-glufosinate via new synthetic routes. Moreover, this finding also opens new possibilities of enantioselective production of LGA from racemic mixtures of **L-(III)** and **D-(III)**, as embodied in the method according to the second aspect of the invention.

In nature, hydantoin racemases catalyze the conversion of one of the two hydantoin enantiomers **H_L** and **H_R** into the other (see the following reaction **<3>**):



It was now surprisingly found that hydantoin racemases also accept substrates in which



Surprisingly, they do not accept substrates in which $\text{R}^* = \text{R}^{\text{Y}} =$

, wherein R = H.

- Suitable hydantoin racemases are described e.g. in WO 01/23582 A1 and by U. Engel, J. Rudat,
 10 C. Syltatk in "The hydantoinase process: recent developments for the production of non-canonical
 amino acids" in the book "Industrial biocatalysis" by P. Grunwald (Ed.), Pan Stanford Series on
 Biocatalysis, **2015**, pages 817 - 862, and by F. J. Las Heras-Vazquez, J. M. Clemente-Jimenez, S.
 Martinez-Rodriguez, F. Rodriguez-Vico in "Hydantoin racemase: the key enzyme for the production
 of optically pure α -amino acids" in chapter 12 of the book "Modern Biocatalysis: Stereoselective
 15 and environmentally friendly reactions" by W. Fessner, T. Anthonsen (Eds), Weinheim: WILEY-
 VCH Verlag GmbH & Co, **2009**, pages 173 - 193.

- A hydantoin racemase E_3 that may be used in optional step (a) of the method according to the first
 aspect of the invention may originate from *Agrobacterium* sp., in particular *Agrobacterium* strain
 20 IP_I-671; *Arthrobacter* sp., in particular *Arthrobacter aurescens*, more in particular *Arthrobacter*
aurescens DSM 3745 or *Arthrobacter* sp. BT801; *Flavobacterium* sp., in particular
Flavobacterium sp. AJ 11199; *Microbacterium* sp., in particular *Microbacterium liquefaciens*,
 preferably *Microbacterium liquefaciens* AJ 3912; *Pasteurella* sp., in particular *Pasteurella* sp.
 AJ11221; *Pseudomonas* sp., in particular *Pseudomonas* sp. NS671; *Pyrococcus* sp., in particular
 25 *Pyrococcus horikoshii* OT3; *Rhodococcus* sp., in particular *Rhodococcus* R04; *Sinorhizobium* sp.,
 in particular *Sinorhizobium meliloti*, more in particular *Sinorhizobium meliloti* CECT 4114, , most
 preferably from *Arthrobacter aurescens* DSM 3745.

A hydantoin racemase **E₃** suitable for the method according to the present invention may be the enzyme HyuR, which originates from *Arthrobacter aureescens* DSM 3745. Another enzyme may be selected from HyuE, Hyu2, HRase, HyuA, PH1054.

- 5 The hydantoin racemase **E₃** that may preferably be used in preferred step (a) of the method according to the first aspect of the invention may be categorized in the EC class 5.1.99.5.

10 The following table 3 gives preferred examples for polypeptide sequences of hydantoin racemase **E₃** that may be preferably used in step (a) of the method according to the first aspect of the invention. The genes encoding the respective hydantoin racemase **E₃** and the respective accession code are indicated as far as known.

Table 3

Hydantoin Racemases (EC 5.1.99.5)			
Strain	Gene name	GenBank/UniProt accession	SEQ ID NO: of the polypeptide
<i>Arthrobacter aureescens</i> DSM 3745	<i>hyuR</i>		SEQ ID NO: 17
<i>Pseudomonas</i> sp. NS671	<i>hyuE</i>	Q00924	SEQ ID NO: 18
<i>Rhodococcus</i> R04	<i>hyu2</i>		SEQ ID NO: 19
<i>Microbacterium liquefaciens</i> AJ 3912	„HRase“		SEQ ID NO: 20
<i>Sinorhizobium meliloti</i> CECT 4114	<i>hyuA</i>	Q6TMG4	SEQ ID NO: 21
<i>Flavobacterium</i> sp. AJ11199	„HRase“		SEQ ID NO: 22
<i>Agrobacterium</i> IP_I-671	<i>hyuA</i>		SEQ ID NO: 23
<i>Pasteurella</i> sp. AJ 11221	<i>not assigned</i>		SEQ ID NO: 24
<i>Pyrococcus horikoshii</i> OT3	<i>PH1054</i>	O58781	SEQ ID NO: 25
<i>Arthrobacter</i> sp. BT801	<i>hyuA</i>	AAL55411	SEQ ID NO: 26

15

In a preferred embodiment of the preferred method according to the second aspect of the present invention, the reaction according to step (a) is catalyzed by a hydantoin racemase **E₃**, wherein the polypeptide sequence of **E₃** is selected from the group consisting of SEQ ID NO: 17 and variants

thereof, SEQ ID NO: 18 and variants thereof, SEQ ID NO: 19 and variants thereof, SEQ ID NO: 20 and variants thereof, SEQ ID NO: 21 and variants thereof, SEQ ID NO: 22 and variants thereof, SEQ ID NO: 23 and variants thereof, SEQ ID NO: 24 and variants thereof, SEQ ID NO: 25 and variants thereof, SEQ ID NO: 26 and variants thereof.

5

4.5.14.3 **Assay G** for determining hydantoin racemase activity

The skilled person is aware of hydantoin racemases, that may be used in preferred step (a) of the method according to the first aspect of the invention.

10

In particular, **Assay G**, described in the following, may be used to determine hydantoin racemase activity of a given enzyme **E_Z** and may advantageously be used according to the invention to determine carbamoylase and L-carbamoylase activity in variants of SEQ ID NO: 17, variants of variants of SEQ ID NO: 18, variants of SEQ ID NO: 19, variants of SEQ ID NO: 20, variants of SEQ ID NO: 21, variants of SEQ ID NO: 22, variants of variants of SEQ ID NO: 23, variants of SEQ ID NO: 24, variants of SEQ ID NO: 25, variants of SEQ ID NO: 26.

15

For the purpose of **Assay G**, the molar mass of the enzyme **E_Z** to be tested is calculated as the molar mass of the polypeptide sequence of **E_Z**.

20

Assay G:

To 0.9 ml of an aqueous reaction solution (phosphate buffer, pH 7.2, 10 mM Mg₂Cl), containing 50 mM of the pure D-enantiomer of an *n*-butyl ester of hydantoin glufosinate of the formula **D-(III)**, wherein R = *n*-butyl, are added 400 nmol of **E_Z** in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM MnCl₂).

25

The resulting solution is incubated at 25 °C, and the pH is held at pH 7.2 by addition of 0.5 M. After 300 minutes, the reaction is stopped by addition of 2 M HCl to achieve a pH of 2.5. The molar amount of the L-enantiomer of formula **L-(III)**, wherein R = *n*-butyl, is measured, at least every 3 minutes (determination by LC-MS, e.g. by the LC-MS method described in the example section, item 5.4, for the detection of LGA).

30

4.5.14.4 **Assay H** for identifying hydantoin racemases

Whether a given enzyme **E_Z** may be considered a hydantoin racemase **E₃**, may be determined in the context of the present invention by the following **Assay H**:

35

H-1 Firstly, **Assay G** as set forth under item 4.5.14.3 is conducted, and the obtained molar amount of **L-(III)** is determined according to **Assay G**.

40

H-2 Then, step H-1 is repeated, except that instead of the addition of 400 nmol E_z in 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM $MnCl_2$), 0.1 ml aqueous phosphate buffer (pH 7.2, 10 mM $MnCl_2$) without E_z is added.

5 4.5.14.5 *Hydantoin racemase activity*

If the molar amount of the compound of formula **L-(III)**, wherein $R = n$ -butyl, that is determined in step H-1 is greater than the molar amount of the compound of formula **L-(III)**, wherein $R = n$ -butyl, that is determined in step H-2, then E_z is deemed to have hydantoin racemase activity, and hence
10 may be considered a hydantoin racemase E_3 in the context of the invention.

4.5.14.6 **Assay J** for identifying preferred hydantoin racemase variants of SEQ ID NO: 17 – 26

An enzyme, the polypeptide sequence of which is selected from the group consisting of SEQ ID
15 NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ
ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, has hydantoin racemase activity.

In a preferred embodiment of the method according to the first aspect of the invention, the
polypeptide sequence of the hydantoin racemase E_3 is selected from the group consisting of SEQ
20 ID NO: 17 and variants thereof, SEQ ID NO: 18 and variants thereof, SEQ ID NO: 19 and variants
thereof, SEQ ID NO: 20 and variants thereof, SEQ ID NO: 21 and variants thereof, SEQ ID NO: 22
and variants thereof, SEQ ID NO: 23 and variants thereof, SEQ ID NO: 24 and variants thereof,
SEQ ID NO: 25 and variants thereof, SEQ ID NO: 26 and variants thereof.

The term "variant" is defined under item 4.3.

25

In the context of the invention, an enzyme, the polypeptide sequence of which is a variant of one of
SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO:
22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, has hydantoin racemase
activity.

30

Whether a given enzyme E_z , the polypeptide sequence of which is a variant of one SEQ ID NO: 17,
SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO:
23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, has hydantoin racemase activity may be
determined as set forth under items 4.5.14.4 and 4.5.14.5.

35

The hydantoin racemase activity of a given hydantoin racemase E_{3v} , the polypeptide sequence of
which is a variant of one of SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ
ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26,
relative to the hydantoin racemase activity of a hydantoin racemase E_{3s} , wherein the polypeptide
40 sequence of E_{3s} is selected from SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO:

20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, may be quantified in the context of the present invention by the following **Assay J**.

Assay J:

5

J-1 Firstly, **Assay G** as set forth under item 4.5.14.3 is conducted, wherein E_{3S} is the enzyme to be tested. The molar amount of the compound according to formula **L-(III)**, wherein R = *n*-butyl, is determined according to **Assay G**.

J-2 Step J-1 is repeated, except that, instead of E_{3S} , E_{3V} is used as the enzyme to be tested.

10 J-3. Then, the molar amount of the compound according to formula **L-(III)**, wherein R = *n*-butyl, is determined in step J-2, is divided by the molar amount of the compound according to formula **L-(III)**, wherein R = *n*-butyl, is determined in step J-1, and the obtained ratio is multiplied by 100, giving the hydantoin racemase activity of hydantoin racemase E_{3V} , relative to the hydantoin racemase activity of the hydantoin racemase E_{3S} , in %.

15

4.5.14.7 Preferred hydantoin racemase variants SEQ ID NO: 17 - 26

In the context of the present invention, hydantoin racemase E_3 , the polypeptide sequence of which is selected from the group consisting of SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, are generally denoted as " E_{3S} ".

Hydantoin racemases E_3 , the polypeptide sequence of which is selected from variants of a sequence selected from the group consisting of f SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, are generally denoted as " E_{3V} ".

In a preferred embodiment of the method according to the first aspect of the present invention, the reaction in step (a) is catalyzed by a hydantoin racemase E_3 , and the polypeptide sequence of the hydantoin racemase E_3 is selected from the group consisting of SEQ ID NO: 17 and variants thereof, SEQ ID NO: 18 and variants thereof, SEQ ID NO: 19 and variants thereof, SEQ ID NO: 20 and variants thereof, SEQ ID NO: 21 and variants thereof, SEQ ID NO: 22 and variants thereof, SEQ ID NO: 23 and variants thereof, SEQ ID NO: 24 and variants thereof, SEQ ID NO: 25 and variants thereof, SEQ ID NO: 26 and variants thereof. More preferably, the reaction in step (a) is catalyzed by a hydantoin racemase E_3 , and the polypeptide sequence of the hydantoin racemase E_3 is selected from the group consisting of SEQ ID NO: 1 and variants of SEQ ID NO: 1.

4.5.14.7.1 Preferred variants of SEQ ID NO: 17

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 17.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 17, is denoted as "**E_{317S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 17, are generally denoted as "**E_{317V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 17 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 17.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 17 is not identical to SEQ ID NO: 17.

- 20 According to the invention, a hydantoin racemase **E_{317V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{317V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{317S}**, wherein the hydantoin racemase activity of **E_{317V}**, relative to the hydantoin racemase activity **E_{317S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{317V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{317S}**, wherein the hydantoin racemase activity of **E_{317V}**, relative to the hydantoin racemase activity of **E_{317S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.2 Preferred variants of SEQ ID NO: 18

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 18.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 18, is denoted as "**E_{318S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 18, are generally denoted as "**E_{318V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 18 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 18.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 18 is not identical to SEQ ID NO: 18.

- 20 According to the invention, a hydantoin racemase **E_{318V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{318V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{318S}**, wherein the hydantoin racemase activity of **E_{318V}**, relative to the hydantoin racemase activity **E_{318S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{318V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{318S}**, wherein the hydantoin racemase activity of **E_{318V}**, relative to the hydantoin racemase activity of **E_{318S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.3 Preferred variants of SEQ ID NO: 19

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 19.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 19, is denoted as "**E_{319S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 19, are generally denoted as "**E_{319V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 19 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 19.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 19 is not identical to SEQ ID NO: 19.

- 20 According to the invention, a hydantoin racemase **E_{319V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{319V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{319S}**, wherein the hydantoin racemase activity of **E_{319V}**, relative to the hydantoin racemase activity **E_{319S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{319V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{319S}**, wherein the hydantoin racemase activity of **E_{319V}**, relative to the hydantoin racemase activity of **E_{319S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.4 Preferred variants of SEQ ID NO: 20

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 20.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 20, is denoted as "**E_{320S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 20, are generally denoted as "**E_{320V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 20 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 20.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 20 is not identical to SEQ ID NO: 20.

- 20 According to the invention, a hydantoin racemase **E_{320V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{320V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{320S}**, wherein the hydantoin racemase activity of **E_{320V}**, relative to the hydantoin racemase activity **E_{320S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{320V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{320S}**, wherein the hydantoin racemase activity of **E_{320V}**, relative to the hydantoin racemase activity of **E_{320S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.5 Preferred variants of SEQ ID NO: 21

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 21.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 21, is denoted as "**E_{321S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 21, are generally denoted as "**E_{321V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 21 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 21.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 21 is not identical to SEQ ID NO: 21.

- 20 According to the invention, a hydantoin racemase **E_{321V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{321V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{321S}**, wherein the hydantoin racemase activity of **E_{321V}**, relative to the hydantoin racemase activity **E_{321S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{321V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{321S}**, wherein the hydantoin racemase activity of **E_{321V}**, relative to the hydantoin racemase activity of **E_{321S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.6 Preferred variants of SEQ ID NO: 22

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 22.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 22, is denoted as "**E_{322S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 22, are generally denoted as "**E_{322V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 22 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 22.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 22 is not identical to SEQ ID NO: 22.

- 20 According to the invention, a hydantoin racemase **E_{322V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{322V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{322S}**, wherein the hydantoin racemase activity of **E_{322V}**, relative to the hydantoin racemase activity **E_{322S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{322V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{322S}**, wherein the hydantoin racemase activity of **E_{322V}**, relative to the hydantoin racemase activity of **E_{322S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.7 Preferred variants of SEQ ID NO: 23

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 23.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 23, is denoted as "**E_{323S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 23, are generally denoted as "**E_{323V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 23 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 23.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 23 is not identical to SEQ ID NO: 23.

- 20 According to the invention, a hydantoin racemase **E_{323V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{323V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{323S}**, wherein the hydantoin racemase activity of **E_{323V}**, relative to the hydantoin racemase activity **E_{323S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{323V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{323S}**, wherein the hydantoin racemase activity of **E_{323V}**, relative to the hydantoin racemase activity of **E_{323S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.8 Preferred variants of SEQ ID NO: 24

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 24.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 24, is denoted as "**E_{324S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 24, are generally denoted as "**E_{324V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 24 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 24.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 24 is not identical to SEQ ID NO: 24.

- 20 According to the invention, a hydantoin racemase **E_{324V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{324V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{324S}**, wherein the hydantoin racemase activity of **E_{324V}**, relative to the hydantoin racemase activity **E_{324S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{324V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{324S}**, wherein the hydantoin racemase activity of **E_{324V}**, relative to the hydantoin racemase activity of **E_{324S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.9 Preferred variants of SEQ ID NO: 25

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 25.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 25, is denoted as "**E_{325S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 25, are generally denoted as "**E_{325V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 25 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 25.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 25 is not identical to SEQ ID NO: 25.

- 20 According to the invention, a hydantoin racemase **E_{325V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{325V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{325S}**, wherein the hydantoin racemase activity of **E_{325V}**, relative to the hydantoin racemase activity **E_{325S}** is determined by **Assay J** described under item 4.5.14.6.
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{325V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{325S}**, wherein the hydantoin racemase activity of **E_{325V}**, relative to the hydantoin racemase activity of **E_{325S}** is determined by **Assay J** described under item 4.5.14.6.
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4.5.14.7.10 Preferred variants of SEQ ID NO: 26

According to the invention, the polypeptide sequence of the hydantoin racemase **E₃** may also be a variant of SEQ ID NO: 26.

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The hydantoin racemase **E₃**, the polypeptide sequence of which is SEQ ID NO: 26, is denoted as "**E_{326S}**". Hydantoin racemases **E₃**, the polypeptide sequence of which is selected from variants of SEQ ID NO: 26, are generally denoted as "**E_{326V}**".

- 10 A variant of the polypeptide sequence of SEQ ID NO: 26 is a polypeptide with sequence identity of at least 60 %, preferably ≥ 65 %, more preferably ≥ 70 %, more preferably ≥ 75 %, more preferably ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 26.
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The polypeptide sequence of a variant of the polypeptide sequence SEQ ID NO: 26 is not identical to SEQ ID NO: 26.

- 20 According to the invention, a hydantoin racemase **E_{326V}** has hydantoin racemase activity, determined as described under item 4.5.14.4 and 4.5.14.5.

- According to the invention, a hydantoin racemase **E_{326V}** preferably has hydantoin racemase activity of at least 1 %, preferably of at least 10 %, more preferably of at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 %, more preferably of at least 100 % the hydantoin racemase of the hydantoin racemase **E_{326S}**, wherein the hydantoin racemase activity of **E_{326V}**, relative to the hydantoin racemase activity **E_{326S}** is determined by **Assay J** described under item 4.5.14.6.
- 25
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- It is even more preferably according to the invention, that a hydantoin racemase **E_{326V}** has hydantoin racemase activity in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % the hydantoin racemase activity of the hydantoin racemase **E_{326S}**, wherein the hydantoin racemase activity of **E_{326V}**, relative to the hydantoin racemase activity of **E_{326S}** is determined by **Assay J** described under item 4.5.14.6.
- 35

40

4.5.15 Preferred method conditions in step (a)

In this preferred embodiment, in which step (a) is catalyzed by a hydantoin racemase **E**₃, the reaction in step (a) may be carried out under conditions known to the skilled person.

5

The reaction medium is preferably aqueous, more preferably an aqueous buffer.

Exemplary buffers commonly used in biotransformation reactions and advantageously used herein include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2-aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-morpholinepropanesulfonic acid ("MOPSO"), choline chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine, 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"), 3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").

In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be added to the reaction.

25 The buffer preferably contains metal salts, more preferably metal salts such as halogenides of monovalent or bivalent metals (e.g. CoCl₂, MnCl₂).

The concentration of these metal salts in the reaction medium is preferably in the range from 1 μM to 1 M, more preferably 1 mM to 100 mM, even more preferably 1 to 10 mM.

30

Preferably, step (a) of the method according to the first aspect of the invention is carried out in a phosphate buffer.

35 The pH of the reaction medium in step (a) of the method according to the first aspect of the invention is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8, more preferably 7.2 to 7.5, most preferably 7.5.

40 Preferably, step (a) of the method according to the first aspect of the invention is carried out at a temperature in the range of from 20 °C to 70 °C, more preferably in the range of from 30 °C to 55 °C, most preferably 50 °C.

In this preferred embodiment, in which step (a) is catalyzed by a hydantoin racemase **E₃**, the preferred reaction conditions in step (a) are the same as described for steps (b) and (c), confer items 4.5.7 and 4.5.13, respectively. It is even more preferred to carry out step (a) concomitantly with steps (b) and (c).

Preferably, the total concentration of all hydantoin racemases **E₃** in the reaction solution in step (a) is in the range of from 1 μ M to 10 mM, preferably 10 μ M to 1 mM, more preferably 0.1 mM to 0.5 mM, most preferably 0.4 mM.

In alternative preferred embodiments, the total concentration of all hydantoin racemases **E₃** in the reaction solution in step (a) is in the range of from 1 μ g/l to 10 g/l, preferably 0.1 mg/l to 5 g/l, more preferably 1 mg/l to 1 g/l, more preferably 5 mg/l to 500 mg/l.

Preferably, step (a) is carried out in the same reaction medium in which steps (c) and (b) are carried out. The advantage is that this allows for a one-pot synthesis in which all the steps (a), (b), and (c) are carried out.

Preferably, the initial concentration of all the compounds according to formula **D-(III)** in the reaction medium in step (a) is in the range of from 1 μ M to 1 M, preferably of from 10 μ M to 0.5 M, more preferably of from 0.1 mM to 0.1 M, more preferably of from 1 mM to 10 mM, most preferably 1.25 mM.

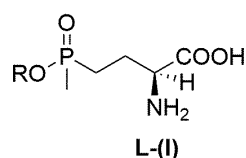
If compounds according to formula **L-(III)** are present in the reaction medium in step (a), the initial concentration of all the compounds according to formula **L-(III)** in the reaction medium is preferably from 1 to a 100 times the concentration of all the compounds according to formula **D-(III)**, more preferably 1 to 10 times the concentration, even more preferably 1 to 2 times even more preferably the same as the concentration of all the compounds according to formula **D-(III)**.

"Initial concentration of all the compounds according to formula **L-(III)/ D-(III)**" refers to the concentration of the respective compound **L-(III)** or **D-(III)**, respectively, in the reaction medium when the respective compounds are employed in step (a).

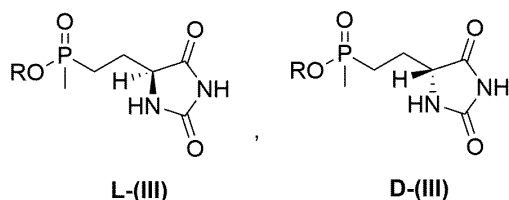
4.6 Second aspect: Method for production of an L-glufosinate *P*-alkyl ester

In a second aspect, the present invention relates to a further method for the production of an

- 5 L-glufosinate *P*-alkyl ester according to formula **L-(I)**:



from a mixture **M_{IIIA}** comprising both enantiomers **L-(III)** and **D-(III)**:



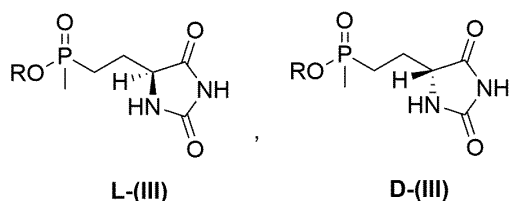
10

wherein R in formulae **L-(I)**, **L-(III)**, **D-(III)** is an alkyl group or aryl group. In particular, R is selected from the group consisting of alkyl group, phenyl group, benzyl group. Preferably, R is an alkyl group, more preferably an alkyl group with 1 to 10, even more preferably with 1 to 6, even more preferably with 1 to 4 carbon atoms. Even more preferably R = ethyl or *n*-butyl, most preferably R = *n*-butyl.

15

In this method according to the second aspect of the invention, a mixture **M_{IIIA}** comprising both enantiomers **L-(III)** and **D-(III)**, wherein:

20



is provided, and subjected to at least two enzymatic reactions (ii) and (iii), giving a composition **M_I** comprising **L-(I)** and optionally **D-(I)**. Therefore, the reaction according to the preferred second aspect of the invention is enzymatic.

25

The method for the production of an L-glufosinate *P*-alkyl ester according to formula **L-(I)**, according to the second aspect of the invention is L-enantioselective, in particular L-enantiospecific, because **M_I** either comprises **L-(I)**, but not **D-(I)** (in which case it is

“L-enantiospecific”) or, when M_I comprises both enantiomers **L-(I)** and **D-(I)**, the molar ratio of **L-(I)** to **D-(I)** in M_I is greater than the molar ratio of **L-(III)** to **D-(III)** in $M_{III A}$ (in which case it is “L-enantioselective, but not L-enantiospecific”).

5 In a particular embodiment of the method according to the second aspect of the invention, M_I comprises both enantiomers **L-(I)** and **D-(I)** and the molar ratio of **L-(I)** to **D-(I)** in M_I is greater than the molar ratio of **L-(III)** to **D-(III)** in $M_{III A}$ (in which case the method according to the second aspect of the invention is “L-enantioselective, but not L-enantiospecific”).

10 For sake of clarity it is pointed out that the feature “both enantiomers **L-(III)** and **D-(III)**” means that **L-(III)** is the enantiomer of **D-(III)**, i.e. the only difference between **L-(III)** and **D-(III)** is the chirality at the sp^3 -hybridized carbon atom of the hydantoin (5-membered) ring.

Likewise, it is pointed out that the feature “both enantiomers **L-(I)** and **D-(I)**” means that **L-(I)** is the enantiomer of **D-(I)**, i.e. the only difference between **L-(I)** and **D-(I)** is the chirality at the α carbon atom (i.e. the carbon atom adjacent to the carbonyl carbon atom).

In the method according to the second aspect of the invention, it is possible to enrich **L-(I)** over its enantiomer **D-(I)** in the product composition M_I , which means that the molar ratio of **L-(I)** to its enantiomer **D-(I)** in M_I obtained in step (iii) is greater than the molar ratio of **L-(III)** to its enantiomer **D-(III)** in mixture $M_{III A}$ provided in step (i-A).

In particular, in case the method according to the second aspect of the invention is “L-enantioselective, but not L-enantiospecific”, the portion of L-enantiomers **L-(I)** per molecule D-enantiomer **D-(I)** in M_I is greater than the portion of L-enantiomers **L-(III)** per molecule D-enantiomer **D-(III)** in $M_{III A}$, in particular by a factor of x_L , wherein x_L is at least 1.1, preferably at least 1.5, more preferably at least 2, more preferably at least 5, even more preferably at least 9, even more preferably at least 99. In another preferred embodiment x_L is in the range of 1.1 to 99, preferably in the range of 1.5 to 9, even more preferably in the range of 2 to 5.

30

4.6.1 Step (i-A)

In a first step of the method according to the second aspect of the invention, a mixture $M_{III A}$ comprising both enantiomers **L-(III)** and **D-(III)** is provided.

35

In a preferred embodiment, the mixture $M_{III A}$ is a racemic mixture of enantiomer **L-(III)** and enantiomer **D-(III)**, meaning that the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** is essentially 1 : 1.

Alternatively, enantiomer **D-(III)** is comprised in an excess to **L-(III)** in mixture **M_{IIIA}**, meaning that, while **L-(III)** is present in mixture **M_{IIIA}**, the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** in mixture **M_{IIIA}** is < 1 : 1, preferably < 0.9 : 1, more preferably < 0.75 : 1, more preferably < 0.5 : 1, more preferably < 0.2 : 1, more preferably < 0.1 : 1, more preferably < 0.01 : 1.

5

In other preferred embodiments, the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** in mixture **M_{IIIA}** is in the range of 3 : 2 to 1 : 99, more preferably in the range 1 : 1 to 1 : 99, more preferably in the range of 1.01 : 1 to 1 : 99, more preferably in the range 1 : 1.01 to 1 : 9, more preferably in the range 1 : 1.01 to 1 : 8, more preferably in the range 1 : 1.01 to 1 : 3.

10

A mixture of these enantiomers can be obtained by the skilled person, for example by organic synthesis as set forth in DE 31 42 036 A1 and by Slomka *et al.*

E. Ware, Chem. Rev. **1950**, 46, 403-470 and C. Avendaño & J. C. Menendez, "Hydantoin and its derivatives" in Kirk-Othmer Encyclopedia of Chemical Technology **2000** give a general overview over hydantoin chemistry.

15

Preferably, the mixture **M_{IIIA}** is provided in step (i-A) in a reaction medium.

Namely, the reaction medium in step (i-A) is preferably aqueous, more preferably an aqueous buffer.

20

Exemplary buffers commonly used in biotransformation reactions and advantageously used in step (i-A) include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2- aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-morpholinepropanesulfonic acid ("MOPSO"), choline chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)- 2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine, 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"), 3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").

25

30

35

In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be added to the reaction.

The buffer preferably contains metal salts, more preferably metal salts such as halogenides of monovalent or bivalent metals (e.g. CoCl₂, MnCl₂).

40

The concentration of these metal salts in the reaction medium in step (i-A) is preferably in the range from 1 μ M to 1 M, more preferably 1 mM to 100 mM, even more preferably 1 to 10 mM.

- 5 Preferably, step (i-A) of the method according to the second aspect of the invention is carried out in a phosphate buffer.

The pH of the reaction medium in step (i-A) of the method according to the first aspect of the invention is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8,
10 more preferably 7.2 to 7.5, most preferably 7.5.

More preferably, the concentration of all the compounds according to formula **D-(III)** in the reaction medium provided in step (i-A) is in the range of from 1 μ M to 1 M, preferably of from 10 μ M to 0.5 M, more preferably of from 0.1 mM to 0.1 M, more preferably of from 1 mM to 10 mM, most
15 preferably 1.25 mM.

Concerning the compounds according to formula **L-(III)** that are present in the reaction medium preferably provided in step (i-A), the concentration of all the compounds according to formula **L-(III)** in the reaction medium is preferably from 1 to a 100 times the concentration of all the compounds
20 according to formula **D-(III)**, more preferably 1 to 10 times the concentration, even more preferably 1 to 2 times even more preferably the same as the concentration of all the compounds according to formula **D-(III)**.

25 4.6.2 Optional Step (i-B)

In an optional step (i-B), at least a part of the compounds **D-(III)** comprised by the mixture **M_{IIIA}** are reacted into **L-(III)**, giving a composition **M_{IIIB}** comprising **L-(III)** and optionally its enantiomer **D-(III)**. It goes without saying that the molar ratio of enantiomer **L-(III)** to enantiomer **D-(III)** in mixture **M_{IIIB}** is greater than in mixture **M_{IIIA}**.

30

In case step (i-B) is carried out, it is preferably carried out as described for step (a) under items 4.5.14 and 4.5.15.

In particular, the reaction according to step (i-B) is catalyzed by a hydantoin racemase **E₃** as
35 described with respect to step (a).

Preferably, the hydantoin racemase **E₃** used in step (i-B) is categorized in the EC class 5.1.99.5. In another embodiment, the polypeptide sequence of the hydantoin racemase **E₃** used in step (i-B) is selected from the group consisting of SEQ ID NO: 17 and variants thereof, SEQ ID NO: 18 and
40 variants thereof, SEQ ID NO: 19 and variants thereof, SEQ ID NO: 20 and variants thereof,

SEQ ID NO: 21 and variants thereof, SEQ ID NO: 22 and variants thereof, SEQ ID NO: 23 and variants thereof, SEQ ID NO: 24 and variants thereof, SEQ ID NO: 25 and variants thereof, SEQ ID NO: 26 and variants thereof, most preferably selected from SEQ ID NO: 17 and variants thereof.

5

Preferably, the hydantoin racemase **E₃** used in step (i-B) is selected from the group consisting of **E_{317S}** and **E_{317V}** (both described under item 4.5.14.7.1), **E_{318S}** and **E_{318V}** (both described under item 4.5.14.7.2), **E_{319S}** and **E_{319V}** (both described under item 4.5.14.7.3), **E_{320S}** and **E_{320V}** (both described under item 4.5.14.7.4), **E_{321S}** and **E_{321V}** (both described under item 4.5.14.7.5), **E_{322S}** and **E_{322V}** (both described under item 4.5.14.7.6), **E_{323S}** and **E_{323V}** (both described under item 4.5.14.7.7), **E_{324S}** and **E_{324V}** (both described under item 4.5.14.7.8), **E_{325S}** and **E_{325V}** (both described under item 4.5.14.7.9), **E_{326S}** and **E_{326V}** (both described under item 4.5.14.7.10), more preferably from **E_{317S}** and **E_{317V}**, even more preferably from **E_{317S}**.

15 The reaction conditions in step (i-B) are preferably as described with respect to step (a) under item 4.5.15.

Namely, the reaction medium in step (i-B) is preferably aqueous, more preferably an aqueous buffer.

20

Exemplary buffers commonly used in biotransformation reactions and advantageously used in step (i-B) include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2-aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-morpholinepropanesulfonic acid ("MOPSO"), choline chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine, 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"), 3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").

35 In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be added to the reaction.

The buffer preferably contains metal salts, more preferably metal salts such as halogenides of monovalent or bivalent metals (e.g. CoCl₂, MnCl₂).

40

The concentration of these metal salts in the reaction medium in step (i-B) is preferably in the range from 1 μ M to 1 M, more preferably 1 mM to 100 mM, even more preferably 1 to 10 mM.

5 Preferably, step (i-B) of the method according to the second aspect of the invention is carried out in a phosphate buffer.

10 The pH of the reaction medium in step (i-B) of the method according to the first aspect of the invention is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8, more preferably 7.2 to 7.5, most preferably 7.5.

15 Preferably, step (i-B) of the method according to the first aspect of the invention is carried out at a temperature in the range of from 20 °C to 70 °C, more preferably in the range of from 30 °C to 55 °C, most preferably 50 °C.

20 Preferably, the total concentration of all hydantoin racemases **E₃** in the reaction solution in step (i-B) is in the range of from 1 μ M to 10 mM, preferably 10 μ M to 1 mM, more preferably 0.1 mM to 0.5 mM, most preferably 0.4 mM.

25 In alternative preferred embodiments, the total concentration of all hydantoin racemases **E₃** in the reaction solution in step (i-B) is in the range of from 1 μ g/l to 10 g/l, preferably 0.1 mg/l to 5 g/l, more preferably 1 mg/l to 1 g/l, more preferably 5 mg/l to 500 mg/l.

30 Preferably, step (i-B) is carried out in the same reaction medium in which steps (ii) and (iii) are carried out. The advantage is that this allows for a one-pot synthesis in which all the steps (i-B), (ii), and (iii) are carried out.

At the end of optional step (i-B), a composition **M_{III B}** comprising **L-(III)** and optionally its enantiomer **D-(III)** is obtained, preferably a mixture **M_{III B}** comprising **L-(III)** and its enantiomer **D-(III)** is obtained.

35 4.6.3 Step (ii)

In step (ii) of the method according to the second aspect of the invention, the mixture **M_{IIIA}** or, in case step (i-B) is carried out, composition **M_{III B}**, is subjected to step (b) as described above under item 4.5.8 – 4.5.13.

In other words: in those embodiments, in which step (i-B) is not carried out, **M_{IIIA}** is subjected to step (b) as described above under item 4.5.8 - 4.5.13.

40 In case step (i-B) is carried out, **M_{III B}** obtained after step (i-B) is subjected to step (b) as described above under item 4.5.8 - 4.5.13.

The reaction according to step (ii) is catalyzed by a hydantoinase **E₂** as described with respect to step (b), preferably by an L-hydantoinase **E₂** as described with respect to step (b).

More preferably, the hydantoinase **E₂**, even more preferably the L-hydantoinase **E₂** used in step (ii) is categorized in the EC class 3.5.2.2.

- 5 In another preferred embodiment, the polypeptide sequence of the hydantoinase **E₂**, even more preferably the L-hydantoinase **E₂** used in step (ii) is selected from the group consisting of SEQ ID NO: 10 and variants thereof, SEQ ID NO: 11 and variants thereof, SEQ ID NO: 12 and variants thereof, SEQ ID NO: 13 and variants thereof, SEQ ID NO: 14 and variants thereof, SEQ ID NO: 15 and variants thereof, SEQ ID NO: 16 and variants thereof, even more preferably from SEQ ID NO: 10 and variants thereof.

- 15 Preferably, the hydantoinase **E₂**, even more preferably the L-hydantoinase **E₂** used in step (ii) is selected from the group consisting of **E_{210S}** and **E_{210V}** (both described under item 4.5.12.1), **E_{211S}** and **E_{211V}** (both described under item 4.5.12.2), **E_{212S}** and **E_{212V}** (both described under item 4.5.12.3), **E_{213S}** and **E_{213V}** (both described under item 4.5.12.4), **E_{214S}** and **E_{214V}** (both described under item 4.5.12.5), **E_{215S}** and **E_{215V}** (both described under item 4.5.12.6), **E_{216S}** and **E_{216V}** (both described under item 4.5.12.7), more preferably from **E_{210S}** and **E_{210V}**, even more preferably from **E_{210S}**.

- 20 The reaction conditions in step (ii) are preferably as described with respect to step (b) under item 4.5.13.

Namely, the reaction medium in step (ii) is preferably aqueous, more preferably an aqueous buffer.

- 25 Exemplary buffers commonly used in biotransformation reactions and advantageously used in step (ii) include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2-aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-morpholinepropanesulfonic acid ("MOPSO"), choline chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine, 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-
35 *N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"), 3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycylglycine, bicine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").
In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be
40 added to the reaction.

The buffer preferably contains metal salts, more preferably metal salts such as halogenides of monovalent or bivalent metals (e.g. CoCl_2 , MnCl_2).

- 5 The concentration of these metal salts in the reaction medium is preferably in the range from 1 μM to 1 M, more preferably 1 mM to 100 mM, even more preferably 1 to 10 mM.

Preferably, step (ii) of the method according to the second aspect of the invention is carried out in a phosphate buffer.

10

The pH of the reaction medium in step (ii) of the method according to the first aspect of the invention is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8, more preferably 7.2 to 7.5, most preferably 7.5.

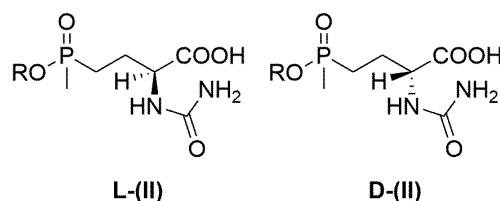
- 15 Preferably, step (ii) of the method according to the first aspect of the invention is carried out at a temperature in the range of from 20 °C to 70 °C, more preferably in the range of from 30 °C to 55 °C, most preferably 50 °C.

- 20 Preferably, the total concentration of all hydantoinases E_2 in the reaction solution in step (ii) is in the range of from 1 μM to 10 mM, preferably 10 μM to 1 mM, more preferably 0.1 mM to 0.5 mM, most preferably 0.4 mM.

- In alternative preferred embodiments, the total concentration of all hydantoinases E_2 in the reaction solution in step (ii) is in the range of from 1 $\mu\text{g/l}$ to 10 g/l, preferably 0.1 mg/l to 5 g/l, more
25 preferably 1 mg/l to 1 g/l, more preferably 5 mg/l to 500 mg/l.

Preferably, step (ii) is carried out in the same reaction medium in which step (iii) is carried out.

- 30 At the end of step (ii), a composition M_{II} comprising **L-(II)** and optionally its enantiomer **D-(II)** is obtained, preferably a mixture M_{II} comprising **L-(II)** and its enantiomer **D-(II)** is obtained:



R in formulae **L-(II)** and **D-(II)** in composition M_{II} has the same meaning as described for **L-(I)**.

35

4.6.4 Step (iii)

In step (iii) of the method according to the second aspect of the invention, **M_{II}** obtained in step (ii) is subjected to step (c) as described above under items 4.5.1 to 4.5.7 wherein the reaction according to step (iii) is catalyzed by an L-carbamoylase **E₁**.

This brings about that the L-enantiomer **L-(I)** is enriched over the D-enantiomer **D-(I)** in **M_I**, i.e., in case **M_I** comprises both enantiomers **L-(I)** and **D-(I)**, the molar ratio of **L-(I)** to **D-(I)** in **M_I** is greater than the molar ratio of **L-(III)** to **D-(III)** in **M_{IIIA}**.

More preferably, the L-carbamoylase **E₁** used in step (iii) is categorized in the EC class 3.5.1.87. In another preferred embodiment, the polypeptide sequence of the L-carbamoylase **E₁** used in step (iii) is selected from the group consisting of SEQ ID NO: 1 and variants thereof, SEQ ID NO: 2 and variants thereof, SEQ ID NO: 3 and variants thereof, SEQ ID NO: 4 and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 6 and variants thereof, SEQ ID NO: 7 and variants thereof, SEQ ID NO: 8 and variants thereof, SEQ ID NO: 9 and variants thereof.

Preferably, the L-carbamoylase **E₁** used in step (iii) is selected from the group consisting of **E_{101S}** and **E_{101V}** (both described under item 4.5.6.1), **E_{102S}** and **E_{102V}** (both described under item 4.5.6.2), **E_{103S}** and **E_{103V}** (both described under item 4.5.6.3), **E_{104S}** and **E_{104V}** (both described under item 4.5.6.4), **E_{105S}** and **E_{105V}** (both described under item 4.5.6.5), **E_{106S}** and **E_{106V}** (both described under item 4.5.6.6), **E_{107S}** and **E_{107V}** (both described under item 4.5.6.7), **E_{108S}** and **E_{108V}** (both described under item 4.5.6.8), **E_{109S}** and **E_{109V}** (both described under item 4.5.6.9), more preferably from **E_{101S}** and **E_{101V}**, even more preferably from **E_{101S}**.

The reaction conditions in step (iii) are preferably as described with respect to step (c) under item 4.5.7.

Namely, the reaction medium in step (iii) is preferably aqueous, more preferably an aqueous buffer.

Exemplary buffers commonly used in biotransformation reactions and advantageously used in step (iii) include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2-aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-morpholinepropanesulfonic acid ("MOPSO"), choline chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine, 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"),

3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").

In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be added to the reaction.

5

The buffer preferably contains metal salts, more preferably metal salts such as halogenides of monovalent or bivalent metals (e.g. CoCl_2 , MnCl_2).

The concentration of these metal salts in the reaction medium is preferably in the range from 1 μM to 1 M, more preferably 1 mM to 100 mM, even more preferably 1 to 10 mM.

10

Preferably, step (iii) of the method according to the second aspect of the invention is carried out in a phosphate buffer.

The pH of the reaction medium in step (iii) of the method according to the first aspect of the invention is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8, more preferably 7.2 to 7.5, most preferably 7.5.

15

Preferably, step (iii) of the method according to the first aspect of the invention is carried out at a temperature in the range of from 20 °C to 70 °C, more preferably in the range of from 30 °C to 55 °C, most preferably 50 °C.

20

Preferably, the total concentration of all carbamoylases E_1 in the reaction solution in step (iii) is in the range of from 1 μM to 10 mM, preferably 10 μM to 1 mM, more preferably 0.1 mM to 0.5 mM, most preferably 0.4 mM.

25

In alternative preferred embodiments, the total concentration of all carbamoylases E_1 in the reaction solution in step (iii) is in the range of from 1 $\mu\text{g/l}$ to 10 g/l, preferably 0.1 mg/l to 5 g/l, more preferably 1 mg/l to 1 g/l, more preferably 5 mg/l to 500 mg/l.

30

At the end of step (i), a composition M_1 comprising L-(I) and optionally its enantiomer D-(I) is obtained, preferably a mixture M_1 comprising L-(I) and its enantiomer D-(I) is obtained.

4.7 Saponification

35

The L-glufosinate *P*-alkyl ester according to formula L-(I) produced with the method according to the first or second aspect can then be saponified to produce LGA, for example in an acidic aqueous medium, preferably at pH < 7, even more preferably at an pH between < 6, more preferably at an pH < 3, even more preferably at an pH of < 1. These saponification conditions are known to the skilled person and described e.g. by H.J. Zeiss, J. Org. Chem. **1991**, *56*, 1783-1788.

40

5. Examples

5.1 Example 1 Identification of suitable enzymes and construction of plasmids

- 5 Genes of different origins encoding a hydantoinase (dihydropyrimidinase, EC 3.5.2.2), L-carbamoylase (*N*-carbamoyl-L-amino-acid hydrolase, EC 3.5.1.87) and hydantoin racemase (EC 5.1.99.5) were tested for their ability to react with different hydantoin substrates according to structure **L-(III)** and **D-(III)** to form the respective enantioselective L-glufosinate derivative according to structure **L-(I)**.

10

5.1.1 Examined enzymes

Details of the strains and genes of the respective enzymes that were used in the examples are summarized in table 4.

15

Table 4

Plasmid	Enzyme	Donor Strain	Gene name	SEQ ID NO: of the poly-nucleotides	SEQ ID NO: of the poly-peptides
pOM22c	L-carbamoylase	<i>Arthrobacter</i> sp. DSM 3747	<i>hyuC</i>	SEQ ID NO: 27	SEQ ID NO: 1
pOM22c	hydantoinase	<i>Arthrobacter</i> sp. DSM 9771	<i>hyuH</i>	SEQ ID NO: 28	SEQ ID NO: 10
pOM21c	hydantoin racemase	<i>Arthrobacter</i> sp. DSM 3745	<i>hyuR</i>	SEQ ID NO: 29	SEQ ID NO: 17

20 5.1.2 Cloning of the enzymes

Cloning of the hydantoin racemase and generation of the plasmid pOM21c (Figure 1) was carried out as described in example 1 of WO 2004/111227 A2. In particular, a polynucleotide was used which comprised the respective gene (SEQ ID NO: 29) and additional sequences for *NdeI* and *PstI* restriction sites.

25

Cloning of hydantoinase and L-carbamoylase into the rhamnose expression vector pJOE4036 was carried out in a plasmid derivative of the rhamnose expression vector pJOE4036.

Polynucleotides comprising the genes of the respective enzymes (SEQ ID NOs: 27, 28) were synthesized by GeneArt (ThermoFisher Scientific (Waltham, USA)). The polynucleotides carried additional sequences for *EcoRI* and *HindIII* restriction sites. Both enzymes were cloned into pJOE4036 using those restriction sites resulting in the plasmid pOM22c, under the control of a rhamnose promoter (Figure 2).

35

5.2 Example 2: Production of strains positive for hydantoinase, L-carbamoylase and hydantoin racemase

Chemically competent *E. coli* ET5 cells (as described in WO 2004/042047 A1) were transformed
5 with 10 ng of the plasmid pOM22c generated according to Example 1.

The generated strain which was positive for hydantoinase- and carbamoylase was rendered chemically competent and transformed with 10 ng of the plasmid pOM21c.

10 An *E. coli* ET5 strain transformed with pOM21c or pOM22c was incubated under shaking (250 U/min) at 30 °C for 18 hours in LB medium containing ampicillin (100 µg/l), chloramphenicol (50 µg/l), and 2 g/l rhamnose.

The biomass was separated by centrifugation, resuspended in 50 mM phosphate buffer (pH 7.2)
15 and applied in biotransformation tests in the following examples. The concentration of the biomass in the solution was 40-50 g/l. The solution was used as catalyst ("*catalyst 1*") in the following.

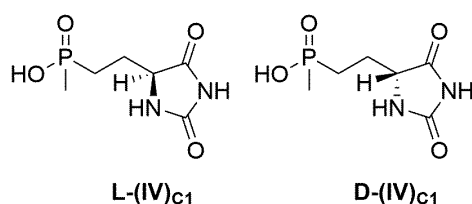
The concentration of the respective polypeptide carbamoylase, hydantoinase and racemase in the obtained solution may be determined by SDS page and analysis of the respective bands via the
20 software GelQuant® (BiochemLabSolutions).

5.3 Inventive Examples I1 and I2 and Comparative Examples C1 and C2 : Production of P-Alkyl phosphinothricin ester using the strains produced in Examples 1 and 2

25 In the following examples, different hydantoin substrates were tested to determine whether the respective polypeptide catalyzed the reaction of the respective substrate enantioselectively to give the corresponding L amino acid.

5.3.1 Comparative Example C1

30 In comparative example C1, a racemic mixture **M_{C1}** of 1.25 mmol **L-(IV)_{C1}** and 1.25 mmol **D-(IV)_{C1}**, wherein **L-(IV)_{C1}** and **D-(IV)_{C1}** are hydantoin with the following formulae, was used as substrate:



35

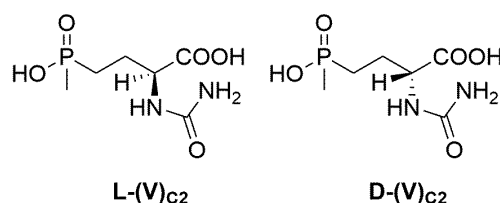
The mixture **M_{C1}** was dissolved in a stirring reactor with 25 ml water. 2.4 g of the *catalyst 1* was added, followed by the addition of 50 µl CoCl₂ solution. The suspension was set to pH 7.5 with

0.5 M NaOH. The total volume was replenished with water to 50 ml, so that the concentration of each enantiomer **L-(IV)_{C1}** and **D-(IV)_{C1}** was 0.025 mol/l and the concentration of CoCl₂ in the final solution was 1 mM. v. The pH was held between 7.0 and 7.5 by HCl-titration or NaOH-titration. The temperature was maintained at 37 °C by a thermostat during the reaction.

- 5 The reaction was stopped after 120 hours by addition of 2 N HCl until pH 2.5 was reached. The biomass was separated by centrifugation or filtration.
The enzyme reaction was monitored by ninhydrin test to determine the formation of amino acids. No formation of amino acids was detected by the ninhydrine test.

10 5.3.2 Comparative Example C2

In comparative example **C2**, a racemic mixture **M_{C2}** of 1.25 mmol **L-(V)_{C2}** and 1.25 mmol **D-(V)_{C2}**, wherein **L-(V)_{C2}** and **D-(V)_{C2}** are carbamoylates with the following formulae, was used as substrate:

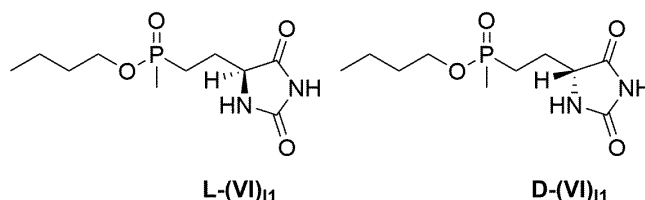


- 15 The mixture **M_{C2}** was dissolved in a stirring reactor with 25 ml water. 2.4 g of the *catalyst 1* was added, followed by the addition of 50 µl CoCl₂ solution. The suspension was set to pH 7.5 with 0.5 M NaOH. The total volume was replenished with water to 50 ml, so that the concentration of each enantiomer **L-(V)_{C2}** and **D-(V)_{C2}** was 0.025 mol/l and the concentration of CoCl₂ in the final solution was 1 mM. The pH was held between 7.0 and 7.5 by HCl-titration or NaOH-titration. The
- 20 temperature was maintained at 37 °C by a thermostat during the reaction.
The reaction was stopped after 120 hours by addition of 2 N HCl until pH 2.5 was reached. The biomass was separated by centrifugation or filtration.
The enzyme reaction was monitored by ninhydrin test to determine the formation of amino acids. No formation of amino acids was detected by the ninhydrine test.

25

5.3.3 Inventive Example I1

In inventive example **I1**, a racemic mixture **M_{I1}** of 1.25 mmol **L-(VI)_{I1}** and 1.25 mmol **D-(VI)_{I1}**, wherein **L-(VI)_{I1}** and **D-(VI)_{I1}** were hydantoinis with the following formulae, was used as substrate:



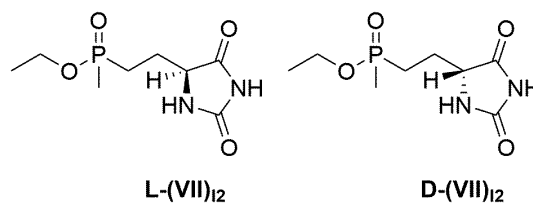
30

- The mixture **M**₁₁ was dissolved in a stirring reactor with 25 ml water. 2.4 g of the *catalyst 1* was added, followed by the addition of 50 µl CoCl₂ solution. The suspension was set to pH 7.5 with 0.5 M NaOH. The total volume was replenished with water to 50 ml, so that the concentration of each enantiomer **L**-(**VI**)₁₁ and **D**-(**VI**)₁₁ was 0.025 mol/l and the concentration of CoCl₂ in the final solution was 1 mM. The pH was held between 7.0 and 7.5 by HCl-titration or NaOH-titration. The temperature was maintained at 37 °C by a thermostat during the reaction.
- The reaction was stopped after 120 hours by addition of 2 N HCl until pH 2.5 was reached. The biomass was separated by centrifugation or filtration.
- The enzyme reaction was monitored by ninhydrin test to determine the formation of amino acids.
- Formation of amino acids was detected by the ninhydrine test.
- In a final step the reaction mixture was saponified at 100 °C for 10 hours by adding 6 M HCl to obtain L-glufosinate and, if present, D-glufosinate. The final reaction mixture was analysed by LC-MS with a CR-I column as described under item 5.4 to determine the enantiomeric excess ("ee") of either D- or L-glufosinate. An ee of LGA of 79 % over the D-enantiomer was detected. The ee of the L-enantiomer ("ee_L") is determined by the following formula in the context of the invention, wherein m_L and m_D are the detected molar masses of L- and D-glufosinate, respectively:

$$ee_L = \frac{(m_L - m_D) \times 100 \% }{(m_L + m_D)}.$$

5.3.4 Inventive Example I2

- In inventive example I2, a racemic mixture **M**₁₂ of 1.25 mmol **L**-(**VII**)₁₂ and 1.25 mmol **D**-(**VII**)₁₂, wherein **L**-(**VII**)₁₂ and **D**-(**VII**)₁₂ were hydantoin with the following formulae, was used as substrate:



- The mixture **M**₁₂ was dissolved in a stirring reactor with 25 ml water. 2.4 g of the *catalyst 1* was added, followed by the addition of 50 µl CoCl₂ solution. The suspension was set to pH 7.5 with 0.5 M NaOH. The total volume was replenished with water to 50 ml, so that the concentration of each enantiomer **L**-(**VII**)₁₂ and **D**-(**VII**)₁₁ was 0.025 mol/l and the concentration of CoCl₂ in the final solution was 1 mM. The pH was held between 7.0 and 7.5 by HCl-titration or NaOH-titration. The temperature was maintained at 37 °C by a thermostat during the reaction.
- The reaction was stopped after 120 hours by addition of 2 N HCl until pH 2.5 was reached. The biomass was separated by centrifugation or filtration.
- The enzyme reaction was monitored by ninhydrin test to determine the formation of amino acids.
- Formation of amino acids was detected by the ninhydrine test.
- In a final step the reaction mixture was saponified at 100 °C for 10 hours by adding 6 M HCl to obtain L-glufosinate and, if present, D-glufosinate. The final reaction mixture was analysed by

LC-MS with a CR-I column as described under item 5.4 to determine the enantiomeric excess ("ee") of either D- or L-glufosinate. An ee of LGA of 77 % over the D-enantiomer was detected.

5.3.5 Results

5

The results of the examples **C1**, **C2**, **I1**, **I2** are summarized in table 5.

Table 5

Example	substrate	Ninhydrine test	ee _L
C1	L-(IV)_{C1} , D-(IV)_{C1}	Negative	-
C2	L-(V)_{C2} , D-(V)_{C2}	Negative	-
I1	L-(VI)_{I1} , D-(VI)_{I1}	Positive	79 %
I2	L-(VII)_{I2} , D-(VII)_{I2}	Positive	77 %

10 5.3.6 Conclusion 1

The results as summarized in table 5 show that certain hydantoin esters such as the alkylated hydantoin esters can be used as substrates for the enzymatic enantioselective synthesis of L-glufosinate (**I1**, **I2**). In contrast, substrates in which the phosphinic acid group is not protected are not accepted by the catalytic system (**C1**, **C2**). In this regard, **C2** suggests that at least one reason for this is that the L-carbamoylase enzyme does not accept the respective substrate in which the phosphinic acid function is not protected by an ester group.

Moreover, in contrast to the process for enantioselective production according to the prior art (CN 111662325 A), the ee excess could be maintained throughout the process, because saponification is carried out not at the hydantoin stage, but at the stage of the amino acid ester.

5.4 Analytical Methods

25 Analysis Method 1

L-glufosinate and D-glufosinate were detected by LC-MS ("Liquid Chromatography - Mass Spectrometry") with a chiral column [Daicel CROWNPAK CR-I(-)] as follows. For hydantoin, a Daicel Chiralpak IA-U column may also be used.

30

This detection method may also be used for detection and quantification of the

- the LGA *P*-(*n*-butyl) ester according to formula **L-(I)**, wherein R = *n*-butyl, according to **Assay A_L** (item 4.5.3.1);
- the D-glufosinate *P*-(*n*-butyl) ester according to formula **D-(I)**, wherein R = *n*-butyl, according to **Assay A_D** (item 4.5.3.2);
- the carbamoyl LGA *P*-(*n*-butyl) ester according to formula **L-(II)**, wherein R = *n*-butyl, according to **Assay D_L** (item 4.5.9.1);

35

- the carbamoyl D-glufosinate *P*-(*n*-butyl) ester according to formula **D-(II)**, wherein R = *n*-butyl, according to **Assay D_D** (item 4.5.9.2);
 - the L-enantiomer of a *n*-butyl ester of hydantoin glufosinate of the formula **L-(III)**, wherein R = *n*-butyl, according to **Assay G** (item 4.5.14.3).
- 5 - the D-enantiomer of a *n*-butyl ester of hydantoin glufosinate of the formula **D-(III)**, wherein R = *n*-butyl, according to **Assay G** (item 4.5.14.3).

5.4.1 Acquisition Method Details

10 CR-I(-), 3.0 mm I.D. x 150 mm, 5 µm, Crownpak, Part.No. 54784
 MS QQQ Mass Spectrometer 6420, Agilent
 ESI+
 MS2 SIM, Glufosinat m/z 182.06 Unit Positive
 Centroid

15

5.4.2 Source Parameter

Parameter Value (+); Gas Temp (°C): 350; Gas Flow (l/min): 12; Nebulizer (psi): 25; Capillary (V): 3000; Sampler Module: G1329B; Injection Volume 1.00 µL; 5°C

20

5.4.3 Binary Pump Module

G1312B

25 Flow: 0.200 mL/min;
 Channel A: H₂O with trifluoroacetic acid (TFA) pH1.15;
 Channel B: ACN (acetonitrile);
 isocratic 80.0 % A / 20.0 % B; stoptime 7.00 min
 Column Comp. Module: G1316A

30 Temperature 5.0 °C

Analysis Method 2

A further analytical method was used to determine the yield of the LGA *P*-(*n*-butyl) ester according to formula **L-(I)**, wherein R = *n*-butyl, and the D-glufosinate *P*-(*n*-butyl) ester according to formula **D-(I)**, wherein R = *n*-butyl. In particular, this method was used to determine the yield of **L-(VIII)₁₃** and **D-(VIII)₁₃**, as summarized in table 6 hereinafter.

35

In this method, LC-MS ("Liquid Chromatography - Mass Spectrometry") with a chiral column
 40 [Chiralpak 3µm ZWIX (+), 150 x 3 mm, DAICEL] was used as follows.

5.4.4 Acquisition Method Details

ZWIX (+), 150 x 3 mm; 3µm

MS QQQ Mass Spectrometer 6420, Agilent

5 ESI+

MS2 SIM, Glufosinat-carbamoyl-butyl ester m/z 238.1 Unit Positive

Centroid

5.4.5 Source Parameter

10

Parameter Value (+); Gas Temp (°C): 350; Gas Flow (l/min): 12; Nebulizer (psi): 50; Capillary (V): 4000; Sampler Module: G1329B; Injection Volume 1.00 µL; 5°C

5.4.6 Binary Pump Module

15

G1312B

Flow: 0.300 mL/min;

isocratic 49.0 % acetonitrile / 49.0 % methanol, 2.0 % H₂O; stoptime 15.00 min

20

Column Comp. Module: G1316A

Temperature 5.0 °C

5.5 Example 3 Identification of further carbamoylase enzymes and construction of plasmids

- 25 In a further test series, genes encoding L-carbamoylases (*N*-carbamoyl-L-amino-acid hydrolases, EC 3.5.1.87) of different origins were tested for their ability to react with different carbamoyl substrates according to structure **L-(II)** and **D-(II)** to form the respective L-glufosinate/ D-glufosinate derivative according to structures **L-(I)** and **D-(I)**, respectively. Cloning and expression of the respective L-carbamoylase gene was essentially carried out as described by B. Wilms, A. Wiese,
- 30 C. Syldatk, R. Mattes, J. Altenbuchner, M. Pietzsch, Journal of Biotechnology **1999**, *68*, 101 – 113 (hereinafter “Wilms *et al.*”), in particular, as set forth in the following.

5.5.1 Examined enzymes

- 35 Details of the strains and genes of the respective L-carbamoylase that were used in the examples are summarized in table 5.

Table 5

Plasmid	Donor Strain	Gene name	SEQ ID NO: of the polypeptides
pOM17c	<i>Arthrobacter</i> sp. DSM 3747	<i>hyuC</i>	SEQ ID NO: 1
pOM17c {Prha}[amaB_Gst]	<i>Geobacillus stearothermophilus</i>	<i>amaB</i>	SEQ ID NO: 2
pOM17c {Prha}[atc_Ps]	<i>Pseudomonas</i> sp. QR-101	<i>atcC</i>	SEQ ID NO: 3
pOM17c {Prha}[hyuC_Pau]	<i>Paenarthrobacter aureus</i>	<i>hyuC</i>	SEQ ID NO: 5
pOM17c {Prha}[hyuC_Asp (co_Ec)]	<i>Arthrobacter</i> sp. BT801	<i>hyuC</i>	SEQ ID NO: 8

5

5.5.2 Cloning of the enzymes

Cloning of the respective L-carbamoylase gene into the rhamnose expression vector pJOE4036 was carried out in a plasmid derivative of the rhamnose expression vector pJOE4036. Polynucleotides comprising the genes of the respective enzymes were synthesized by GeneArt (ThermoFisher Scientific (Waltham, USA)). The polynucleotides carried additional sequences for *ndel* and *HindIII* restriction sites. Each one of the genes encoding the enzymes was cloned into pJOE4036 using those restriction sites resulting in the respective plasmid, i.e. pOM17c for expression of SEQ ID NO: 1 (shown in Figure 3), pOM17c{Prha}[amaB_Gst] for expression of SEQ ID NO: 2 (shown in Figure 4), pOM17c{Prha}[atcC_Ps] for expression of SEQ ID NO: 3 (shown in Figure 5), pOM17c{Prha}[hyuC_Pau] for expression of SEQ ID NO: 5 (shown in Figure 6), pOM17c {Prha}[hyuC_Asp (co_Ec)] for expression of SEQ ID NO: 8 (shown in Figure 7), each under the control of a rhamnose promoter.

20

5.6 Example 4: Production of strains positive for L-carbamoylase

Chemically competent *E. coli* ET5 cells (as described in WO 2004/042047 A1) were transformed with 10 ng of the respective plasmid generated according to Example 3.

25

An *E. coli* ET5 strain transformed with the respective plasmid was incubated under shaking (250 U/min) at 30 °C for 18 hours in LB medium containing ampicillin (100 µg/l), and 2 g/l rhamnose.

The biomass was separated by centrifugation, resuspended in 50 mM phosphate buffer (pH 7.2) and applied in biotransformation tests in the following examples. The concentration of the biomass in the solution was 12.2 g/l. The solution was used as catalyst ("catalyst 2") in the following.

30

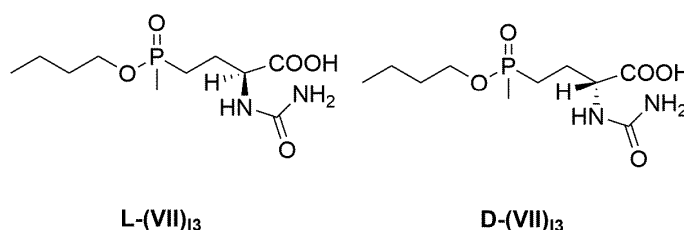
The concentration of the respective polypeptide carbamoylase in the obtained solution may be determined by SDS page and analysis of the respective bands via the software GelQuant® (BiochemLabSolutions).

- 5 In the following examples, the different carbamoylases were tested to determine whether the respective polypeptide catalyzed the reaction of the carbamoyl substrate enantioselectively to give the corresponding L amino acid.

5.6.1 Inventive Example I3

10

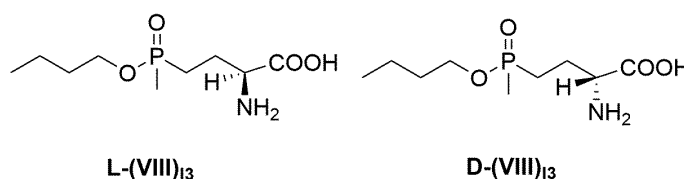
In inventive example I3, a racemic mixture **M**₁₃ of 0.25 mmol **L-(VII)**₁₃ and 0.25 mmol **D-(VII)**₁₃, wherein **L-(VII)**₁₃ and **D-(VII)**₁₃ were carbamoyles with the following formulae, was used as substrate:



- 15 The mixture **M**₁₃ was dissolved in a stirring reactor with 25 ml water. 2.4 g of the *catalyst* 2 was added, followed by the addition of CoCl₂ solution. The suspension was set to pH 7.4 with 0.5 M NaOH. The total volume was replenished with water to 50 ml, so that the concentration of each compound **L-(VII)**₁₃ and **D-(VII)**₁₃ was 5 mmol/l and the concentration of CoCl₂ in the final solution was 1 mM. The pH was held between 7.4 by HCl-titration or NaOH-titration. The
- 20 temperature was maintained at 37 °C by a thermostat during the reaction. The reaction was stopped after 48 hours by addition of 2 N HCl until pH 2.5 was reached. The biomass was separated by centrifugation or filtration.

The enzyme reaction was monitored by ninhydrin test to determine the formation of amino acids.

25



- The overall conversion of **L-(VII)**₁₃ to the respective product **L-(VIII)**₁₃ and of **D-(VII)**₁₃ to the respective product **D-(VIII)**₁₃ after 48 hours was quantified and is shown in table 6. For this analysis,
- 30 the column: Chiralpak 3μm ZWIX (+), 150 x 3 mm, DAICEL was used.

Table 6 gives the yield of L-(VIII)₁₃ relative to the compound L-(VII)₁₃ employed and the yield of D-(VIII)₁₃ relative to the compound D-(VII)₁₃ employed.

Table 6

5

Donor Strain	Gene name	SEQ ID NO:	Conversion of L-(VII) ₁₃ → L-(VIII) ₁₃ , in %	Conversion of D-(VII) ₁₃ → D-(VIII) ₁₃ , in %
<i>Arthrobacter</i> sp. DSM 3747	<i>hyuC</i>	SEQ ID NO: 1	86.4	n.d.
<i>Geobacillus stearothermophilus</i>	<i>amaB</i>	SEQ ID NO: 2	61.8	n.d.
<i>Pseudomonas</i> sp. QR-101	<i>atcC</i>	SEQ ID NO: 3	42.6	n.d.
<i>Paenarthrobacter aureus</i>	<i>hyuC</i>	SEQ ID NO: 5	77.6	n.d.
<i>Arthrobacter</i> sp. BT801	<i>hyuC</i>	SEQ ID NO: 8	36.2	n.d.

n.d. = "not detectable".

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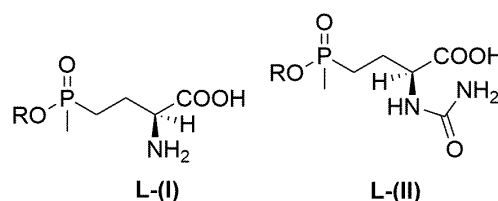
5.6.2 Conclusion 2

The results as summarized in table 6 are further evidence that certain carbamoylate esters such as the alkylated carbamoylate esters can be used as substrates for the enzymatic enantioselective synthesis of L-glufosinate. It further shows that these carbamoylases specifically catalyse the conversion of the L-glufosinate carbamoylate, but not the D-glufosinate carbamoylate.

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Claims

1. A method for the production of an L-glufosinate *P*-alkyl ester according to formula **L-(I)**,
 5 comprising a step (c) in which a compound according to formula **L-(II)** is reacted to give a compound according to formula **L-(I)**:



wherein the reaction according to step (c) is catalyzed by a carbamoylase **E₁**,
 and wherein R is an alkyl group or an aryl group.

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2. The method according to Claim 1, wherein R is an alkyl group.

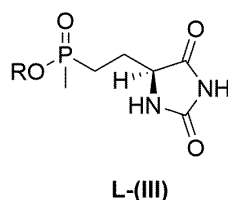
3. The method according to Claim 1 or 2, wherein the reaction according to step (c) is catalyzed by an L-carbamoylase **E₁**.

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4. The method according to Claim 3, wherein the L-carbamoylase **E₁** is categorized in the EC class 3.5.1.87.

5. The method according to Claim 3 or 4, wherein the polypeptide sequence of the L-carbamoylase **E₁** is selected from the group consisting of SEQ ID NO: 1 and variants thereof, SEQ ID NO: 2 and variants thereof, SEQ ID NO: 3 and variants thereof, SEQ ID NO: 4 and variants thereof, SEQ ID NO: 5 and variants thereof, SEQ ID NO: 6 and variants thereof, SEQ ID NO: 7 and variants thereof, SEQ ID NO: 8 and variants thereof, SEQ ID NO: 9 and variants thereof.

- 25 6. The method according to one of Claims 1 to 5, wherein the compound according to formula **L-(II)** is obtained by a step (b) in which a compound according to formula **L-(III)** is reacted to give a compound according to formula **L-(II)**:



wherein the reaction according to step (b) is catalyzed by a hydantoinase **E₂**,
 and wherein R has the same meaning as described for **L-(I)**.

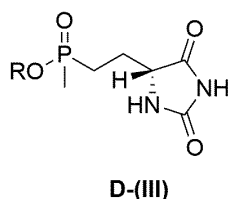
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7. The method according to Claim 6, wherein the hydantoinase E_2 is categorized in the EC class 3.5.2.2.

8. The method according to Claim 6 or 7, wherein the polypeptide sequence of the hydantoinase E_2 is selected from the group consisting of SEQ ID NO: 10 and variants thereof, SEQ ID NO: 11 and variants thereof, SEQ ID NO: 12 and variants thereof, SEQ ID NO: 13 and variants thereof, SEQ ID NO: 14 and variants thereof, SEQ ID NO: 15 and variants thereof, SEQ ID NO: 16 and variants thereof.

9. The method according to one of Claims 6 to 8, wherein the hydantoinase E_2 is an L-hydantoinase E_2 .

10. The method according to one of Claims 6 to 9, wherein the compound according to formula L-(III) is obtained by a step (a) in which a compound according to formula D-(III) is reacted to give a compound according to formula L-(III):



and wherein R has the same meaning as described for L-(I).

11. The method according to Claim 10, wherein the reaction according to step (a) is catalyzed by a hydantoin racemase E_3 .

12. The method according to Claim 11, wherein the hydantoin racemase E_3 is categorized in the EC class 5.1.99.5.

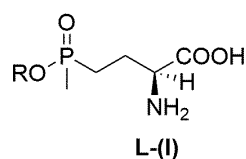
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13. The method according to Claim 11 or 12, wherein the polypeptide sequence of the hydantoin racemase E_3 is selected from the group consisting of SEQ ID NO: 17 and variants thereof, SEQ ID NO: 18 and variants thereof, SEQ ID NO: 19 and variants thereof, SEQ ID NO: 20 and variants thereof, SEQ ID NO: 21 and variants thereof, SEQ ID NO: 22 and variants thereof, SEQ ID NO: 23 and variants thereof, SEQ ID NO: 24 and variants thereof, SEQ ID NO: 25 and variants thereof, SEQ ID NO: 26 and variants thereof.

14. A method for the production of an L-glufosinate *P*-alkyl ester according to formula L-(I), wherein R is an alkyl group or aryl group:

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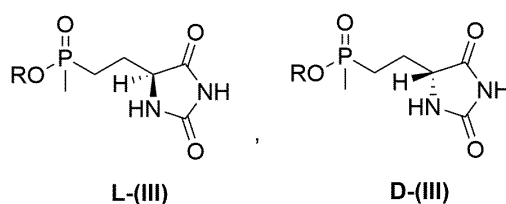
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comprising the following steps:

(i-A) providing a mixture **M_{III A}** comprising both enantiomers **L-(III)** and **D-(III)**, wherein R has the same meaning as described for **L-(I)**:

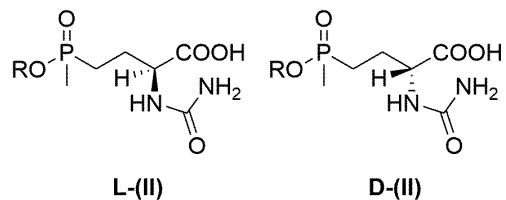
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(i-B) optionally reacting at least a part of the compounds **D-(III)** comprised by the mixture **M_{III A}** into **L-(III)** by a step (a) according to one of Claims 10 to 13, giving a composition **M_{III B}** comprising **L-(III)** and optionally its enantiomer **D-(III)**;

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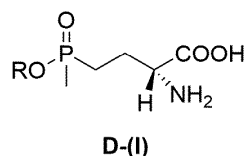
(ii) subjecting mixture **M_{III A}** or, in case step (i-B) is carried out, composition **M_{III B}**, to a reaction according to step (b) according to one of Claims 6 to 9, giving a composition **M_{II}** comprising **L-(II)** and optionally its enantiomer **D-(II)**, wherein **L-(II)** and **D-(II)** have the following formulae and wherein R in formulae **L-(II)** and **D-(II)** has the same meaning as described for **L-(I)**:



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(iii) subjecting **M_{II}** to a reaction according to step (c) according to Claims 3 to 5, giving a composition **M_I** comprising **L-(I)** and optionally its enantiomer **D-(I)**, wherein, in case **M_I** comprises both enantiomers **L-(I)** and **D-(I)**, the molar ratio of **L-(I)** to **D-(I)** in **M_I** is greater than the molar ratio of **L-(III)** to **D-(III)** in **M_{III A}**, wherein **D-(I)** has the following formula and wherein R in formula **D-(I)** has the same meaning as described for **L-(I)**:

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15. Method according to one of Claims 1 to 14, wherein the compound according to formula **L-(I)** is saponified to give L-glufosinate.