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(54) Title: MICROORGANISMS AND A METHOD FOR THE PRODUCTION OF LACTONES AND THEIR SECONDARY PRODUCTS BY CONVERTING CYCLOALKANES

(57) Abstract: The present invention relates to microorganisms or progeny thereof having biosynthetic capability to convert cycloalkanes and to produce lactones using complex composed of cytochrome P450 monooxygenase, two reductases ferredoxin (Fd) and ferredoxin reductase (FdR) together with alcohol (cyclohexanol) dehydrogenase and Bayer-Villager type (cyclohexanone) monooxygenase; all from Acidovorax CHX 100. Further, secondary products can be produced which are aliphatic monomers of large industrial polymers such as w- hydroxy fatty acid, dicarboxylic acids and w-amino acids (an analogue of lactams). The invention refers further to the biotechnological production of lactones and their secondary products using the microorganisms or progeny thereof.

Microorganisms and a method for the production of lactones and their secondary products by converting cycloalkanes

The present invention relates to microorganisms or progeny thereof having biosynthetic capability to convert cycloalkanes and to produce lactones and their secondary products which are aliphatic monomers of large industrial polymers such as ω-hydroxy fatty acid, dicarboxylic acids and ω-amino acids (an analogue of lactams). The invention refers further to the biotechnological production of lactones and their secondary products using the microorganisms or progeny thereof.

The cycloalkane such as cyclohexane is mainly used for the industrial production of cyclohexanone, ε-caprolactone, ε-caprolactam, and adipic acid e.g. precursors to nylon. Lactones and ω-hydroxy fatty acids form polyester building blocks whereas dicarboxylic acids and ω-amino acids are applied as polyamide building blocks yielding polymers such as Nylon 4,6; Nylon 4,10; Nylon 5; Nylon 5,10; Nylon 6; Nylon 6,4; Nylon 6,5; Nylon 6,6 and Nylon 6,10, and polyesters. Polyamides are a class of plastics extensively used as textiles and mechanical parts as they incorporate mechanical strength and durability with chemical resistance. The most commercial applied polyamides are PA-6 (Nylon 6), which is a homopolymer of 6-aminohexanoic acid, and PA-66 (Nylon 6,6) a co-polymer of adipic acid and 1,6-hexanediamine which accounts for more than 90 to 95% of the global market of polyamides.

Key-monomers for these are adipic acid and Ɛ-caprolactam. In 2012, the world-wide demand for adipic acid was approx. 2.3 million tons, mainly produced via nitric acid-driven oxidation of cyclohexanol or a mixture of cyclohexanol–cyclohexanone. This process generates significant amounts of nitrous oxide waste.

The most common synthetic route used to prepare Ɛ-caprolactam is based on the conversion of cyclohexanone to cyclohexanone oxime. This reaction typically occurs in the presence of hydroxylamine sulfate under pH buffered conditions at 85°C. Subsequently, the oxime is converted to ε-caprolactam by Beckmann rearrangement in the presence of fuming sulfuric acid, which leads to large amounts of salts as by-product.

Due to critical environmental issues connected to their production processes, there is a pressing demand for alternative production routes for plastics. Current efforts focus on the utilization of naturally occurring bioplastics as produced by species of *Ralstonia* and others. The utilization of

naturally occurring biomonomers, which are either further processed ex situ or are directly suitable for polymerization into bioplastics is another alternative route towards bioplastics. Very recently the first example of a completely fermentative approach to 6-aminocaproic acid produced from glucose was published and a process for the production of Polyamide 12 from plant-oil-derived lauric acid methyl ester has been developed (WO2009/077461A1).

Accordingly, it is an object of the present invention to find a simple and direct procedure for preparing lactones and aliphatic monomers thereof. In particular, it is an object of the present invention to provide organisms having biosynthetic capability to the biotechnological production of the lactones and/or ω-hydroxy fatty acid, dicarboxylic acids and/or ω-amino acids as their secondary products using readily available and cheap substrates.

These objects of the present invention are in general accomplished according to the claims.

According to the present invention metabolic pathways can be designed in engineered cells or microorganisms to obtain monomers for high-end polymers. The present invention is based, at least in part, on the discovery that lactones and also their secondary products can be directly produced from cycloalkanes. At present bioprocesses converting cycloalkanes are not known. Cycloalkanes (hydrocarbons with a ring-like structure) are readily available and cheap substrates.

The present invention provides microorganisms or progeny thereof synthesizing enzymes or enzymatic activities being able to produce lactones and further (when indicated) their secondary products by using cycloalkanes. The inventive microorganism or progeny thereof can convert especially C_5 to C_{11} - cycloalkanes, preferably cyclohexane.

In one embodiment of the present invention microorganisms or progeny thereof (as host cells) are provided which comprise a biosynthetic pathway to a cyclic alcohol compound and further enzyme activities that convert the cyclic alcohol compound resulting in the production of the corresponding lactones (e.g., ε-caprolactone, δ-valerolactone, 2-oxocanone, 2-oxonanone and oxacycloundecan-2-one, oxacyclododecan-2-one) by using the corresponding cycloalkanes as substrate. Furthermore the inventive microorganisms or progeny thereof can comprise nucleic acid sequences encoding an enzyme having an activity that converts the lactone and thereby results in the production of the corresponding ω-hydroxy fatty acid(s) (e.g., 6-hydroxyhexanoic

acid) and/or nucleic acid sequences encoding an enzyme having an activity that converts ω-hydroxy fatty acid(s) to dicarboxylic acid(s) (e.g., adipic acid).

Additionally the microorganisms or progeny thereof can comprise nucleic acid sequences encoding an enzyme having an activity that converts the lactone and thereby results in the production of the corresponding ω-amino acid(s) (e.g., ω-aminohexanoic acid).

The terms "enzyme" or “enzyme activity” encompass a single enzyme, mixtures comprising one or more enzymes, or enzyme complexes. The term “nucleic acid sequences” includes DNA (deoxyribonucleic acids) and RNA (ribonucleic acids) which are able to encode polypeptides having enzymatic activities. A "polypeptide" is a polymer comprising two or more amino acid residues (e.g. a peptide or a protein). The polymer can additionally comprise non-amino acid elements such as labels, quenchers, blocking groups, or the like and can optionally comprise modifications such as glycosylation or the like. The amino acid residues of the polypeptide can be natural or non-natural and can be unsubstituted, unmodified, substituted or modified. The skilled person is well aware of means and methods to determine whether a given polypeptide has the desired enzymatic activity or not and to determine the level of the enzymatic activity of a particular polypeptide or probe in absolute values and/or relative to another polypeptide or probe.

The term “progeny” denotes cells and microorganisms which result from proliferation of the microorganisms of the invention and which express the enzymatic pathways as defined in the claims. According to the invention the microorganism can be a prokaryotic organism, preferably a bacterial cell or algae, more preferably a bacterial cell of the genus *Acidovorax,* or *Pseudomonas,* or *E.coli*; the genus *Pseudomonas* is more preferred. Even more preferably microorganisms are *Acidovorax* CHX 100, *Pseudomonas taiwanensis* VLB120 or *P. taiwanensis* VLB120T7, *P.putida* KT2440, *P.putida* KT2440 (T7), *P. putida* GPo1, *P. fluorescens*, *P. putida* mt-2, *P. aeruginosa* PAO1, *P. putida* GPo12, *P. taiwanensis* VLB120ΔC, *P. taiwanensis* VLB 130, *P. taiwanensis* VLB 140, *P. taiwanensis* VLB 150, *P. taiwanensis* VLB 160, *P. putida* S12, *P. acidovorans*, *P. putida* PpS81, *P. putida* PpG1, *P. putida* 86, *P. kilonensis*, *P. monteilii*, *P. graminis*, *P. plecoglossicida*, *E. coli* JM101, *E. coli* JM109, *E. coli* JM110, *E. coli* W3110, *E. coli* BL21 (DE3)

P. taiwanensis VLB120 and *Acidovorax* CHX 100 are the most preferred organisms.

In one embodiment of the invention microorganisms or progeny thereof are represented which express the following enzymatic pathway:

- enzyme E1 catalyzing conversion of a cycloalkane to the corresponding cyclic alcohol;
- enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
- enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone
- enzyme E4 catalyzing conversion of said lactone to the corresponding ω-hydroxy fatty acid(s);
- enzyme E5 catalyzing conversion of said ω-hydroxy fatty acid(s) to ω--oxo fatty acid(s) and
- enzyme E6 catalyzing conversion of said ω-oxo fatty acid(s) to linear saturated dicarboxylic acid(s);

wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, E3, E4, E5, and/or E6.
In particular the inventive microorganism expresses E1, E2, E3, E4, E5 and E6.

In another embodiment of the invention microorganisms or progeny thereof are represented which express the following enzymatic pathway:

- enzyme E1 catalyzing conversion of a cycloalkane to the corresponding cyclic alcohol;
- enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
- enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone and
- enzyme E4 catalyzing conversion of said lactone to the corresponding ω-hydroxy fatty acid(s);
- enzyme E5 catalyzing conversion of said ω-hydroxy fatty acid(s) to ω--oxo fatty acid(s) and
- enzyme E7 catalyzing conversion of said ω-oxo fatty acid(s) to linear saturated ω-aminocarboxylic acid(s);

wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, E3, E4, E5, and/or E7.
In particular the inventive microorganism expresses E1, E2, E3, E4, E5 and E7.

In another embodiment of the invention microorganisms or progeny thereof are represented which express the following enzymatic pathway:

- enzyme E1 catalyzing conversion of a cycloalkane to the corresponding cyclic alcohol;
- enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
- enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone and
- enzyme E4 catalyzing conversion of said lactone to the corresponding ω-hydroxy fatty acid(s);

wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, E3, and/or E4.
In particular the inventive microorganism expresses E1, E2, E3 and E4.

In another embodiment of the invention microorganisms or progeny thereof are represented expressing the following enzymatic pathway:

- enzyme E1 catalyzing conversion of a cycloalkane to the corresponding cyclic alcohol;
- enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
- enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone;

wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, and/or E3.
In particular the inventive microorganism expresses E1, E2 and E3.

In another embodiment of the invention microorganisms or progeny thereof are represented expressing the following enzymatic pathway:

- enzyme E1 catalyzing conversion of a cycloalkane to the corresponding cyclic alcohol;
- enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;

wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1 and/or E2.
In particular the inventive microorganism expresses E1 and E2.

Preferred microorganisms or progeny thereof of the present invention are represented comprising nucleic acid sequences encoding cytochrome P450 monooxygenase activity as

enzyme E1 and nucleic acid sequences encoding an alcohol dehydrogenase activity as enzyme E2. Further the microorganism can comprise nucleic acid sequences encoding a Bayer-Villager type monooxygenase activity as enzyme E3 and/or nucleic acid sequences encoding a lactonase activity as enzyme E4. Furthermore, the present invention can provide microorganisms or progeny thereof comprising additionally nucleic acid sequences encoding ω-hydroxy fatty acid dehydrogenases E5 and/or ω-oxo fatty acid dehydrogenases E6. The microorganisms or progeny thereof can further comprise nucleic acid sequences encoding a ω-transaminase activity as enzyme E7.

A “cytochrome P450 monooxygenase (CYP)” as an enzyme E1 as used herein refers to an enzyme that uses a variety of small and large molecules as substrates in enzymatic reactions. They are, in general, the terminal oxidase enzymes in electron transport chains. CYP enzymes have been identified in all domains of life. Preferably the term “cytochrome P450 monooxygenase” encompasses the activity of a given polypeptide to catalyse a reaction with at least one cycloalkane as substrate.

An “alcohol dehydrogenase (ADH)” as an enzyme E2 as used herein refers to a group of dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between alcohols and aldehydes or ketones. Preferably the term “alcohol dehydrogenases” encompasses the activity of a given polypeptide to catalyse a reaction with at least one cyclic alcohol as substrate to the corresponding cyclic ketone.

A “Baeyer-Villiger monooxygenase (BVMO)” as an enzyme E3 as used herein refers to a biocatalyst that offer the prospect of high chemo-, regio-, and enantioselectivity in the organic synthesis of lactones or esters from a variety of ketones. Preferably the term “Baeyer-Villiger monooxygenase” encompasses the activity of a given polypeptide to catalyse a reaction with at least one cyclic ketone as substrate to the corresponding lactone(s).

A “lactonase” as an enzyme E4 as used herein refers to a metalloenzyme produced by certain species of bacteria, which targets and converts lactones. Preferably the term “lactonase” encompass the activity of a given polypeptide to catalyse a reaction with at least one lactone as substrate to the corresponding ω-hydroxy fatty acid(s).

An “omega-hydroxy fatty acid dehydrogenase” as an enzyme E5 as used herein refers to a group of dehydrogenase enzymes that occur in many organisms and convert linear hydroxy

fatty acids. Preferably the term “ω-hydroxy fatty acid dehydrogenases” encompasses the activity of a given polypeptide to catalyse a reaction with at least one ω-hydroxy fatty acid as substrate to the ω-oxo fatty acid.

An “omega-oxo fatty acid dehydrogenase” as an enzyme E6 as used herein refers to a group of dehydrogenase enzymes that occur in many organisms and convert linear ω-oxo fatty acids. Preferably the term “ω-oxo fatty acid dehydrogenases” encompasses the activity of a given polypeptide to catalyse a reaction with at least one ω-oxo fatty acid as substrate to the corresponding linear saturated dicarboxylic acid(s).

An “omega-transaminase” as an enzyme E7 as used herein refers to an enzyme capable of producing chiral amines. Preferably the term “ω-transaminase” encompasses the activity of a given polypeptide to catalyse a reaction with at least one cyclic lactone as substrate to the corresponding ω-aminocarboxylic acid(s). Furthermore a “ω-transaminase” E7 can catalyse a reaction of an ω-oxo-fatty acid to the corresponding linear saturated ω-aminocarboxylic acid.

In one embodiment of the invention the cytochrome P450 monooxygenase activity E1 is a cyclohexane cytochrome P450 monooxygenase system which comprises a cytochrome P450 (CYP) activity, a ferredoxin(Fd) activity and a ferredoxin reductase (FdR) activity. In particular, the enzyme E1 is a cyclohexane cytochrome P450 monooxygenase system derived from *Acidovorax* CHX100.

Most preferably the enzyme E1 is classified as a cyclohexane cytochrome P450 monooxygenase system comprising a cytochrome P450 protein (CHX) with an amino acid sequence of SEQ ID NO: 1 or amino acid sequences which share at least 85% sequence identity, a ferredoxin protein (Fd) with an amino acid sequence of SEQ ID NO: 2 or amino acid sequences which share at least 85% sequence identity and a ferredoxin reductase protein (FdR) with an amino acid sequence of SEQ ID NO: 3 or amino acid sequences which share at least 85% sequence identity.
Further amino acid sequences of SEQ ID NO 1, 2 and 3 can have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the respective above sequences SEQ ID NO 1 to 3 fulfilling the function of enzyme E1.

Enzyme E2 is preferably a cyclohexanol dehydrogenase (CDH) activity; more preferably enzyme E2 is a cyclohexanol dehydrogenase (CDH) activity derived from *Acidovorax* CHX 100. Most preferably the enzyme E2 is a cyclohexanol dehydrogenase protein (CDH) with an amino acid sequence of SEQ ID NO: 4 or amino acid sequences which share at least 85% sequence identity.
Further amino acid sequences E2 can have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the respective above sequence SEQ ID NO 4 and have the same function.

Enzyme E3 is preferably a cyclohexanone monooxygenase (CHXON). More preferably enzyme E3 is a cyclohexanone monooxygenase (CHXON) derived from *Acidovorax* CHX 100. Most preferably the enzyme E3 is a cyclohexanone monooxygenase protein (CHXON) with an amino acid sequence of SEQ ID NO: 5 or amino acid sequences which share at least 85% sequence identity.
Further amino acid sequences E3 can have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the respective above sequences SEQ ID NO 5 and have the same function.

Enzyme E4 is preferably a lactonase. More preferably enzyme E4 is an endogenous lactonase or a 1-oxa-2-oxo-cycloheptane lactonase derived from *Acidovorax* CHX 100. Most preferably the enzyme E4 is a lipase or lactonase protein with an amino acid sequence of SEQ ID NO: 6 or amino acid sequences which share at least 85% sequence identity.
Further amino acid sequences E4 can have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the respective above sequences SEQ ID NO 6 and have the same function.

Enzyme E5 is preferably a dehydrogenase. More preferably enzyme E5 is an endogenous dehydrogenase or a 6-hydroxy-hexanoic acid dehydrogenase derived from *Acidovorax* CHX

100. Most preferably the enzyme E5 is a dehydrogenase protein with an amino acid sequence of SEQ ID NO: 7 or amino acid sequences which share at least 85% sequence identity.
Further amino acid sequences E5 can have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the respective above sequences SEQ ID NO: 7 and have the same function.

Enzyme E6 is preferably a dehydrogenase. More preferably enzyme E6 is an endogenous dehydrogenase or a 6-oxo-hexanoic acid dehydrogenase derived from *Acidovorax* CHX 100. Most preferably the enzyme E6 is a dehydrogenase protein with an amino acid sequence of SEQ ID NO: 8 or amino acid sequences which share at least 85% sequence identity.
Further amino acid sequences E6 can have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the respective above sequences SEQ ID NO: 8 and have the same function.

An ω-transaminase derived from *Chromobacterium violaceum* is the preferred enzyme E7; more preferably the enzyme E7 is a ω-transaminase with an amino acid sequence of SEQ ID NO: 9 or amino acid sequences which share at least 85% sequence identity.
Further amino acid sequences E7 can have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the respective above sequences SEQ ID NO 9 and have the same function.

That means the invention is not limited to the above exemplified enzymes. Nucleic acid sequences encoding the enzymes having the above described capacity would be routine to screen and these sequences from similar organisms or unrelated organisms as described above may be used in the methods of the invention.

In some embodiments, the enzyme will be a wild-type enzyme and in other embodiments it will be a mutant or an engineered variant of the wild-type enzyme. In the embodiments, the mutant or engineered variants of the wild-type enzymes SEQ ID NO 1 to SEQ ID NO 9 and corresponding polynucleotides encoding such engineered enzymes can be obtained using

methods used by those skilled in the art. The engineered enzymes described herein can be obtained by subjecting the naturally occurring polynucleotide encoding the naturally occurring enzymes to mutagenesis and/or directed evolution methods.

A preferred microorganism of the present invention according to the disclosure will be engineered to comprise the following nucleic acid sequences:

Nucleic acid sequences for a cytochrome P450 enzyme system (CHX) comprising the monooxygenase (CYP) and the two reductases (Fd and FdR) (Genbank Accession No.: kb686056.1), namely:
SEQ ID NO: 18 – encoding the monooxygenase (CYP) of SEQ ID NO: 1. Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 18 and possess the same properties.
SEQ ID NO: 19 – encoding the ferredoxin protein (Fd) of SEQ ID NO: 2 which is part of the electron-transfer chain necessary for cytochrome P450 monooxygenase activity. Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 19 and possess the same properties.
SEQ ID NO: 20 – encoding the ferredoxin reductase protein (FdR) of SEQ ID NO: 3 which is part of the electron-transfer chain necessary for cytochrome P450 monooxygenase activity.
Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 20 and possess the same properties.

SEQ ID NO: 21 - encoding the cyclohexanol dehydrogenase (CDH) of SEQ ID NO: 4. Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 21 and possess the same properties.

SEQ ID NO: 22 encoding the cyclohexane monooxygenase (CHXON) of SEQ ID NO: 5. Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 22 and possess the same properties.

SEQ ID NO: 23 – encoding the lactonase of SEQ ID NO: 6. Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 23 and possess the same properties.

SEQ ID NO: 24 - encoding the 6-hydroxy-hexanoic acid dehydrogenase of SEQ ID NO: 7. Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 24 and possess the same properties.

SEQ ID NO: 25 - encoding the 6-oxo-hexanoic acid dehydrogenase of SEQ ID NO: 8. Further nucleic acid sequences can be comprised having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 25 and possess the same properties.

Optionally SEQ ID NO: 26 – encoding the ω-transaminase of SEQ ID NO: 9. Further nucleic acid sequences can be used having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above SEQ ID NO: 26 and possess the same properties.

A nucleic acid sequence (polynucleotide) or polypeptide has a certain percent "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same, and in the same relative position, when comparing the

two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the world wide web at ncbi.nlm.nih.gov/BLAST. See, e.g., Altschul et al. (1990), J. Mol. Biol. 215:403-10. Another alignment algorithm is FASTA, available in the Genetics Computing Group (GCG) package, from Madison, Wisconsin, USA, a wholly owned subsidiary of Oxford Molecular Group, Inc. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, California, USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. See J. Mol. Biol. 48: 443-453 (1970).

The term “polynucleotide sequence” or “nucleic acid sequence” or “gene” used interchangeably herein is said to "encode" a sense or antisense RNA molecule, or RNA silencing or interference molecule or a polypeptide, if the polynucleotide sequence can be transcribed (in spliced or unspliced form) and/or translated into the RNA or polypeptide, or a subsequence thereof. The skilled person is well aware of the degeneracy of the genetic code, allowing for a number of different nucleic acid sequences encoding for the same amino acid sequence or polypeptide and has no difficulties in determining whether a given nucleic acid sequence encodes for a given amino acid sequence or polypeptide.

In some embodiments, a preferred microorganism or a progeny thereof of the present invention can be one that has been genetically modified with one or more heterologous nucleic acid sequences encoding a CYP450 monooxygenase system E1, an alcohol dehydrogenase E2, a Bayer-Villager type monooxygenase E3, a lactonase E4, an ω-hydroxy fatty acid dehydrogenase E5, an ω-oxo fatty acid dehydrogenase E6 and/or also an ω-transaminase E7.

An example of a microorganism of the invention is a microorganism of the genus *Pseudomonas* that has been genetically modified with a construct comprising heterologous nucleic acid sequences as set forth in SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21 and SEQ ID NO: 22 and optionally SEQ ID NO: 23 and/or SEQ ID NO: 24 and/or SEQ ID NO: 25. Such a microorganism of the genus *Pseudomonas* can produce the intrinsic enzymes lactonase

E4 of SEQ ID NO: 6, ω-hydroxy fatty acid dehydrogenase E5 of SEQ ID NO: 7 and/or ω-oxo fatty acid dehydrogenase E6 of SEQ ID NO: 8. Such an inventive microorganism is preferably capable of producing ε-caprolactone, 6-hydroxyhexanoic acid and adipic acid.

A further example of a microorganism of the present invention is a microorganism of the genus *Pseudomonas* that has been genetically modified with a construct comprising heterologous nucleic acid sequences as set forth in 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 and SEQ ID NO: 26. Such an inventive microorganism is preferably capable of producing ε-caprolactone, 6-hydroxyhexanoic acid and ω-aminohexanoic acid.

A further example of a microorganism of the present invention is a microorganism of the genus *Pseudomonas* that has been genetically modified with a construct comprising heterologous nucleic acid sequences as set forth in SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23. Such an inventive microorganism is preferably capable of producing ε-caprolactone, and 6-hydroxyhexanoic acid.

A further example of a microorganism of the present invention is a microorganism of the genus *Pseudomonas* that has been genetically modified with a construct comprising heterologous nucleic acid sequences as set forth in SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21 and SEQ ID NO: 22. Such an inventive microorganism is preferably capable of producing ε-caprolactone.

An additional example of a microorganism of the present invention is a microorganism of the genus *Pseudomonas* that has been genetically modified with a construct comprising heterologous nucleic acid sequences as set forth in SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, and SEQ ID NO: 21. Such an inventive microorganism is preferably capable of producing cyclohexanone.

More preferred is a microorganism of *Pseudomonas taiwanensis* VLB120. A further preferred microorganism of the invention can be genetically engineered Acidovorax CHX100.

The term "heterologous nucleic acid," as used herein, refers to a nucleic acid wherein at least one of the following is true: (a) the nucleic acid is foreign ("exogenous") to (i.e., not naturally found in) a given host microorganism; (b) the nucleic acid comprises two or more nucleotide

sequences that are not found in the same relationship to each other in nature, e.g., the nucleic acid is recombinant. An example of a heterologous nucleic acid is a nucleic acid encoding CYP, where a host microorganism that does not normally produce CYP is genetically modified with the nucleic acid encoding CYP; because CYP-encoding nucleic acids are not naturally found in the host microorganism, the nucleic acid is heterologous to the genetically modified microorganism.

"Recombinant," as used herein, means that a particular nucleic acid (DNA or RNA) is the product of various combinations of cloning, restriction, and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems. Generally, DNA sequences encoding the structural coding sequence can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of synthetic oligonucleotides, to provide a synthetic nucleic acid which is capable of being expressed from a recombinant transcriptional unit contained in a cell or in a cell-free transcription and translation system. Such sequences can be provided in the form of an open reading frame uninterrupted by internal non-translated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA comprising the relevant sequences can also be used in the formation of a recombinant gene or transcriptional unit. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions, and may indeed act to modulate production of a desired product by various mechanisms (see "DNA regulatory sequences", below).

Thus, e.g., the term "recombinant" polynucleotide or nucleic acid refers to one which is not naturally occurring, e.g., is made by the artificial combination of two otherwise separated segments of sequence through human intervention. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

By "construct" is meant a recombinant nucleic acid, generally recombinant DNA, which has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences.

In a preferred embodiment the invented technology provides especially a biocatalytic pathway for the production of ε-caprolactone, 6-hydroxyhexanoic acid and adipic acid and optionally ω-aminohexanoic acid by using a microorganism of *Pseudomonas taiwanensis* VLB120. This microorganism of the present invention comprises heterologous enzymes from *Acidovorax* CHX100 coupled to its intrinsic occurring enzymes. These respective heterologous enzymes include the cyclohexane cytochrome P450 monooxygenase (CHX) E1 from *Acidovorax* CHX 100, the cyclohexanol dehydrogenase (CDH) E2 from *Acidovorax* CHX100 and the cyclohexanone monooxygenase (CHXON) E3 from *Acidovorax* CHX100.

Furthermore the heterologous enzymes of *Pseudomonas taiwanensis* VLB120 can optionally comprise the ω-transaminase E7 of *Chromobacterium violaceum.*

The method to produce the inventive microorganisms is characterized by transformation of the microorganism with at least one of an expression cassette of above and/or a vector of above. The methods are known to the skilled person. To generate a subject genetically modified microorganism, one or more nucleic acid sequences encoding an desired enzyme(s) is introduced stably or transiently into the microorganism, using established techniques, including, but not limited to, electroporation, calcium phosphate precipitation, DEAE-dextran mediated transfection, liposome-mediated transfection, and the like. For stable transformation, a nucleic acid will generally further include a selectable marker, e.g., any of several well-known selectable markers such as neomycin resistance, ampicillin resistance, tetracycline resistance, chloramphenicol resistance, kanamycin resistance, and the like.

The term "transformation" refers to a permanent or transient genetic change induced in a cell of the microorganism following introduction of new nucleic acid (i.e., DNA exogenous to the microorganism cell). Genetic change ("modification") can be accomplished either by incorporation of the new DNA into the genome of the microorganism, or by transient or stable maintenance of the new DNA as an episomal element. In prokaryotic cells, permanent changes can be introduced into the chromosome or via extrachromosomal elements such as plasmids and expression vectors, which may contain one or more selectable markers to aid in their maintenance in the recombinant host microorganism cell of invention.

A nucleic acid is hybridizable to another nucleic acid, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid can anneal to the other nucleic acid under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein; and Sambrook, J. and Russell, W., Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001).

Therefore the present invention provides also an expression cassette for the manufacture of a microorganism of the invention, wherein the expression cassette comprises the nucleic acid sequences encoding the desired enzymes.

For examples, the expression cassette can comprise nucleic acid sequences encoding at least two of enzymes E1, E2, E3, E4, E5, and E6. Preferably comprises the expression cassette nucleic acid sequences encoding at least three of the enzymes E1, E2, E3, E4, E5, and E6; more preferably the enzymes E1, E2 and E3. Especially the expression cassette can comprise nucleic acid sequences encoding enzyme E1 with amino acid sequences of SEQ ID NO: 1 and 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, enzyme E4 with an amino acid sequence of SEQ ID NO: 6, enzyme E5 with an amino acid sequence of SEQ ID NO: 7, and enzyme E6 with an amino acid sequence of SEQ ID NO: 8.

In particular, an inventive expression cassette can comprises a nucleic acid sequence encoding at least two of enzymes E1, E2, E3, and E4; preferably at least three of the enzymes E1, E2, E3, and E4; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, enzyme E4 with an amino acid sequence of SEQ ID NO: 6.

Furthermore an expression cassette for the manufacture of an inventive microorganism can comprise a nucleic acid sequence encoding at least two of enzymes E1, E2, and E3; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5.

In another embodiment an expression cassette for the manufacture of an inventive microorganism can comprise a nucleic acid sequence encoding at least two of enzymes E1 and E2; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4.

Furthermore, in another embodiment of the present invention an expression cassette for the manufacture of a microorganism of invention is provided, wherein the expression cassette comprises a nucleic acid sequence encoding at least two of enzymes E1, E2, E3, E4, E5, and E7; preferably at least three of the enzymes E1, E2, E3, E4, E5, and E7, preferably enzymes E1, E2 and E3. Especially preferred comprises the expression cassette nucleic acid sequences encoding enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, enzyme E4 with an amino acid sequence of SEQ ID NO: 6, enzyme E5 with an amino acid sequence of SEQ ID NO: 7, and enzyme E7 with an amino acid sequence of SEQ ID NO: 9 **or** nucleic acid sequences encoding enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, and enzyme E7 with an amino acid sequence of SEQ ID NO: 9.

Subject matter of the invention is also a vector comprising an above described expression cassette.

An "expression cassette" is a linear or cyclic, single or double stranded nucleic acid construct, e.g. as a part of a vector, such as a plasmid, a viral vector, etc., capable of producing transcripts and, potentially, polypeptides encoded by a polynucleotide sequence. An expression cassette is capable of producing transcripts in an exogenous cell, e.g. a bacterial cell, *in vivo* or *in vitro*. Expression of a product can be either constitutive or inducer dependent, e.g. on the promoter selected. Antisense, sense or RNA interference or silencing configurations that are not or cannot be translated are expressly included by this definition. In the context of an expression cassette, a promoter is said to be "operably linked" or “functionally linked” to a polynucleotide sequence if it is capable of regulating expression of the associated polynucleotide sequence. The term also applies to alternative exogenous gene constructs, such as expressed or integrated transgenes. Similarly, the term operably or functionally linked applies equally to

alternative or additional transcriptional regulatory sequences such as enhancers, associated with a polynucleotide sequence.

The term "vector" refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophage, pro-viruses, phagemids, transposons, and artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome- conjugated DNA, or the like, that are not autonomously replicating.

"Expression" means transcription of DNA into RNA (optionally including modification of the RNA, e.g. splicing), translation of RNA into a polypeptide (possibly including subsequent modification of the polypeptide, e.g. posttranslational modification), or both transcription and translation, as indicated by the context.

In a further aspect, the disclosure relates to a method for making lactones and/or their secondary products comprising providing a microorganism as set forth above and transformed with nucleic acid sequences encoding the enzymes E1, E2, E3, E4, E5, E6 and/or E7. The microorganism is cultured under sufficient culture conditions in the presence of a carbon source to promote the gene expression of the enzymes and the production of lactones and/or their secondary products like hydroxylic acids, ω-oxo acids, and/or linear saturated dicarboxylic acids and/or ω-aminocarboxylic acid in the presence of a cycloalkane.

The method for the production of lactones and/or their secondary products from cyclic alkanes comprises the step of:

- contacting a microorganism of above with a cycloalkane, preferably with a C_5 to C_{11} -cycloalkane;
- culturing the mixture of microorganism and cycloalkane; and
- extracting corresponding lactones and/or hydroxylic acids, ω-oxo acids and/or linear saturated dicarboxylic acids and/or ω-aminocarboxylic acid from the culture product.

Preferably the culturing is performed in a medium and under conditions appropriate to allow for growth of said microorganism; preferably the culture of the mixture of microorganism and cycloalkane is performed in a culture medium containing the necessary growth nutrients and the cycloalkane either in solution or as an organic liquid phase or as an organic gaseous phase, which enable the cellular catalyst to form lactones, ω-hydroxy fatty acid ω-oxo acids, and linear saturated dicarboxylic acids and optionally ω-aminocarboxylic acid.

In particular a cell stock of a microorganism of the present invention was taken and incubated. The biotransformation was initiated by adding respective cycloalkanes. For gas-chromatography analysis, the reaction was stopped for example by adding of ice cold diethyl ether containing decane as internal standard to an equal amount of the reaction mixture phase. Both phases were mixed on a vortex for product extraction and then centrifuged to separate phases. The ether phase was dried over anhydrous Na_2SO_4 and analyzed by gas chromatography.

High pressure liquid chromatography (HPLC) analysis was used to determine ω-hydroxy fatty acid, ω-oxo acids, and linear saturated dicarboxylic acids, the samples are centrifuged at room temperature. Supernatant was used for HPLC analysis while cell pellets were discarded.

The reaction is preferably transforming C_5 to C_{11} - cycloalkanes as the substrate using one whole cell of the microorganism of invention especially in aqueous medium. This single cell reactions together with the increased kinetics of the enzymes and can occur at ambient conditions utilizing molecular oxygen (e.g. from air) as oxidant, without dangerous chemicals, and significantly reduces waste and emissions.

The culture of the mixture of microorganism and cycloalkane can be performed in a bioreactor, in a stirred tank, or in a capillary micro-reactor. Stirred tank is the most preferred reactor format. The microorganism can be provided as immobilized biofilm. Biofilms offers several advantages compared to their suspended counterpart as they present a self-immobilizing, self-regenerating biocatalyst format. In addition they are resistant against many otherwise toxic chemical substances and can be operated for long term with activities ranging from days to months

In particular a method for producing ε-caprolactone, 6-hydroxyhexanoic acid and adipic acid via an enzymatic pathway in *Pseudomonas taiwanensis* VLB120 is provided.
The method comprises:

(i) culturing in a suitable medium and in presence of cyclohexane a *Pseudomonas taiwanensis* VLB120 cell genetically modified to produce heterologous cytochrome P450 enzyme system (CHX) comprising the monooxygenase (CYP) and the two reductases (Fd and FdR), cyclohexane monooxygenase (CHXON) and cyclohexanol dehydrogenase, and wherein the genetically modified *Pseudomonas taiwanensis* VLB120 cell is further genetically modified or able to produce one or more additional enzymes like e.g.: a lactonase being 1-oxa-2-oxo-cycloheptane lactonase or others having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% sequence identity to the above and possess the same properties

(ii) recovering the produced ε-caprolactone, 6-hydroxyhexanoic acid and adipic acid.

The preferred inventive microorganisms produce ε-caprolactone, 6-hydroxyhexanoic acid, adipic acid and/or ω-aminohexanoic acid by using cyclohexane as cycloalkane in good amounts.

Summing up, the current invention provides a method, which includes microorganism culturing, expression of exogenous nucleic acids encoding the enzymes E1, E2, E3, E4, E5, E6 and/or E7 in a sufficient amount to produce respective products such as lactones, ω-hydroxy fatty acid, and linear saturated dicarboxylic or ω-aminocarboxylic acid from corresponding cyclic alkanes, and reaction conditions for biotransformations yielding the desired products.

In particular this biocatalytic transformation of the preferred used cyclohexane catalyzed by the inventive catalyst allows the synthesis of the three key products caprolactone, 6-hydroxyhexanoic acid and adipic acid as central monomers for large polymers by using the inventive microorganism *Pseudomonas taiwanensis* VLB120 comprising the heterologous enzymes cyclohexane cytochrome P450 monooxygenase (CHX) E1, the cyclohexanol dehydrogenase (CDH) E2 and the cyclohexanone monooxygenase (CHXON) E3 from *Acidovorax* CHX100.
Furthermore, the introduction of the heterologous enzyme ω-transaminase E7 from *Chromobacterium violaceum* in *Pseudomonas taiwanensis* VLB120T7 results in ω-aminohexanoic acid as product in addition to caprolactone.

Examples

Figure 1: Construction of plasmid pCom10_capro encoding the respective recombinant genes from *Acidovorax* CHX100: CypP450 enzyme system (CHX): Cytochrome P450 monooxygenase (CYP) with the corresponding Fd:Ferredoxin FdR: NADH:Ferredoxin reductase, CHXON: Bayer-Villiger type cyclohexanone monooxygenase, and CDH: cyclohexanol dehydrogenase

Figure 2: Synthesis of the recombinant enzymes encoded by pCom10_capro in *P. taiwanensis* VLB120 visualized via SDS-PAGE. Respective enzymes are marked with the arrows. CHXON: cyclohexanone monooxygenase; CYP: Cytochrome P450 monooxygenase; CDH: cyclohexanol dehydrogenase.

Figure 3: Product profile of the three main products ε-caprolactone, adipic acid, and 6-hydroxyhexanoic acid in a resting cell assay.

Figure 4: GC data for biotransformation of cyclopentane, cyclohexane, cycloheptane and cyclooctane to δ-Valerolactone, ε-caprolactone, oxocan-2-one and oxonan-2-one, respectively.

Figure 5: GC data for biotransformation of cyclodecane to oxacycloundecan-2-one.

Figure 6: LC-MS data of adipic acid and 6-hydrohexanoic acid formation from cyclohexane biotransformation.

Figure 7: GC-MS data of δ-Valerolactone formation from cyclopentane biotransformation.

Figure 8: GC-MS data of epsilon-caprolactone formation from cyclohexane biotransformation.

Figure 9: GC-MS data of oxocan-2-one formation from cycloheptane biotransformation.

Figure 10: GC-MS data of oxonan-2-one formation from cyclooctane biotransformation.

Figure 11: GC-MS data of oxacycloundecan-2-one formation from cyclodecane biotransformation.

Figure 12: GC-MS data of oxacyclododecan-2-one formation from cycloundecane biotransformation.

Example 1

a) Construction of plasmid pCom10_capro encoding the respective recombinant genes from *Acidovorax* CHX100

Cloning strategy: The construction of pCom10_capro plasmid, the genes coding for the Cytochrome P450 enzyme complex (*chx*), cyclohexanol dehydrogenase enzyme (*cdh*) and cyclohexanone monooxygenase (*chxon*) was conducted in several steps. In the first step, pCom10_CHX plasmid was constructed by amplifying cytochrome P450 monooxygenase (CYP)

and the two reductases (Fd and FdR) genes from the genomic DNA of *Acidovorax* CHX100 by polymerase chain reaction PCR (Sambrook and Russel 2001) using Phusion high fidelity polymerase (Life Technologies GmbH, Darmstadt, Germany) and the primers (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) given in Table 1. The resulting PCR products were cloned into the pCom10 backbone via restriction sites NdeI and AscI, respectively, yielding plasmid pCom10_chx. The product was introduced into *E.coli* DH5α or *P. taiwanensis* VLB120 and after overnight growth on LB agar plates containing kanamycin, the desired plasmid was isolated from obtained transformants. The verification of the correct insertion of the genes was performed with different restriction enzymes and PCR amplification of the insert.

In the second step, cyclohexanol dehydrogenase (*cd*h) and cyclohexanone monooxygenase (*chxon*) have been coupled on plasmid pCom10_chxon_cdh. Respective genes have been amplified from the genomic DNA of Acidovorax CHX100 by standard polymerase chain reaction PCR (Sambrook and Russel 2001). Primers have been are listed in Table 1. Subsequently the resulting PCR products were cloned into the pCom10 backbone via restriction sites AscII/HindIII and NdeI/AscI, respectively, yielding plasmid pCom10_chxon_cdh. This was introduced into P. taiwanensis VLB120 and after overnight growth on LB agar plates containing kanamycin, pCom10_chxon_cdh was isolated from the obtained transformants. Correct insertion of the genes was verified using restriction digest analysis and PCR. To couple *chxon_cdh* to the existing pCom10_CHX restriction sites needed to be adapted. This was accomplished via PCR using degenerated primers carrying the respective sequences (Table 1). Finally, *chxon_cdh* encoding the dehydrogenase and the BVMO were coupled to *chx* located on pCom10_CHX using standard cloning procedures. The resulting plasmid pCom10_Capro was introduced into *P. taiwanensis* VLB120 and after overnight growth on LB agar plates containing kanamycin, the desired plasmid was isolated from obtained transformants. Correct insertion of the genes was verified using restriction digest analysis and PCR. The plasmid map is given in the supporting information.

The gene sequence for Cytochrome P450 enzyme system (CHX) comprising the monooxygenase (CYP) and the two reductases (Fd and FdR) is represented by SEQ ID NO 18, 19 and 20. The gene sequence for cyclohexanol dehydrogenase (CDH) is represented by SEQ ID NO: 21. The gene sequence for cyclohexane monooxygenase (CHXON) is represented by SEQ ID NO: 22.

Table 1: Primers used for cloning respective genes

CHX forward	SEQ ID NO: 10… 5'- AGCGGCATATGACTCAGACTGCTGCG-3'	NdeI
CHX reverse	SEQ ID NO: 11 … 5'- AAAAAAAGGCGCGCCTCAGTGCTGCCCTTGCG-3'	AscI
CDH forward	SEQ ID NO: 12 … 5'- AACACGGCGCGCCGGAGAATTCGATATGAAACGCGTAGAAAACAAAGTG-3'	AscI
CDH reverse	SEQ ID NO: 13 … 5'- AACAGAAGCTTTCAATTGGCCGTGTAGCCGCCATC-3'	HindIII
CHXON forward	SEQ ID NO: 14… 5'- GCCGCGCATATGAAAAAAACCCAACATCTG-3'	NdeI
CHXON reverse	SEQ ID NO:15 … 5'- GAACAAGGCGCGCCTTACTGGAATACGAAACCCTC-3'	AscI
CHXON_CDH forward	SEQ ID NO: 16… 5'- GAGACAAGCTTTGGCATGCTTGCGGCCAG-3'	HindIII
CHXON_CDH reverse	SEQ ID NO:17 … 5'- CGGACGGAAAATCTTCTCAATTGGCCGTGTAGC-3'	XmnI

Microbial strains, plasmids and related cultivation and transformation methods: All organisms used have been cultivated in Luria-Bertani (LB) medium (DSMZ_medium 381) or M9*-medium (DSMZ_medium 382) supplemented with 0.5% (w/v) citrate as a carbon source, US* trace elements (Emmerling et al. 2002, J. Bacteriol. 184:152), and kanamycin 50 µg mL^{-1}. Pre-cultures were grown overnight in 5-10 mL LB-medium or M9*-medium in a horizontal shaker (30°C and 200 rpm, Multitron, Infors HT, Bottmingen, Switzerland).
Figure 1 shows the construction of plasmid pCom10_capro.

b) Transformation of the plasmid into *P. taiwanensis* VLB120
The plasmid was introduced into *P. taiwanensis* VLB120 using electroporation (2500 V, Equibio EasyjecT Prima, Kent, UK). All transformation methods were performed as described in Sambrook and Russell, 2001.
Biotransformation of cycloalkanes: Cells were grown on M9* mineral media containing 0.5% (w/v) citrate to an OD_{450} of 0.9 to 1.0 and then induced by the addition of (0,025% v/v)

dicyclopropylketone (DCPK). During growth, cells were sampled after each hour for SDS PAGE. After 5-6 h of induction, cells were harvested by centrifugation (Thermo Electron Corporation, Langenselbold, Germany) at 20°C, 4595 g, 15 min, washed and re-suspended in 100 mM of Kpi buffer pH 7.4 supplemented with 1% of citrate to a cell concentration of 0.5-2.0 g_{CDW} L^{-1}. This cell stock (working volume mentioned wherever necessary) was distributed in baffled Erlenmeyer flasks with the screw caps and incubated in a rotary shaker at 30°C and 300 rpm for 10 min. The biotransformation was initiated by adding cycloalkanes (5 to 10 mM) to the respective flasks.

For gas-chromatography analysis, the reaction was stopped by adding 1 mL of ice cold diethyl ether containing 0.2 mM decane as internal standard to an equal amount of the reaction mixture phase. Both phases were mixed for 2 min on a vortex for product extraction and then centrifuged (13000 rpm; 2 min) to separate phases. The ether phase was dried over anhydrous Na2SO4 and analyzed by gas chromatography.

For high pressure liquid chromatography (HPLC) analysis, the samples were centrifuged for 10 min at 13000 g, and room temperature. Supernatant was used for HPLC analysis while cell pellets were discarded. Specific activities were calculated as U g_{CDW}^{-1}, where 1 U is equal to 1 µmol cyclohexanol produced min^{-1}.

Analytical methods: Proteins were detected using SDS-PAGE according to (Laemmli 1970, *Nature* **227**, 680 - 685) loading 10 µg of total protein per lane. Cycloalkanes concentrations were measured by GC (Trace 1310, Thermo Scientific, Waltham, USA) equipped with a TG-5MS GC Column (Thermo Scientific, Waltham, USA) and a flame ionization detector with molecular nitrogen as carrier gas and 1 µl in splittless injection mode. The temperature profile setting was as follows:

Retention time [min]	**Heating rate [°C/min]**	**Target value [°C]**	**Hold time [min]**
3	0	40	3
11.67	15	170	0
17.97	100	300	5

The detection was based using flame ionization detector (FID). GC-MS analysis were performed using by ISQ™ LT Single Quadrupol GC-MS-System (Thermo Scientific, Waltham, USA)

equipped with a TG-5MS GC Column (30m×0.25mm×0.25µm) (Thermo Scientific, Waltham, USA). The temperature profile is as described above.

6-hydroxyhexanoic acid, 8-hydroxyoctanoic acid, 10-hydroxydecanoic acid were determined by HPLC Dionex Ultimate 3000 (Thermo Scientific, Waltham, USA) equipped with Acclaim® OA column (Thermo Scientific, Waltham, USA). Mobile phase A consisted of 10 mM sodium sulfate containing 0.1% (v/v) formic acid. Acetonitrile was used as mobile phase B. 10-20 µL of sample was injected, while flow and column temperature was kept constant at 0.34 mL/min and 30°C, respectively. Detection was accomplished by using a UV spectrometer. Flow profile was: 5%B for 3 min, 5 to 80%B in 17 min, 80-5%B in 5 min and 5%B for 5 min.

For HPLC-MS analysis, a Agilent 1260 G1312B (Agilent Technologies,Waldbronn, Germany) equipped with a Acclaim Organic acid (OA) column (Thermo Scientific) and Agilent 1260 G1367E autosampler was used. The oven temperature was set to 30°C and the flow rate to 300 µL/min. The mobile phase consisted of water 0.1 mM formic acid (A), and acetonitril 0.1 mM formic acid (B), all solvents from Biosolve with UPLC grade. Following flow profile was applied for measurements:

Step	**Total Time (min)**	**Flow Rate (µl/min)**	**A (%)**	**B (%)**
0	0.01	300	97.5	2.5
1	5.00	300	97.5	2.5
2	10.00	300	10.0	90.0
3	14.00	300	97.5	2.5
4	16.00	300	97.5	2.5

For mass spectrometer analysis, Mass spectrometer QTRAP 5500 (Sciex, Toronto, CAN), source temperature 450°C and by using positive electrospray ionization +5 kV.
Ion transitions (m/z):

Adipic acid:	145 – 101	CE=-12 V
	145 – 83	CE= -18V
	145 – 81	CE=-25 V
6-hydroxyhexanoic acid:	131 – 85	CE= -18 V
	131 – 87	CE= -18 V
	131 – 45	CE = -30 V

Figure 2: shows the synthesis of the recombinant enzymes encoded by pCom10_capro in *P. taiwanensis* VLB120 visualized via SDS-PAGE.

c) Production of the three main products caprolactone, adipic acid, and 6-hydroxyhexanoic acid in a resting cell assay by using cyclohexane

Assay condition: 20 mL in 300 mL shake flask, substrate 5 mM, 30°C at 200 rpm, *P. taiwanensis* VLB120 p10_capro: 1.22 $g_{CDW}L^{-1}$

Figure 3 shows the product profile. Average product concentrations of 0.5 to 0.6 g/L for caprolactone (0.1 g/L/h), 0.2 to 0.25 g/L for 6-hydroxyhexanoic acid (0.01 g/L/h), 0.02 g/L adipic acid were achieved.

CLAIMS:

1. Microorganism or progeny thereof expressing genes and synthesizing enzymes which are able to produce lactones and/or their secondary products by converting cycloalkanes.

2. Microorganism or progeny thereof according to claim 1 expressing the following enzymatic pathway:
 enzyme E1 catalyzing conversion of a cycloalkane, preferably a C5 to C11 cycloalkane, to the corresponding cyclic alcohol;
- enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
- enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone;
- enzyme E4 catalyzing conversion of said lactone to ω-hydroxy fatty acid(s);
- enzyme E5 catalyzing conversion of said ω-hydroxy fatty acid(s) to ω-oxo fatty acid(s ;
- enzyme E6 catalyzing conversion of said ω-oxo fatty acid(s) to linear saturated dicarboxylic acid(s);
wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, E3, E4, E5 and/or E6.

3. Microorganism or progeny thereof according to claim 1 expressing the following enzymatic pathway:
- enzyme E1 catalyzing conversion of a cycloalkane, preferably a C5 to C11 cycloalkane, to the corresponding cyclic alcohol;
- enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
- enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone;
- enzyme E4 catalyzing conversion of said lactone to ω-hydroxy fatty acid(s);
- enzyme E5 catalyzing conversion of said ω-hydroxy fatty acid(s) to ω-oxo fatty acid(s ;
- enzyme E7 catalyzing conversion of said ω-oxo fatty acid(s) to ω-aminocarboxylic acid;
wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, E3, E4, E5 and/or E7.

4. Microorganism or progeny thereof according to claim 1 or 2 expressing the following enzymatic pathway:
   - enzyme E1 catalyzing conversion of a cycloalkane, preferably a C5 to C11 cycloalkane, to the corresponding cyclic alcohol;
   - enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
   - enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone;
   - enzyme E4 catalyzing conversion of said lactone to ω-hydroxy fatty acid(s);

   wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, E3, and/or E4.

5. Microorganism or progeny thereof according to claim 1 or 2 expressing the following enzymatic pathway:
   - enzyme E1 catalyzing conversion of a cycloalkane, preferably a C5 to C11 cycloalkane, to the corresponding cyclic alcohol;
   - enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;
   - enzyme E3 catalyzing conversion of said cyclic ketone to the corresponding lactone;

   wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1, E2, and/or E3.

6. Microorganism or progeny thereof according to claim 1 or 2 expressing the following enzymatic pathway:
   - enzyme E1 catalyzing conversion of a cycloalkane, preferably a C5 to C11 cycloalkane, to the corresponding cyclic alcohol;
   - enzyme E2 catalyzing conversion of said cyclic alcohol to the corresponding cyclic ketone;

   wherein the microorganism is genetically modified to express at least one, more than one, either or all of the enzymes E1 and/or E2.

7. Microorganism or progeny thereof of claim 2, wherein enzyme E1 is a cytochrome P450 monooxygenase and/or enzyme E2 is an alcohol dehydrogenase and/or enzyme E3 is a Bayer-Villager type monooxygenase and/or enzyme E4 is a lactonase and/or

E5 is a ω -hydroxy fatty acid dehydrogenase and/or E6 is a ω -oxo fatty acid dehydrogenase.

8. Microorganism or progeny thereof of claim 3, wherein enzyme E1 is a cytochrome P450 monooxygenase and/or enzyme E2 is an alcohol dehydrogenase and/or enzyme E3 is a Bayer-Villager type monooxygenase and/or enzyme E4 is a lactonase and/or E5 is a ω - hydroxy fatty acid dehydrogenase and/or E7 is a ω-transaminase.

9. Microorganism or progeny thereof of claim 4, wherein enzyme E1 is a cytochrome P450 monooxygenase and/or enzyme E2 is an alcohol dehydrogenase and/or enzyme E3 is a Bayer-Villager type monooxygenase and/or enzyme E4 is a lactonase.

10. Microorganism or progeny thereof of claim 5, wherein enzyme E1 is a cytochrome P450 monooxygenase and/or enzyme E2 is an alcohol dehydrogenase and/or enzyme E3 is a Bayer-Villager type monooxygenase.

11. Microorganism or progeny thereof of claim 6, wherein enzyme E1 is a cytochrome P450 monooxygenase and/or enzyme E2 is an alcohol dehydrogenase.

12. Microorganism or progeny thereof of one of the preceding claims, wherein enzyme E1 is a cyclohexane cytochrome P450 monooxygenase system comprising a cytochrome P450 (CYP), a ferredoxin (Fd) and a ferredoxin reductase (FdR); preferably the enzyme E1 is a cyclohexane cytochrome P450 monooxygenase system derived from *Acidovorax* CHX 100; more preferably the enzyme E1 is a cyclohexane cytochrome P450 monooxygenase system comprising a cytochrome P450 protein (CYP) with an amino acid sequence of SEQ ID NO: 1, a ferredoxin protein (Fd) with an amino acid sequence of SEQ ID NO: 2, and a ferredoxin reductase protein (FdR) with an amino acid sequence of SEQ ID NO: 3.

13. Microorganism or progeny thereof of one of the preceding claims, wherein enzyme E2 is a cyclohexanol dehydrogenase (CDH); preferably enzyme E2 is a cyclohexanol dehydrogenase (CDH) derived from *Acidovorax* CHX 100; more preferably the enzyme

E2 is a cyclohexanol dehydrogenase protein (CDH) with an amino acid sequence of SEQ ID NO: 4.

14. Microorganism or progeny thereof of one of the preceding claims, wherein enzyme E3 is a cyclohexanone monooxygenase (CHXON); preferably enzyme E3 is a cyclohexanone monooxygenase (CHXON) derived from *Acidovorax* CHX 100; more preferably the enzyme E3 is a cyclohexanone monooxygenase protein (CHXON) with an amino acid sequence of SEQ ID NO: 5.

15. Microorganism or progeny thereof of one of the preceding claims, wherein enzyme E4 is a lactonase; preferably enzyme E4 is an endogenous lactonase or lactonase derived from *Acidovorax* CHX 100; more preferably the enzyme E4 is a lactonase protein with an amino acid sequence of SEQ ID NO: 6.

16. Microorganism or progeny thereof of one of the preceding claims, wherein enzyme E5 is a ω-hydroxy fatty acid dehydrogenase, preferably is enzyme E5 a ω-hydroxy fatty acid dehydrogenase derived from *Acidovorax* CHX 100; more preferably the enzyme E5 is a ω-hydroxy fatty acid dehydrogenase with an amino acid sequence of SEQ ID NO: 7.

17. Microorganism or progeny thereof of one of the preceding claims, wherein enzyme E6 is a ω-oxo fatty acid dehydrogenase, preferably is enzyme E6 a ω-oxo fatty acid dehydrogenase derived from *Acidovorax* CHX 100; more preferably the enzyme E6 is a ω-oxo fatty acid dehydrogenase with an amino acid sequence of SEQ ID NO: 8.

18. Microorganism or progeny thereof of one of the preceding claims, wherein enzyme E7 is ω-transaminase, preferably is enzyme E7 a ω-transaminase derived from *Chromobacterium violaceum*; more preferably the enzyme E5 is a ω-transaminase with an amino acid sequence of SEQ ID NO: 9.

19. Microorganism or progeny thereof of one of the preceding claims, wherein the microorganism is a prokaryotic organism, preferably a bacterial cell or algae, more preferably a bacterial cell of the genus *Acidovorax* or *Pseudomonas*, even more

preferably *Acidovorax* CHX 100 or *Pseudomonas taiwanensis* VLB120 or *Pseudomonas taiwanensis* VLB120T7, most preferably *Pseudomonas taiwanensis* VLB120.

20. An expression cassette for the manufacture of a microorganism of one of claims 1 to 19, wherein the expression cassette comprises nucleic acid sequences encoding at least two of enzymes E1, E2, E3, E4, E5, and E6; preferably at least three of the enzymes E1, E2, E3, E4, E5, and E6; more preferably the enzymes E1, E2 and E3; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, enzyme E4 with an amino acid sequence of SEQ ID NO: 6, enzyme E5 with an amino acid sequence of SEQ ID NO: 7, and enzyme E6 with an amino acid sequence of SEQ ID NO: 8.

21. An expression cassette for the manufacture of a microorganism of one of claims 1 to 19, wherein the expression cassette comprises nucleic acid sequences encoding at least two of enzymes E1, E2, E3, E4, E5, and E7; preferably at least three of the enzymes E1, E2, E3, E4, E5, and E7; more preferably the enzymes E1, E2 and E3; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, enzyme E4 with an amino acid sequence of SEQ ID NO: 6, enzyme E5 with an amino acid sequence of SEQ ID NO: 7, and enzyme E7 with an amino acid sequence of SEQ ID NO: 9.

22. Expression cassette of claim 20, wherein the expression cassette comprises nucleic acid sequences encoding at least two of enzymes E1, E2, E3, and E4; preferably at least three of the enzymes E1, E2, E3, and E4; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO:1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, enzyme E4 with an amino acid sequence of SEQ ID NO: 6.

23. Expression cassette of claim 20, wherein the expression cassette comprises nucleic acid sequences encoding at least two of enzymes E1, E2, and E3; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an

amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5.

24. Expression cassette of claim 20, wherein the expression cassette comprises nucleic acid sequences encoding at least two of enzymes E1 and E2; even more preferably enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4.

25. Expression cassette of claim 21, wherein the expression cassette comprises nucleic acid sequences encoding at least two of the enzymes E1, E2, E3 and E7; preferably at least three of the enzymes E1, E2, E3, and E7; even more enzyme E1 with an amino acid sequence of SEQ ID NO: 1, 2 and 3, enzyme E2 with an amino acid sequence of SEQ ID NO: 4, enzyme E3 with an amino acid sequence of SEQ ID NO: 5, and enzyme E7 with an amino acid sequence of SEQ ID NO: 9.

26. A vector comprising an expression cassette of any one of claims 20 to 25.

27. Method of producing a microorganism of one of claims 1 to 19, wherein a microorganism is transfected with at least one of an expression cassette of claims 20 to 25 and/or a vector of claim 26.

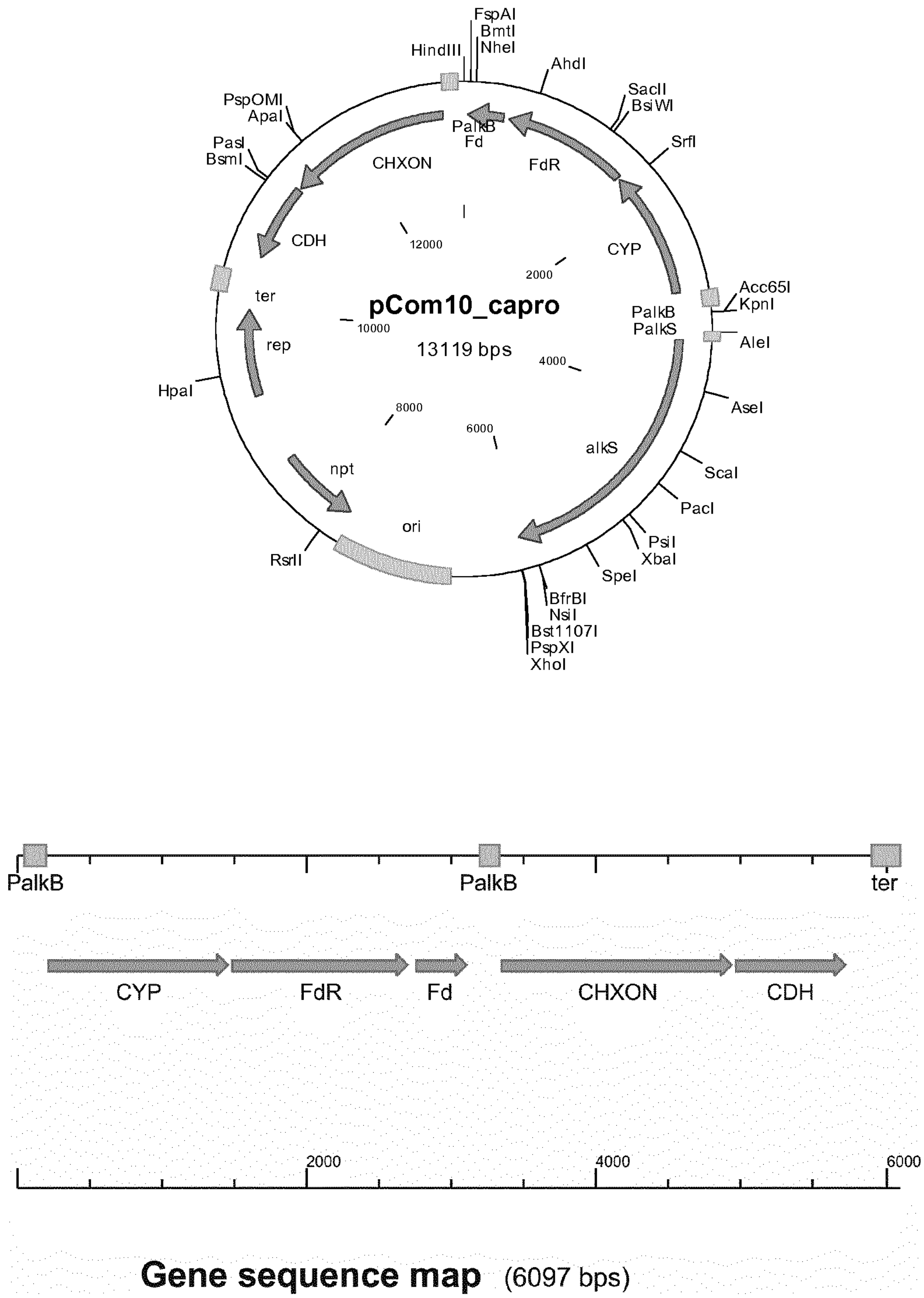
28. Use of a microorganism or a progeny thereof of one of claims 1 to 19 for the production of lactones, ω–hydroxy acids, and/or dicarboxylic acids and/or ω-aminohexanoic acids from cyclic alkanes.

29. Method for the production of lactones, ω–hydroxy acids, and/or dicarboxylic acids and/or ω-aminohexanoic acids from cycloalkanes, the method comprising the step of:
- contacting a microorganism of one of claims 1 to 19 with a cycloalkane, preferably with a C5 to C11 cycloalkane;
- culturing the mixture of microorganism and cycloalkane; and
- extracting lactones, ω–hydroxy acids, and/or dicarboxylic acids from the culture product.

30. Method of claim 29, wherein culture of the mixture of microorganism and cyclic alkane is performed in solution or liquid phase; preferably culture is performed in a medium and under conditions appropriate to allow for growth of said microorganism.

31. Method of one of claims 29 and 30, wherein culture of the mixture of microorganism and cyclic alkane is performed in a bioreactor, in a stirred tank, or in a capillary micro-reactor.

32. Method of one of claims 29 to 31, wherein the microorganism is provided as immobilized biofilm.



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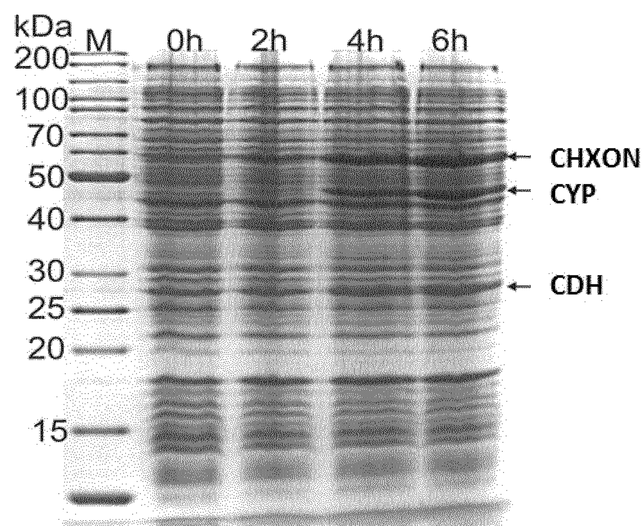


Figure 2:

Biotransformation of cyclohexane:

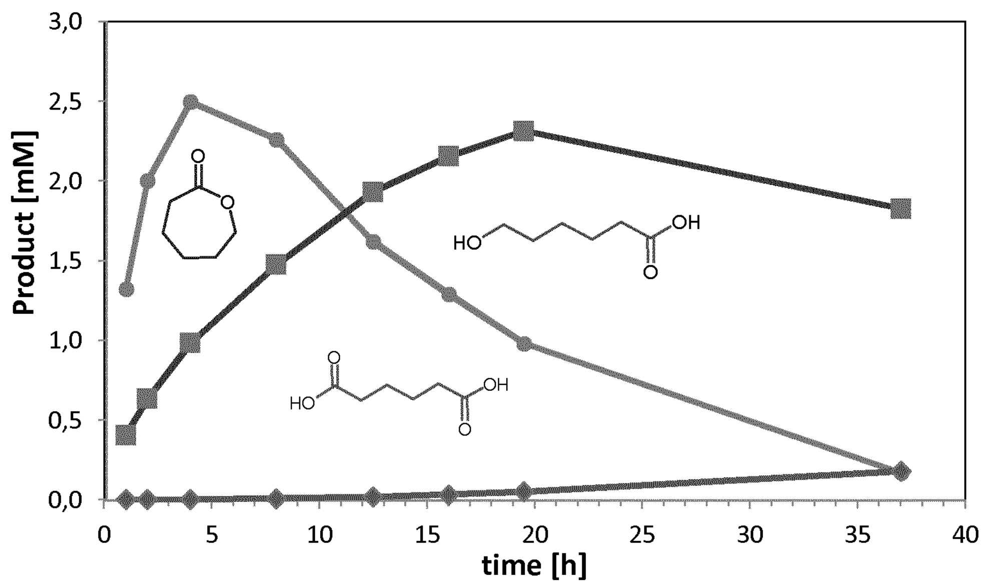


Figure 3:

Figure 4

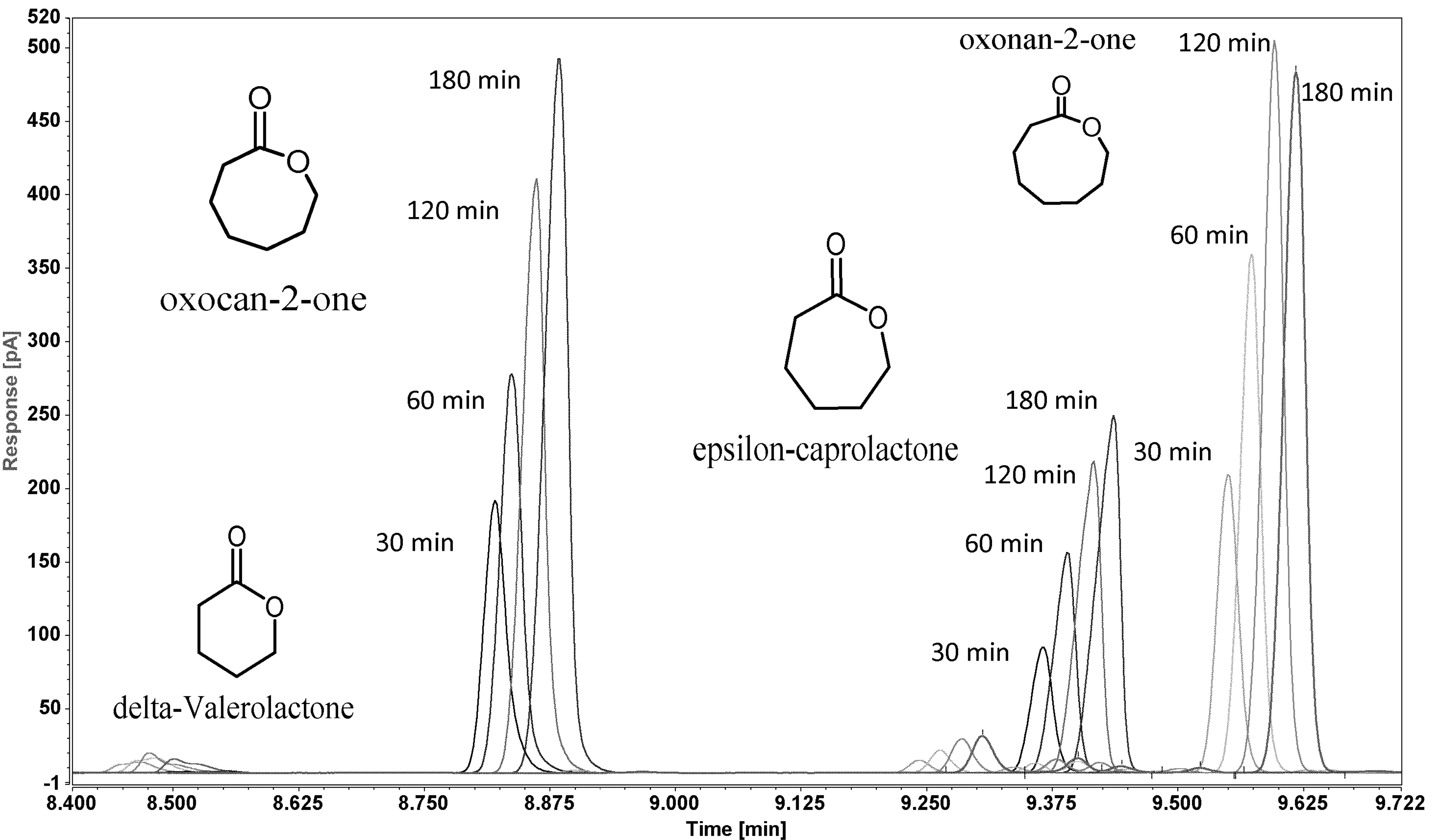


Figure 5

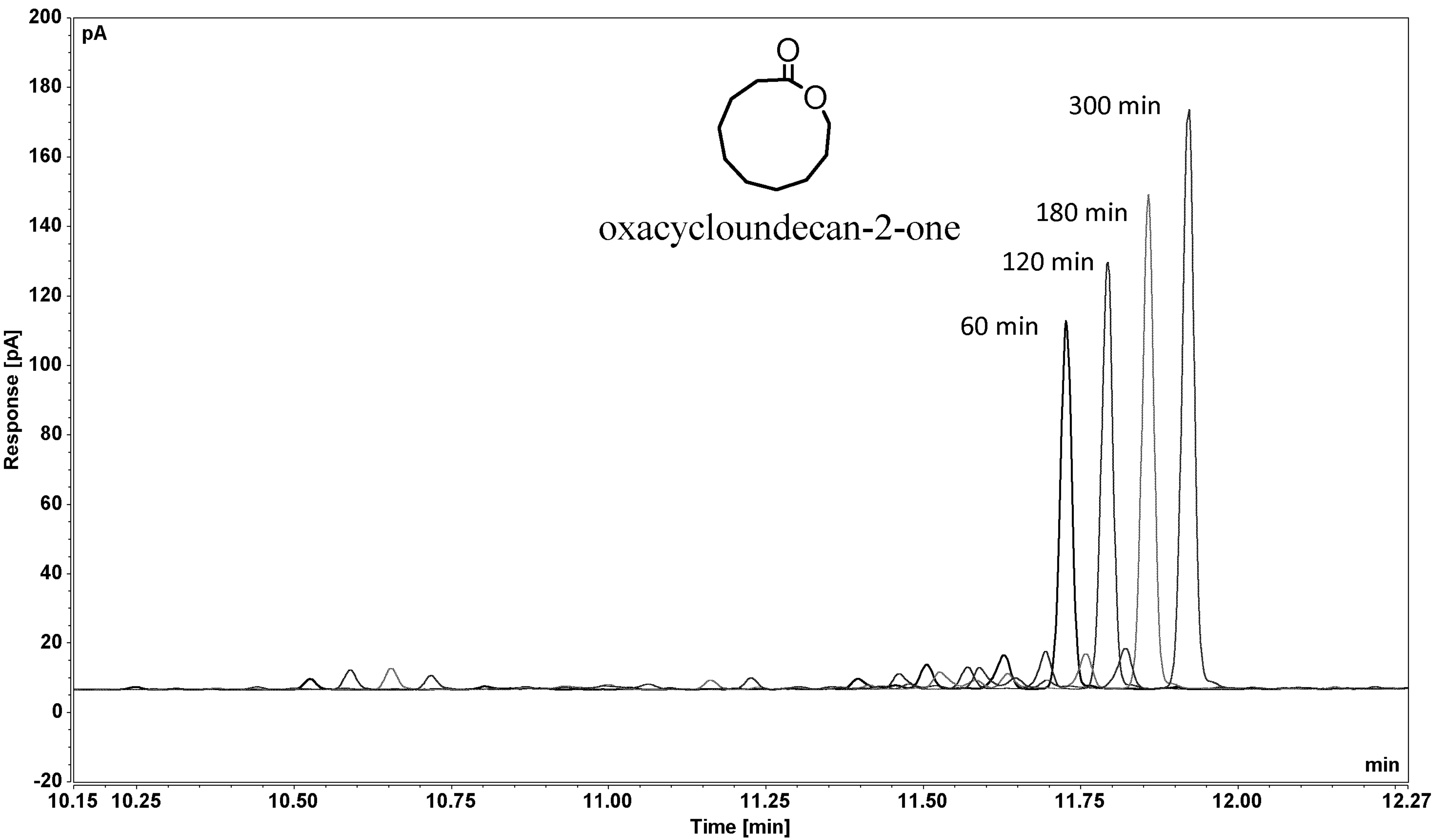


Figure 6

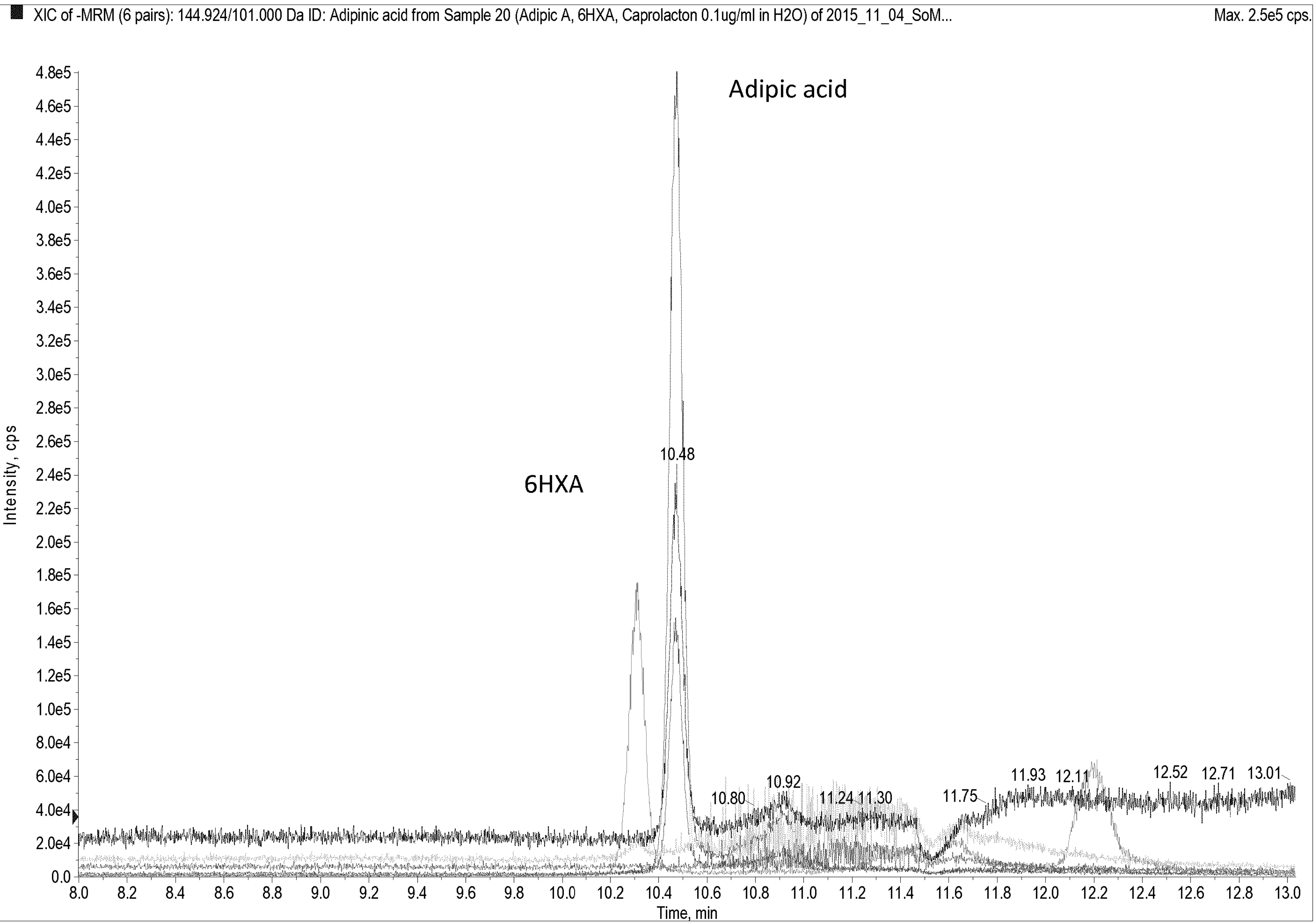


Figure 7

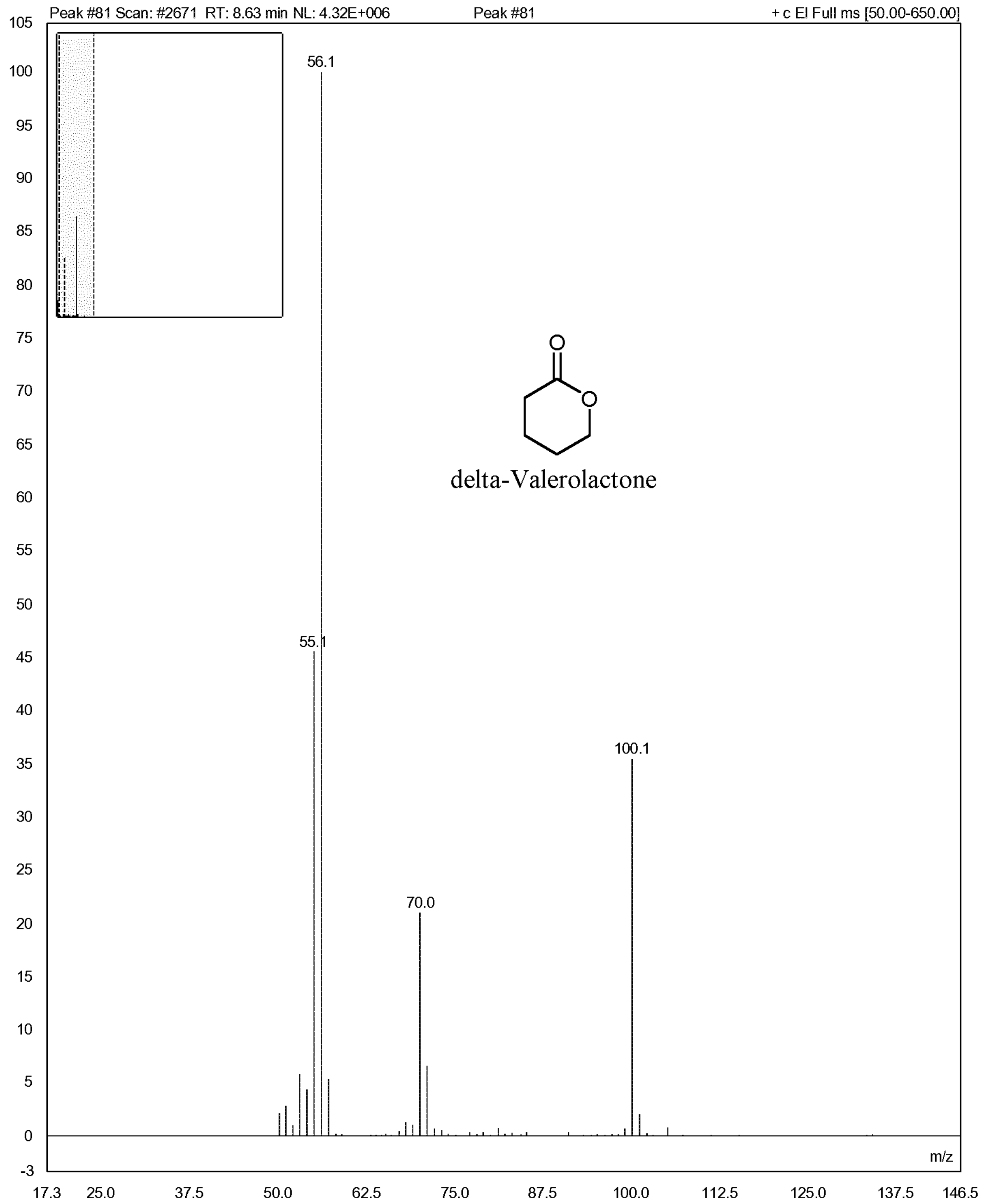


Figure 8

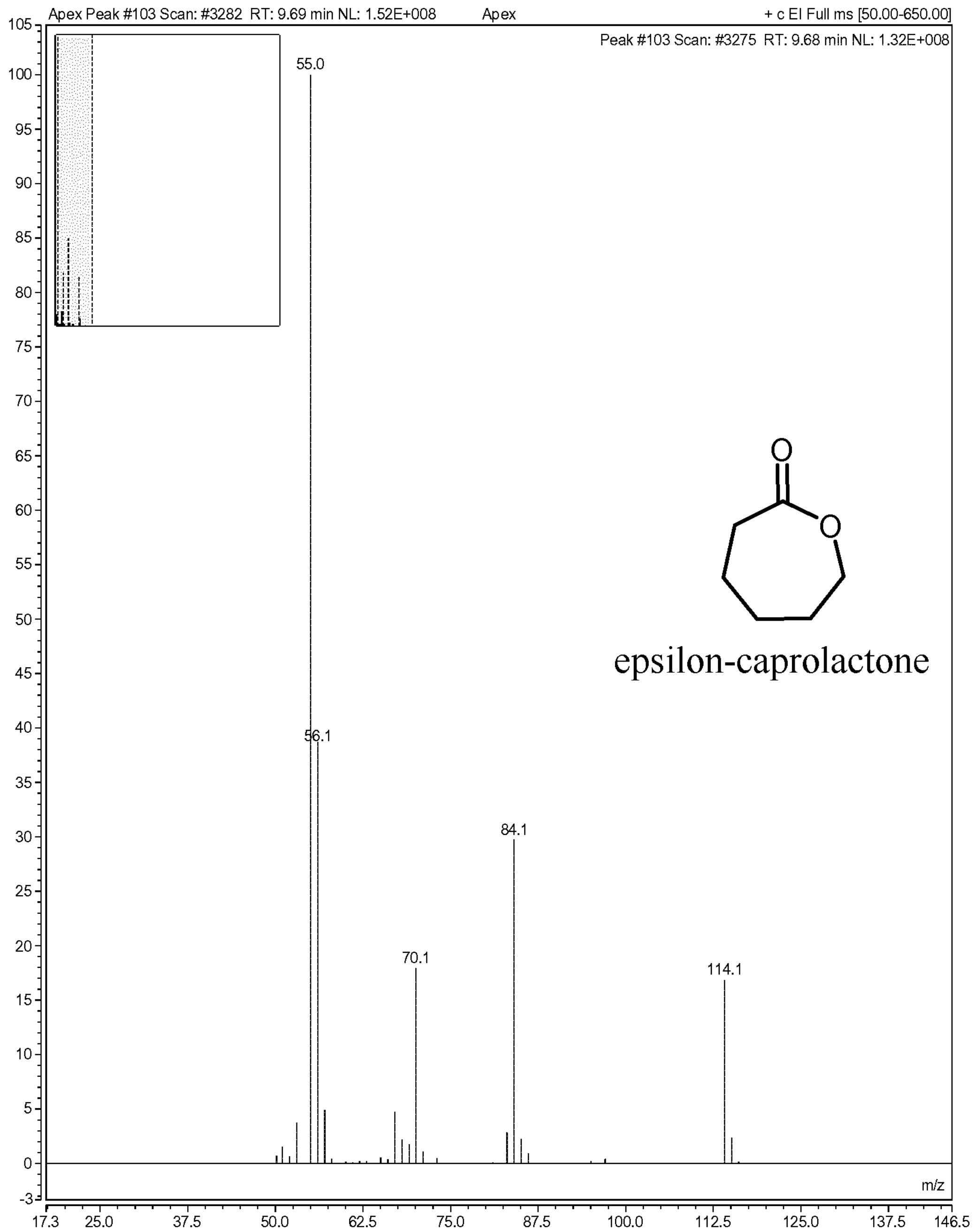


Figure 9

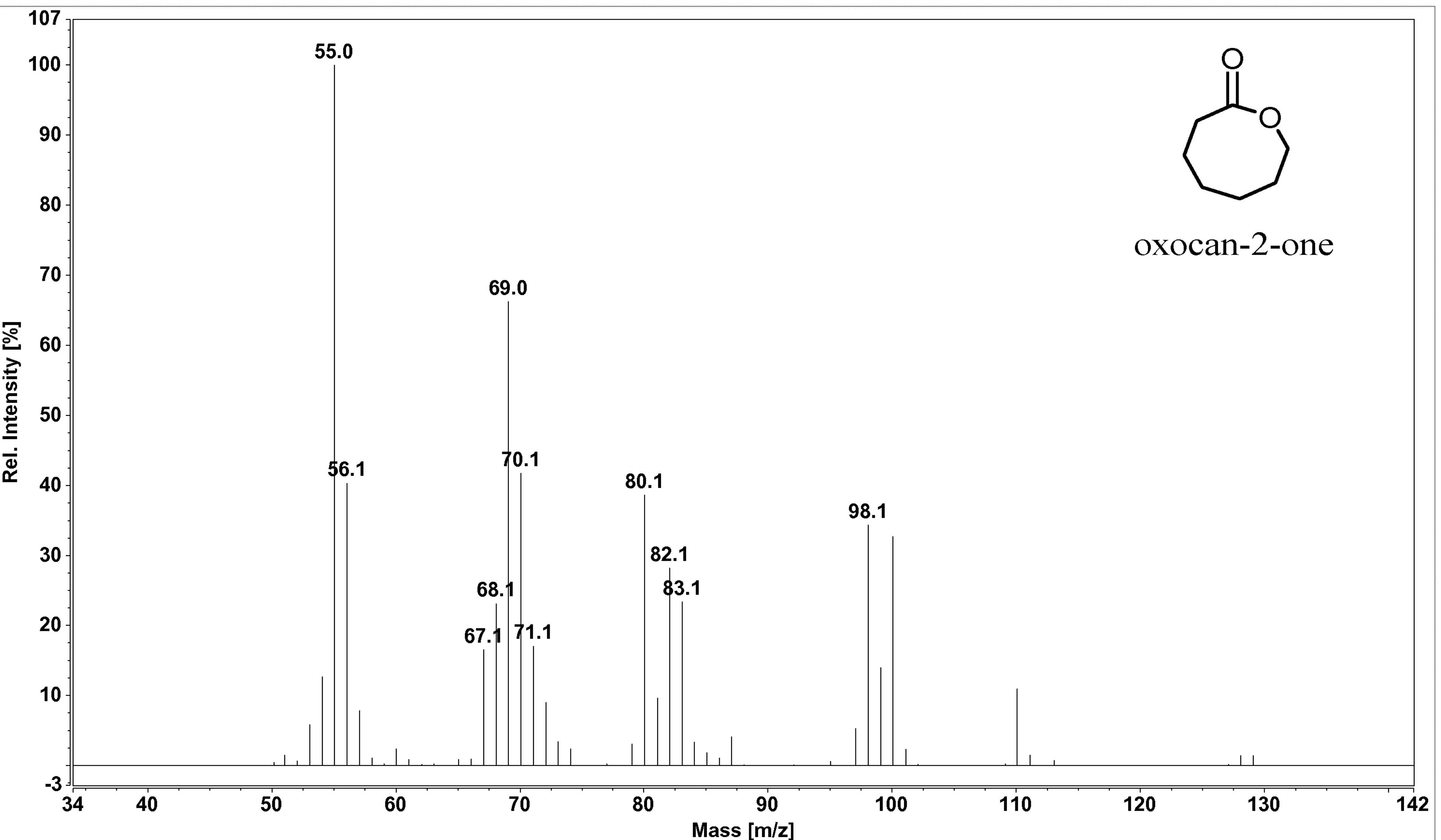


Figure 10

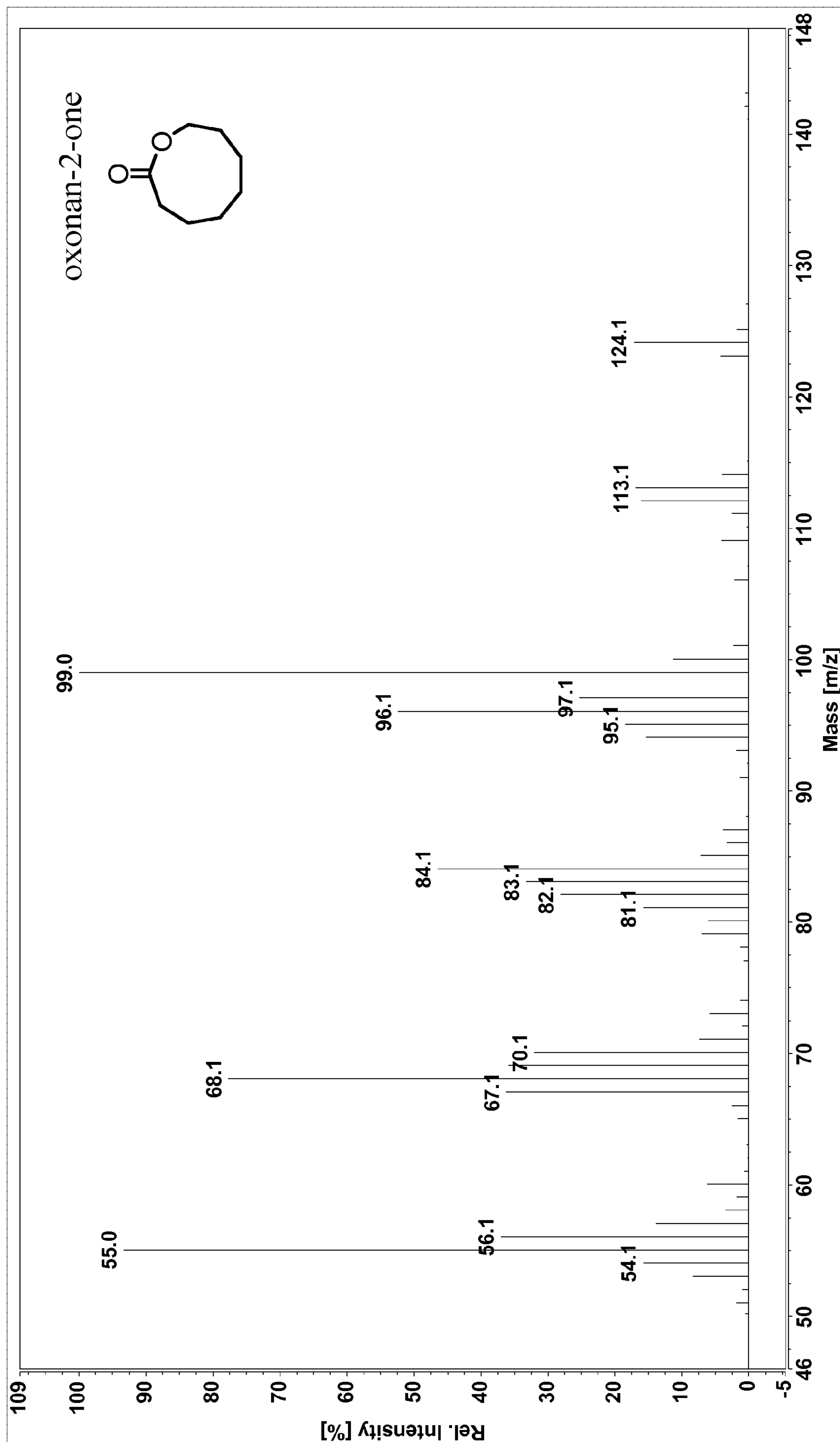


Figure 11

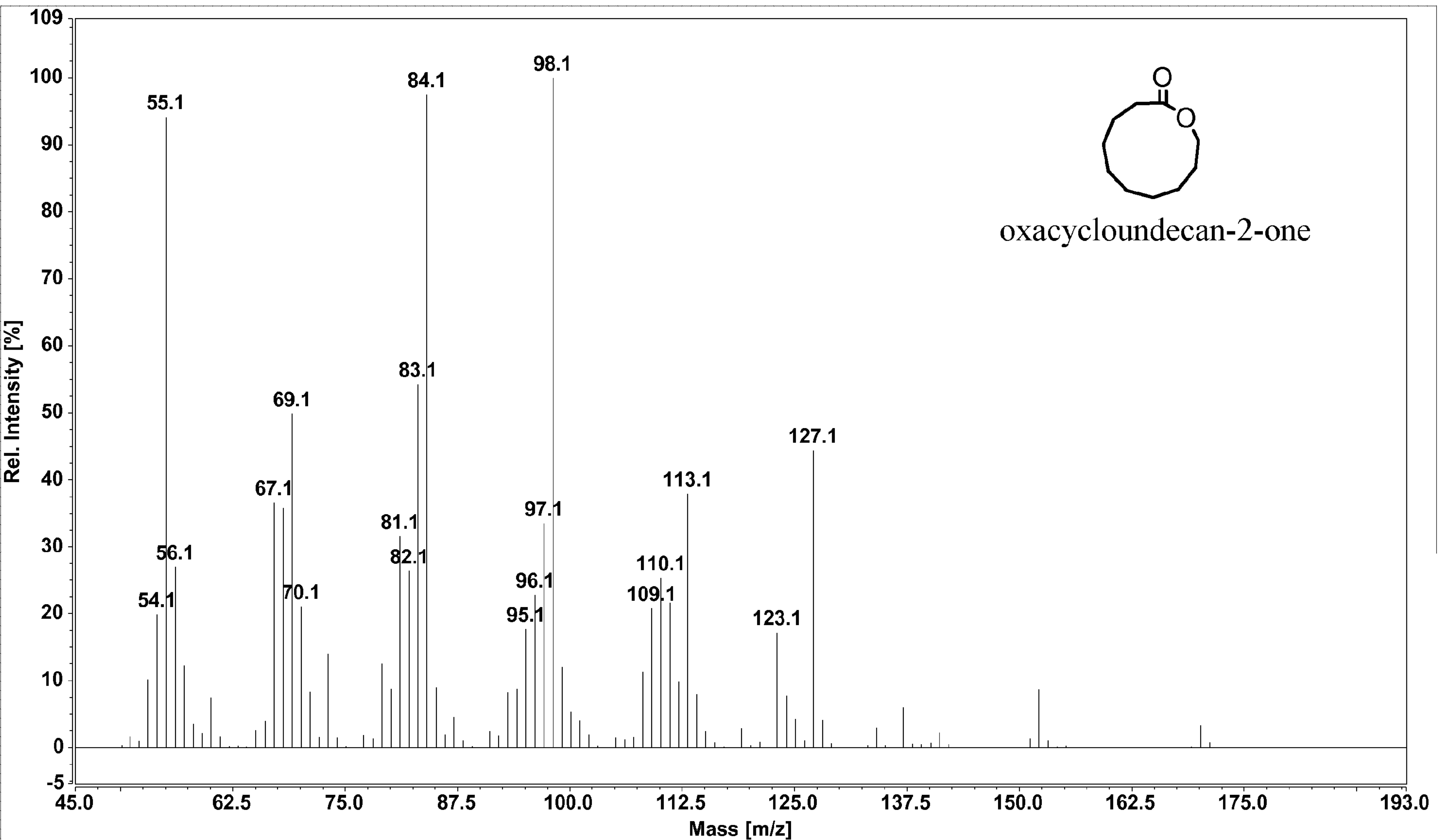
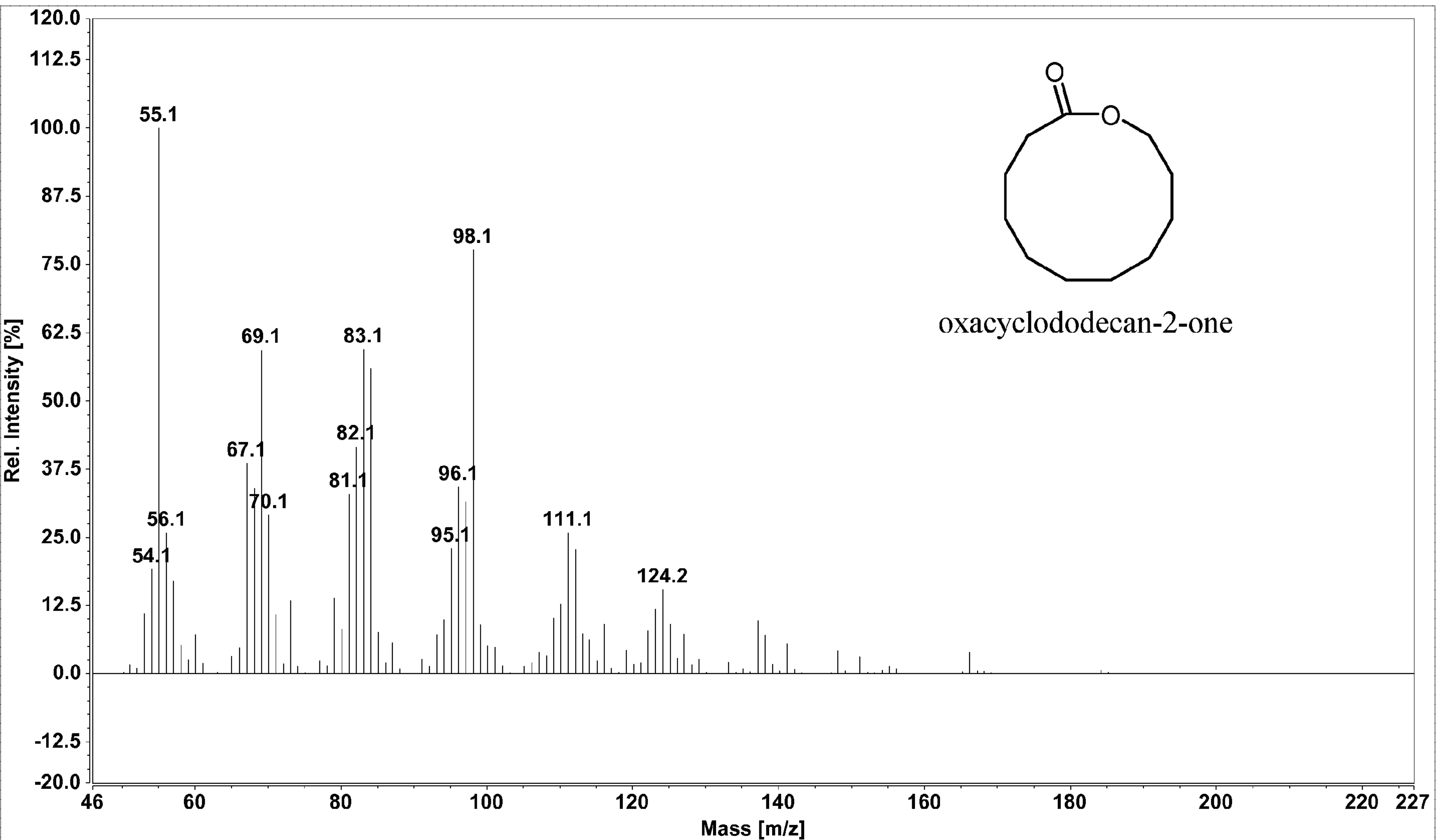


Figure 12



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/071388

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P17/08 C12P7/42 C12P7/44 C12N9/02 C12N9/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/224807 A1 (PEARLMAN PAUL S [US] ET AL) 29 August 2013 (2013-08-29) paragraphs [0127], [0130] -----	1-32
Y	SALAMANCA DIEGO ET AL: "Novel cyclohexane monooxygenase fromAcidovoraxsp. CHX100", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, DE, vol. 99, no. 16, 3 May 2015 (2015-05-03), pages 6889-6897, XP035521105, ISSN: 0175-7598, DOI: 10.1007/S00253-015-6599-9 [retrieved on 2015-05-03] the whole document -----	1-32

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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
9 May 2017	23/05/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schneider, Patrick

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Rohan Karande ET AL: "Continuous cyclohexane oxidation to cyclohexanol using a novel cytochrome P450 monooxygenase from Acidovorax sp. CHX100 in recombinant P. taiwanensis VLB120 biofilms", Biotechnology and Bioengineering, 18 September 2015 (2015-09-18), pages 52-61, XP055369825, United States DOI: 10.1002/bit.25696 Retrieved from the Internet: URL:http://epo.summon.serialssolutions.com/2.0.0/link/0/eLvHCXMwpV3Pa9swFBbtoKw7rF06umwdvEMZvbi1Hcmyj0lYf0APpaSlOxlJllZDYoXa6ZI_aP_nnuQkzaCHwW4CGf_gPT193_PT9wg5NpoqXRgZCJMkAe7HDONgQQOFhCiiIuaJ8DndIR1d8NsLev1Sm9PKQ6zzbW5h-HC9ppphfCZL19k6yZIzIV06QDUYgaMkdAVdgx8vWuE8bf9UOs7cY1m80hXavMVruPJvmOr3mfO9_3zDffJ- [retrieved on 2017-05-05] the whole document -----	1-32
Y	DIEGO SALAMANCA ET AL: "Isolation and characterization of two novel strains capable of using cyclohexane as carbon source", ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH INTERNATIONAL, vol. 21, no. 22, 28 June 2014 (2014-06-28), pages 12757-12766, XP055369118, DE ISSN: 0944-1344, DOI: 10.1007/s11356-014-3206-z the whole document -----	1-32

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/071388

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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