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(54) Title: MODIFIED DACS/DAOC ENZYMES

(57) Abstract: Deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) modified to reduce the hydroxylation activity and in particular DAOC/DACS modified to reduce the hydroxylation activity and further modified to improve the ring expanding activity.

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MODIFIED DACS/DAOC ENZYMES

Field of the invention

The present invention relates to a modified deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) and polynucleotides encoding the modified DAOC/DACS and in particular to DAOC/DACS having reduced hydroxylation activity.

Background of the Invention

Penicillins and cephalosporins have long been used as antibiotics in the treatment of infectious diseases. Many semisynthetic derivatives based on these compounds have been tried and a large number of the compounds are in medical use. Typically, both penicillins and cephalosporins are produced by fermentation. The biosynthesis of penicillin and cephalosporin antibiotics in microorganisms requires the formation of the bicyclic nucleus of penicillin. Isopenicillin N synthase (IPNS) catalyses the reaction of a tripeptide –(L--aminoadipyl)-L-cysteinyl-D-valine, (ACV) to form isopenicillin N.

A wide variety of organisms can produce antibiotics. Examples include *Aspergillus*, *Streptomyces, Bacillus, Monospora, Cephalosporium, Penicillium* and *Nocardia* species. Many of these organisms express additional enzymes that result in the conversion of isopenicillin N into a variety of different antibiotics such as penicillin N, penicillin G and penicillin V. Typically, penicillins are produced industrially by fermentation.

Cephalosporins may be produced by expansion of the 5-membered thiazolidine ring of penicillin to the 6-membered dihydrothiazine ring of cephalosporins. In particular, cephalosporins may be derived from 7-aminodeacetoxycephalosporanic acid (7-ADCA), 7-aminocephalosporianic acid (7-ACA) or deacetylcephalosporin C (DAC). Ring expansion enzymes are expressed by a number of organisms.

In some organisms, for example *Cephalosporium acremonium*, penicillin N is converted into DAC by the bifunctional enzyme deacetoxy/deacetylcephalosporin C

synthase (DAOC/DACS). DAOC/DACS expands the ring of penicillin N to form deacetoxycephalosporin C (DAOC) and subsequently carries out the hydroxylation of DAOC to produce DAC. In some organisms, for example *Cephalosporium acremonium*, DAC may be subsequently converted to cephalosporin C.

Summary of the Invention

The present invention provides a modified DAOC/DACS having a reduced hydroxylation activity. In particular, the present invention provides a modified DAOC/DACS enzyme wherein the DAOC/DACS enzyme is SEQ ID NO: 2 or a variant of SEQ ID NO: 2. In one embodiment, the invention provides a modified DAOC/DACS enzyme wherein the methionine at position 306 of SEQ ID NO: 2 is substituted or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the methionine at position 306 of SEQ ID NO: 2 is substituted. The methionine at position 306 of SEQ ID NO: 2 or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the methionine at position 306 of SEQ ID NO: 2 may substituted by an amino acid with an aliphatic side chain, for example isoleucine. In another embodiment, the tryptophan at position 82 of SEQ ID NO: 2 is substituted or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the tryptophan at position 82 of SEQ ID NO: 2 is substituted. The tryptophan at position 82 of SEQ ID NO: 2 or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the tryptophan at position 82 of SEQ ID NO: 2 may be substituted by an amino acid with a non-aromatic side chain, for example serine.

The invention also provides a modified DAOC/DACS having reduced hydroxylation activity further modified to improve the ring expanding activity. In one embodiment, the invention provides a DAOC/DACS enzyme modified as discussed above to reduce hydroxylation activity wherein the asparagine at position 305 of SEQ ID NO: 2 is substituted or the amino acid residue in the a variant of SEQ ID NO: 2 that is at an equivalent position to asparagine at position 305 of SEQ ID NO: 2 is substituted. The asparagine at position 305 of SEQ ID NO: 2 or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the asparagine at position 305 of SEQ ID NO: 2 may be substituted by a hydrophobic

residue, for example leucine. In another embodiment, the invention provides a DAOC/DACS enzyme modified as discussed above to reduce hydroxylation activity further comprising a deletion within the C terminus.

The invention further provides:

- a polynucleotide encoding a modified DAOC/DACS according to the invention;
- an expression vector comprising a polynucleotide of the invention;
- a host cell transformed with a polynucleotide or a vector of the invention;
- use of a modified DAOC/DACS enzyme of the invention in a method of ring expansion;
- a method of ring expanding penicillin G utilising a modified DAOC/DACS enzyme or a host cell of the invention;
- a method of producing 7-ADCA utilising a host cell of the invention; and
- a method of producing adipyl-7-ADCA utilising a host cell of the invention.

Description of the Figures

Figure 1 shows the circular dichroism (CD) spectra of wild type DAOC/DACS, and W82A, W82S and M306I mutants.

Figure 2 shows the CD spectra of wild type, Δ310 and Δ328 DAOC/DACS mutants.

Figure 3 shows the initial rate of penicillin G oxidation in the presence of 6 μM wild type DAOC/DACS.

Figure 4 shows the absorbance at 260 nm following incubation of wild type DAOC/DACS and penicillin G under the standard reaction conditions.

Figure 5 shows the absorbance at 260 nm following incubation of N305L and penicillin G under the standard reaction conditions.

Figure 6 shows absorbance at 260 nm following incubation of M306I, Δ310/M306I and Δ310/N305L/M306I and penicillin G under the standard reaction conditions.

Figure 7 shows the absorbance at 260 nm following incubation of Δ310 and penicillin G under the standard reaction conditions.

Figure 8 shows the Michaelis-Mention curve (dependence of rate on substrate concentration) for penicillin G oxidation by the N305L mutant.

Figure 9 shows the Michaelis-Mention curve (dependence of rate on substrate concentration) for penicillin G oxidation by the M306I mutant.

Figure 10 shows the Michaelis-Mention curve (dependence of rate on substrate concentration) for penicillin G oxidation by the Δ310/M306I mutant.

Figure 11 shows the Michaelis-Mention curve (dependence of rate on substrate concentration) for penicillin G oxidation by the Δ310/N305L/M306I mutant.

Description of the Sequences

SEQ ID NO: 1 is the nucleic acid sequence which encodes DAOC/DACS from *Cephalosporium acremonium*.

SEQ ID NO: 2 is the amino acid sequence of DAOC/DACS from *Cephalosporium acremonium*.

Detailed Description of the Invention

The present invention provides a modified DAOC/DACS which shows reduced hydroxylation activity.

A modified DAOC/DACS according to the invention may comprise a DAOC/DACS derived from *Cephalosporium acremonium* or a DAOC/DACS derived from other bacterial or fungal species. A DAOC/DACS for use in accordance with the invention may be one isolated from *Aspergillus*, *Streptomyces*, *Bacillus*, *Monospora*, *Cephalosporium*, *Penicillium* or *Nocardia* species. A modified DAOC/DACS according to the invention may comprise a non-naturally occurring DAOC/DACS which has been, for example, artificially synthesized or expressed in a cell which does not normally express a DAOC/DACS using techniques well known in the art.

The amino acid sequence of DAOC/DACS from *Cephalosporium acremonium* is set out in SEQ ID NO: 2. Variations in the sequence of SEQ ID NO: 2 may be present in DAOC/DACS obtained from other isolates or strains of

Cephalosporium acremonium. DAOC/DACS from other *Cephalosporium* strains or other fungal or bacterial species can be isolated following standard cloning techniques, for example, using the polynucleotide sequence of SEQ ID NO: 2 or a fragment thereof as a probe.

A variant polypeptide having an amino acid sequence which varies from that of SEQ ID NO: 2 may be modified in accordance with the present invention. A variant for use in accordance with the invention is one having DAOC/DACS activity.

A variant of SEQ ID NO: 2 may be a naturally occurring variant which is expressed by another strain of *Cephalosporium*. Such variants may be identified by looking for DAOC/DACS activity in those strains which have a sequence which is highly conserved compared to SEQ ID NO: 2. Such proteins may be identified by analysis of the polynucleotide encoding such a protein isolated from an alternative strain of *Cephalosporium*, for example, by carrying out the polymerase chain reaction using primers derived from portions of SEQ ID NO: 2.

Variants of SEQ ID NO: 2 include sequences which vary from SEQ ID NO: 2 but are not necessarily naturally occurring DAOC/DACS. Over the entire length of the amino acid sequence of SEQ ID NO: 2, a variant will preferably be at least 80% homologous to that sequence based on amino acid identity. More preferably, the polypeptide is at least 85% or 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 2 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 40 or more, for example 60, 100 or 120 or more, contiguous amino acids ("hard homology").

Amino acid substitutions may be made to the amino acid sequence of SEQ ID NO: 2, for example from 1, 2, 3, 4 or 5 to 10, 20 or 30 substitutions. Conservative substitutions may be made, for example, according to the following table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

NON-AROMATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		H K R
AROMATIC		H F W Y

One or more amino acid residues of the amino acid sequence of SEQ ID NO: 2 may alternatively or additionally be deleted. From 1, 2, 3, 4 or 5 to 10, 20 or 30 residues may be deleted, or more. Polypeptides of the invention also include fragments of the above-mentioned sequences. Such fragments retain DAOC/DACS activity. Fragments may be at least from 200 or 250 amino acids in length. Such fragments may be used to produce chimeric enzymes as described in more detail below.

Variants of SEQ ID NO: 2 include chimeric proteins comprising fragments or portions of SEQ ID NO: 2. DAOC/DACS from *Cephalosporium acremonium* (SEQ ID NO: 2) comprises three domains. The N-terminal (amino-terminal) domain comprises residues 1 to 155 and is believed to have little functional importance. The jelly-roll motif comprises residues 156 to 266 and contains the catalytic residues. The C-terminal (carboxy-terminal) domain comprises residues 267 to 332 and plays an important role in the activity of the enzyme. A chimeric enzyme typically comprises the jelly-roll motif and the C-terminal domain of SEQ ID NO: 2. Part or all of the N-terminal domain may be replaced by an alternative sequence. Typically, the N-terminal domain is replaced by the N-terminal domain of an enzyme other than DAOC/DACS, for example deacetoxycepahlosporin C synthase (DAOCS), isopenicillin N synthase (IPNS), 1-aminocyclopropane-1-carboxylate oxidase, flavanone 3-β-hydroxylase, hysocyamine 6-β-hydroxylase, flavanol synthase and giberrellin synthase.

Variants of SEQ ID NO: 2 also include enzymes that have at least 80% homology over the jelly-roll motif and C-terminal domain to SEQ ID NO: 2 based on amino acid identity. More preferably, the polypeptide is at least 85% or 90% and

more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 2 over the jelly-roll motif and C-terminal domain.

One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the N-terminus or C-terminus of the amino acid sequence of SEQ ID NO: 2 or polypeptide variant or fragment thereof. The extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer. A carrier protein may be fused to an amino acid sequence according to the invention. A fusion protein incorporating the polypeptides described above can thus be provided.

A polypeptide to be modified in connection with the present invention is one which has DAOC/DACS activity, namely, the ability to catalyse both ring expansion and subsequently hydroxylation. Ring expansion is the conversion of a 5-membered thiazolidine ring, for example, of a penicillin to a 6-membered ring, for example, of DAOC. Hydroxylation is the introduction of a hydroxyl group (-OH) onto the substrate as, for example, during the conversion of DAOC to DAC. Preferably, a polypeptide suitable for modification is one which has DAOC/DACS activity prior to modification.

A modified DAOC/DACS in accordance with the invention is one which demonstrates a reduced hydroxylation activity when compared to the DAOC/DACS enzyme not so modified. A modified variant in accordance with the invention is one which demonstrates a reduced hydroxylation activity when compared to a variant sequence not so modified. A reduction in hydroxylation activity is a reduction of the ability of the enzyme to introduce a hydroxyl group (-OH) onto the substrate, for example DAOC or 7-ADCA. This results in reduction in the production of a hydroxylated product, for example DAC. The hydroxylation activity may be reduced by at least 5, 10, 20, 30, 40, 50, 60, 70, 80 or 90%. Preferably the hydroxylation activity is reduced by 70 or 80%. In some embodiments the hydroxylation activity is completely abolished such that it is undetectable using standard methods.

The hydroxylation activity of the enzyme can be monitored *in vitro* or *in vivo* for example in accordance with the methods which are described in more detail below. In particular, assays may be carried out to monitor activity of the enzyme by monitoring for the oxidation of the substrate such as DAOC, for the presence of

hydroxyl (-OH) groups on the substrate and/or the production a hydroxylated product such as DAC. Assays may also be carried out to monitor activity of the enzyme by monitoring for the ring expansion of the substrate, such as DAOC or penicillin G, without subsequent hydroxylation. In particular, assays may be carried out to monitor activity of the enzyme by monitoring for production of phenylacetyl-7-ADCA production from penicillin G. Assays may also be carried out to measure the conversion of the cosubstrate 2-oxoglutarate (Lee, H. J. *et al,* J. Biochem. Biophys. Res. Commun., 2000; 267: 445-448). Control experiments using the DAOC/DACS or variant not so modified can be carried out to establish whether a modified enzyme demonstrates reduced hydroxylation activity.

Methods known in the art may be used to monitor the hydroxylation activity of the enzyme. Typically, High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR) and/or mass spectrometric analysis are used (Lloyd, M. D. *et al*, J. Mol. Biol., 1999; 287: 943-960).

Preferably, DAOC/DACS is modified by substitution within the hydroxylation active site of the enzyme. Preferably, the modified polypeptide comprises the amino acid sequence of SEQ ID NO: 2 which is modified within the hydroxylation active site of the enzyme or a variant sequence of SEQ ID NO: 2 having a similar modification.

Modification of Methionine at position 306

In one aspect of the invention, a DAOC/DACS polypeptide incorporates mutation of methionine at position 306 of SEQ ID NO: 2 to reduce the hydroxylation activity. The methionine at position 306 of SEQ ID NO: 2 may be substituted by a different amino acid with an aromatic side chain. Different amino acids with an aromatic side chain are phenylalanine and tyrosine. Preferably, the methionine at position 306 of SEQ ID NO: 2 is substituted by an amino acid with an aliphatic side chain. An aliphatic side chain is non-aromatic and contains only carbon and hydrogen atoms. Amino acids with an aliphatic side chain are glycine, alanine, valine, leucine, isoleucine and proline. For example methionine may be substituted with isoleucine. Alternative substitutions include alanine or valine. In addition, the invention relates to a variant of SEQ ID NO: 2 having an equivalent modification. A

variant of SEQ ID NO: 2 may be a naturally occurring variant seen in alternative strains of *Cephalosporium acremonium* or a DAOC/DACS derived from other bacterial or fungal species. A variant may also be a non-naturally occurring variant or chimeric enzyme as described in more detail above. The equivalent amino acid to methionine at position 306 of SEQ ID NO: 2 can be identified by aligning a variant polypeptide with the sequence of SEQ ID NO: 2 and thus to identify the equivalent amino acid of any such variant to methionine at position 306 of SEQ ID NO: 2. Computer programs that may be used to align a polypeptide with SEQ ID NO: 2 are discussed in more detail below. The equivalent amino acid is modified to reduce the hydroxylation activity of the DAOCS/DACS enzyme, for example methionine may be substituted by isoleucine or alanine.

As before, a modified DAOC/DACS polypeptide demonstrates reduced hydroxylation activity compared with the polypeptide not so modified.

Modification of Tryptophan at position 82

In one aspect of the invention, a DAOC/DACS polypeptide incorporates mutation of tryptophan at position 82 of SEQ ID NO: 2 to reduce the hydroxylation activity. The tryptophan at position 82 of SEQ ID NO: 2 may be substituted by a different amino acid with an aromatic side chain. Different amino acids with an aromatic side chain are phenylalanine and tyrosine. Preferably, the tryptophan at position 82 of SEQ ID NO: 2 is substituted by an amino acid with a non-aromatic side chain. Amino acids with a non-aromatic side chain are alanine, cysteine, aspartic acid, glutamic acid, glycine, isoleucine, lysine, leucine, methionine, asparagines, proline, glutamine, arginine, serine, threonine and valine. For example tryptophan may be substituted with serine. Alternative substitutions include alanine or valine. In addition, the invention relates to a variant of SEQ ID NO: 2 having an equivalent modification. A variant of SEQ ID NO: 2 may be a naturally occurring variant seen in alternative strains of *Cephalosporium acremonium* or a DAOC/DACS derived from other bacterial or fungal species. A variant may also be a non-naturally occurring variant or chimeric enzyme as described in more detail above. The equivalent amino acid to tryptophan at position 82 of SEQ ID NO: 2 can be identified by aligning a variant polypeptide with the sequence of SEQ ID NO: 2 and

thus to identify the equivalent amino acid of any such variant to tryptophan at position 82 of SEQ ID NO: 2. Computer programs that may be used to align a polypeptide with SEQ ID NO: 2 are discussed in more detail below. The equivalent amino acid is modified to reduce the hydroxylation activity of the DAOCS/DACS enzyme, for example tryptophan may be substituted by serine or alanine.

As before, a modified DAOC/DACS polypeptide demonstrates reduced hydroxylation activity compared with the polypeptide not so modified.

In a preferred embodiment, a DAOC/DACS polypeptide incorporates mutation of methionine at position 306 of SEQ ID NO: 2 and mutation of tryptophan at position 82 of SEQ ID NO: 2 to reduce the hydroxylation activity.

A DAOC/DACS modified in accordance with the present invention to reduce the hydroxylation activity may be further modified such that the ring expanding activity is increased when compared with a DAOC/DACS not so modified. A variant modified in accordance with the present invention to reduce the hydroxylation activity may be further modified such that the ring expanding activity is increased when compared with the variant not so modified. An increase in the ring expanding activity is an increase in the ability of the enzyme to convert of a 5-membered thiazolidine ring, for example, of a penicillin to a 6-membered ring, for example, of DAOC. The ring expanding activity may be increased to at least 105%, 150%, 200%, 250%, 300%, 350% or 400% of the enzyme not so modified.

The activity of the DAOC/DACS for its natural substrate or a substrate other than its natural substrate may be increased. For example, the modification enhances the activity of the enzyme for its natural substrate penicillin N. The modification may also introduce or enhance the activity of the enzyme for a substrate other that its natural substrate such as penicillin G, penicillin V or adipyl-7-aminopenicillanoic acid (adipyl-6-APA). The substrate specificity of the enzyme may therefore be altered. In particular, the side chain selectivity of the enzyme may be altered. Typically, the side chain selectivity is broadened so that the enzyme accepts an increased number of substrates with different side chains. In another embodiment, the side chain selectivity is changed so that the enzyme accepts a substrate with a different side chain. Examples of modifications that alter the substrate specificity or side chain selectivity of the equivalent DAOCS enzyme may be found in the art (Lee, H. J. *et al,* J. Biochem. Biophys. Res. Commun., 2000; 267: 445-448; Dubus, A. *et*

al, Cell. Mol. Life Sci., 2001; 58: 835-843; and Wei, C.-L. *et al*, Appl. Microbiol. Biotech., 2003; 69: 2306-2312).

The modification may increase the activity of an enzyme for a substance other than its natural substrate when compared with the activity of the enzyme not so modified for the substance other than its natural substrate. The modified DAOC/DACS in accordance with the invention may have enhanced catalytic activity or increased specificity for another substrate such as penicillin G or adipyl-6-APA.

The activity of an enzyme can be monitored *in vitro* or *in vivo* for example in accordance with the methods which are described in more detail below. In particular, assays may be carried out to monitor activity of the enzyme by monitoring for production of the ring-expanded product such as phenylacetyl-7-ADCA from the substrate such as penicillin G. Assays may also be carried out to measure the conversion of the cosubstrate 2-oxoglutarate (Lee, H. J. *et al,* J. Biochem. Biophys. Res. Commun., 2000; 267: 445-448). Control experiments using the DAOC/DACS or variant not so modified can be carried out to establish whether a modified enzyme demonstrates increased ring expanding activity of the substrate such as penicillin G by monitoring for higher levels of the ring expanded product such as phenylacetyl-7-ADCA.

Methods known in the art may be used to monitor the hydroxylation activity of the enzyme. Typically, High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR) and/or mass spectrometric analysis are used (Lloyd, M. D. *et al*, J. Mol. Biol., 1999; 287: 943-960). A spectrophotometric assay may also be used (Dubus, A. *et al*, Cell. Mol. Life Sci., 2001; 58: 835-843).

Preferably, the ring expanding activity of DAOC/DACS is modified by mutation or deletion within the C-terminal region of the polypeptide or through substitution within the ring expanding active site of the enzyme. Preferably, the modified polypeptide comprises the amino acid sequence of SEQ ID NO: 2 which is modified by mutation or deletion within the C-terminal region of the polypeptide or through substitution within the ring expanding active site of the enzyme, for example to improve the specificity for penicillin G as a substrate or a variant sequence of SEQ ID NO: 2 having a similar modification.

Modification of Asparagine at position 305

In one aspect of the invention, a DAOC/DACS polypeptide modified to reduce the hydroxylation activity in accordance with the invention further incorporates mutation of asparagine at position 305 of SEQ ID NO: 2 to increase the ring expanding activity. The asparagine may be substituted with glutamine. Preferably, the asparagine is substituted with a hydrophobic amino acid. Hydrophobic amino acids are glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, cysteine, methionine, serine and threonine. For example asparagine may be substituted with leucine. Alternative substitutions may be made. In addition, the invention relates to a variant of SEQ ID NO: 2 having an equivalent modification. A variant of SEQ ID NO: 2 may be a naturally occurring variant seen in alternative strains of *Cephalosporium acremonium* or a DAOC/DACS derived from other bacterial or fungal species. A variant may also be a non-naturally occurring variant or chimeric enzyme as described in more detail above. The equivalent amino acid to asparagine as position 305 of SEQ ID NO: 2 can be identified by aligning a variant polypeptide with the sequence of SEQ ID NO: 2 and thus to identify the equivalent amino acid of any such variant to asparagine at position 305 of SEQ ID NO: 2. Computer programs that may be used to align a polypeptide with SEQ ID NO: 2 are discussed in more detail below. The equivalent amino acid is modified to increase the ring expanding activity of the modified DAOCS/DACS enzyme, for example asparagine may be substituted by leucine.

As before, the modified DAOC/DACS polypeptide demonstrates improved capacity to ring expand a natural or non-natural substrate, for example penicillin N or penicillin G, compared to the polypeptide not so modified.

C-Terminal Deletion

In another aspect, a DAOC/DACS modified to reduce hydroxylation activity in accordance with the invention further incorporates a deletion within the C-terminal region of the polypeptide. The deletion may comprise deletion of from 1 to 26 amino acids, preferably deletion of from 5 to 20 amino acids from the very C-terminus of the polypeptide. The deletion may be within the C-terminal region such

that the very C-terminal amino acid or acids are retained but that one or more amino acids N-terminal to the C-terminal amino acid are deleted within a region up to 26 amino acids more preferably up to 25, 20, 15, 10 or 5 amino acids from the very C-terminus of the polypeptide.

In more detail, with reference to the amino acid sequence of SEQ ID NO: 2, preferred modifications include deletion of the C-terminal amino acids to result in a polypeptide of 310, 312, 317, 320 or 328 amino acids in length. Alternatively, the deletion may comprise a deletion within the C-terminal, for example, deletion of the amino acids 310 to 315 or 310 to 323.

The C-terminal deletion typically increases the ring expanding activity of the enzyme. In addition to this deletion, the C-terminus of the enzyme may be modified by the attachment of additional amino acids. For example, the extension can improve the stability of the enzyme or improve purification of the enzyme. The extension is typically short, for example from 1 to 5 amino acids in length. The extension may be a homopolymer, for example of alanine or leucine, or comprise different amino acids. Typical examples include AAAAA, LLLLL and AALAA.

Additionally, the invention relates to a variant of SEQ ID NO: 2 having an equivalent C-terminal modification to those described above. A variant of SEQ ID NO: 2 may be a naturally occurring variant seen in alternative strains of *Cephalosporium acremonium* or a DAOC/DACS derived from other bacterial or fungal species. A variant may also be a non-naturally occurring variant or chimeric enzyme as described in more detail above. The deletions and modifications described above may be made at or within the C-terminus of such variant polypeptides.

A modified DAOC/DACS demonstrates improved activity in the ring-expansion of a natural or non-natural substrate, for example penicillin N or penicillin G, compared to the corresponding amino acid sequence not so modified.

In an especially preferred embodiment, DAOC/DACS incorporates mutation of asparagine at position 305 of SEQ ID NO: 2 and C-terminal deletion to increase the ring expanding activity.

A modified peptide in accordance with the present invention may incorporate one or more of the modifications described for example modification of methionine at position 306, modification of asparagine at position 305 and deletion within the C-

terminus. In a particularly preferred embodiment, a modified polypeptide in accordance with the invention incorporates substitution of methionine at position 306 with isoleucine, substitution of asparagine at position 305 with leucine and a deletion of 23 amino acids from the very C-terminus.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 85%, e.g. 80%, 95%, 98% or 99%, by weight of the polypeptide in the preparation is a polypeptide of the invention.

Polypeptides of the invention may be modified for example by the addition of histidine residues to assist their identification or purification or by the addition of a signal sequence to promote their secretion from a cell where the polypeptide does not naturally contain such a sequence. It may be desirable to provide the polypeptides in a form suitable for attachment to a solid support. For example the polypeptides of the invention may be modified by the addition of a cysteine residue.

A polypeptide of the invention above may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g. ^{125}I, ^{35}S, enzymes, antibodies, polynucleotides and linkers such as biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of a polypeptide of the invention in a sample.

The proteins and peptides of the invention may be made synthetically or by recombinant means. The amino acid sequence of proteins and polypeptides of the invention may be modified to include non-naturally occurring amino acids or to increase the stability of the compound. When the proteins or peptides are produced by synthetic means, such amino acids may be introduced during production. The proteins or peptides may also be modified following either synthetic or recombinant production.

The proteins or peptides of the invention may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the

C to N orientation. This is conventional in the art for producing such proteins or peptides.

A number of side chain modifications are known in the art and may be made to the side chains of the proteins or peptides of the present invention. Such modifications include, for example, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$, amidination with methylacetimidate or acylation with acetic anhydride.

The polypeptides of the invention may be introduced into a cell by *in situ* expression of the polypeptide from a recombinant expression vector. The expression vector optionally carries an inducible promoter to control the expression of the polypeptide.

Such cell culture systems in which polypeptides of the invention are expressed may be used in assay systems.

A polypeptide of the invention may be produced in large scale following purification by any protein liquid chromatography system after recombinant expression as described below. Preferred protein liquid chromatography systems include FPLC, AKTA systems, the Bio-Cad system, the Bio-Rad BioLogic system and the Gilson HPLC system.

A polynucleotide of the invention encodes a polypeptide according to the invention. A polynucleotide of the invention encodes a DAOC/DACS or variant modified to reduce the hydroxylation activity. A polynucleotide of the invention is typically a contiguous sequence of nucleotides which is capable of hybridising selectively with the coding sequence of SEQ ID NO: 2 or to the sequence complementary to that coding sequence. Polynucleotides of the invention include variants of the coding sequence of SEQ ID NO: 1 which encode the amino acid sequence of SEQ ID NO: 2. Such polynucleotides additionally incorporate one or more modification to encode a modified polypeptide as described in more detail above.

A polynucleotide for use in the invention and the coding sequence of SEQ ID NO: 1 can hybridize at a level significantly above background. Background hybridization may occur, for example, because of other cDNAs present in a cDNA library. The signal level generated by the interaction between a polynucleotide of the invention and the coding sequence of SEQ ID NO: 1 is typically at least 10 fold,

preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of SEQ ID NO: 1. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P. Selective hybridization is typically achieved using conditions of medium to high stringency (for example 0.03M sodium chloride and 0.003M sodium citrate at from about 50°C to about 60°C).

A nucleotide sequence capable of selectively hybridizing to the DNA coding sequence of SEQ ID NO: 1 or to the sequence complementary to that coding sequence will be generally at least 80%, preferably at least 90% and more preferably at least 95%, homologous to the coding sequence of SEQ ID NO: 1 or its complement over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides or, indeed, over the full length of the coding sequence. Thus there may be at least 85%, at least 90% or at least 95% nucleotide identity over such regions.

Any combination of the above mentioned degrees of homology and minimum size may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher homology over longer lengths) being preferred. Thus for example a polynucleotide which is at least 85% homologous over 25, preferably over 30, nucleotides forms one aspect of the invention, as does a polynucleotide which is at least 90% homologous over 40 nucleotides.

For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology, for example used on its default settings (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (such as identifying equivalent residues or corresponding sequences (typically on their default settings)), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S.F *et al* (1990) J Mol Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold

(Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSP's containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

Polynucleotides of the invention may be used to produce a primer, e.g a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40

nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as a DNA polynucleotide and primers according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques. The polynucleotides are typically provided in isolated and/or purified form.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the DAOC/DACS gene which it is desired to clone, bringing the primers into contact with DNA obtained from a suitable cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al*, 1989.

Polynucleotides or primers of the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using techniques known *per se*.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector such as a cloning vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus polynucleotides of the invention may be made by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered

from the host cell. Suitable host cells are described below in connection with expression vectors.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. Such expression vectors can be used to express the polypeptide of the invention.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. Multiple copies of the same or different modified DAOC/DACS genes may be introduced into the vector.

Such vectors may be transformed into a suitable host cell to provide for expression of a polypeptide of the invention. Thus, a polypeptide according to the invention can be obtained by cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression of the polypeptide, and recovering the expressed polypeptide. More preferably, such host cells may be used in the production of 7-ADCA.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example a tetracycline resistance gene. Promoters and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. Multiple copies of the same or different modified DAOC/DACS gene in a single expression vector, or more than one expression vector each including a modified DAOC/DACS gene which may be the same or different may be transformed into the host cell.

In a preferred aspect of the invention, the promoter sequence is a promoter sequence derived from an antibiotic-producing organism and in particular a fungal organism such as from *Cephalosporium, Aspergillus* or *Penicillium*. In a preferred embodiment, a promoter sequence derived from *Cephalosporium acremonium* is used. In another preferred embodiment, a promoter sequence derived from *Penicillium chrysogenum* is used such as the *ipnA*, *pcbC* or *pIPNS* promoter

sequence. In further embodiments, other fungal promoters such as *alcR*, *pacC* or *gpd* are used. For expression in bacterial cells, a T7 or *trc* promoter is typically used.

Suitable expression vectors for the expression of the modified DAOC/DACS gene include: pPenFTSO, pTS, pPen/Ceph-1 and pTS-8 (Crawford, L., *et al*, Biotechnology, 1995; 13(1): 58-62); pALC88 (Valesco, J., *et al*, Nature Biotechnology, 2000; 18: 857-861); pAMEX, pSAF, pAMEX*lat* or pSAF*lat* (Chary, V. K., *et al*, Appl. Microbiol. Biotechnol., 2000; 53:282-288); and pJB500, pJS63, pRH1090, pRH103, pRH107 and pRH1091 (Baldwin, J. E., et al, Bioorg. Med. Chem. Lett., 1992; 2: 663-668).

Host cells transformed (or transfected) with the polynucleotides or vectors for the replication and expression of polynucleotides of the invention will be chosen to be compatible with the said vector. Preferably, the host cells will be antibiotic-producing cells such as fungal cells for example *Cephalosporium acremonium (Acremonium chrysogenum)*, *Streptomyces, Nocardia or Penicillium* cells. Typically, *Penicillium chrysogenum*, *Streptomyces clavuliger*us, *Streptomyces lividans*, *Cephalosporium acremonium, Acremonium nidulans* or *Nocardia lactamdurans* cells are used. Preferably, *Penicillium chrysogenum* cells are used. Alternatively, they may be cells of bacterial origin such as *E.coli*, particularly, for the production *in vitro* of the modified polypeptide of the invention. Any cell with a λ DE3 lysogen, for example BL21 (DE3), JM109 (DE3) andB834 (DE3), can express a vector comprising the T7 promoter. *E. coli* JM109 and NM554 are preferably used for vectors comprising the *trc* promoter. Other expression systems known in the art, for example pichia yeast, may also be used.

An enzyme modified in accordance with the invention to reduce the hydroxylation activity is useful in the ring-expansion of penicillin, such as penicillin G. Such ring-expansion may be carried out *in vitro* or *in vivo*. Such ring-expansion may be used as part of a process for the production of 7-ADCA and derivatives thereof.

An enzyme modified in accordance with the invention to reduce the hydroxylation activity may be used to ring expand semisynthetic penicillins to their cephalosporin form. For example, amoxycillin could be converted to cephadroxil and ampicillin could be converted to cephalexin. Alternatively an enzyme modified in accordance with the invention to reduce the hydroxylation activity can be used in

the conversion 6-APA to 7-ADCA. An enzyme modified in accordance with the invention to reduce the hydroxylation activity can be used to convert penicillin G to cephalosporin G or penicillin V to the corresponding cephalosporin. An enzyme modified according to the invention to reduce hydroxylation activity may be used to convert adipyl-6-APA to produce adipyl-7-ADCA. The enzymes can be used as part of a sequence of reactions or enzymes to produce compounds of interest such as 7-ACA.

An enzyme in accordance with the invention can be used *in vitro*, for example, bound to an immobile substrate. The enzyme can be immobilised through the addition of a binding sequence such as a His-tag or maltose binding site or by using a general immobiliser. The immobilised enzyme can then be used in the ring expansion and conversion reactions described above.

In an alternative aspect of the invention, host cells are provided such as *Cephalosporium or Penicillium* cells as described above, transformed with polynucleotide encoding a modified DAOC/DACS of the invention for use in one or more of the conversions described above.

In a particularly preferred aspect of the present invention, the modified enzyme is expressed in a host cell which is capable of producing penicillin so that the penicillin within the host cell is converted to 7-ADCA. In another preferred embodiment, the modified enzyme is expressed in a host cell that produces penicillin G so that the penicillin G is converted into phenylacetyl-7-ADCA. The side chain of phenylacetyl-7-ADCA may be subsequently removed by hydrolysis to produce 7-ADCA. This hydrolysis reaction may be carried out enzymatically using standard methods in the art (Crawford, L., *et al*, Bio-technology, 1995; 13(1): 58-62; Sio, C. F. *et al*, Eur. J. Biochem., 2002; 269(18): 4495-4504; Robin J., *et al*, Appl. Microbiol. Biotechnol., 2001; 57(3): 357-362; and Xie, Y. I. *et al*, J. Chromatogr. A., 2001; 908(1-2): 273-291).

In yet another preferred embodiment, the modified enzyme is expressed in a host cell which is capable of producing penicillin, for example penicillin G, and is fed on adipic acid (adipate feed; Crawford, L., *et al*, Bio-technology, 1995; 13(1): 58-62). The adipate feed is typically incorporated into the cell as the penicillin side chain precursor, adipyl-6-APA. The prefix adipyl- used herein is interchangeable with the prefix adipoyl-. Expression of the modified enzyme of the invention results

in the ability of the cell to convert adipyl-6-APA to adipyl-7-ADCA. The side chain of adipyl-7-ADCA may be subsequently removed by hydrolysis to produce 7-ADCA. This hydrolysis reaction may be carried out enzymatically using standard methods in the art (Crawford, L., *et al*, Bio-technology, 1995; 13(1): 58-62; Sio, C. F. *et al*, Eur. J. Biochem., 2002; 269(18): 4495-4504; Robin J., *et al*, Appl. Microbiol. Biotechnol., 2001; 57(3): 357-362; and Xie, Y. I. *et al*, J. Chromatogr. A., 2001; 908(1-2): 273-291), for example by acylase.

Host cells may be selected which naturally produce penicillin. Such host cells may be cultured in media which promote production of penicillin. Such host cells may additionally be transformed with expression vectors encoding additional enzymes required for the production of penicillin which may subsequently be ring-expanded using a modified enzyme in accordance with the present invention.

The activity of a modified enzyme in accordance with the invention may be monitored by carrying out assays *in vitro* or *in vivo*, that is within a host cell, to monitor for ring-expanding activity of the enzyme. Such assays may include monitoring for the production of 7-ADCA from penicillin, for example phenylacetyl-7-ADCA from penicillin G, either *in vitro* or using an organism which is capable of producing penicillin such as *Penicillium chrysogenum*.

The activity of the enzyme may, for example, be monitored by monitoring the conversion of a co-factor (2-oxoglutarate) to the oxidized product succinate (Lee, H. J. *et al,* J. Biochem. Biophys. Res. Commun., 2000; 267: 445-448). Alternatively, after reaction with the enzyme, any residual penicillin could be digested away using a penicillinase (Baldwin, J. E. *et al,* Biochem. J., 1987; 245: 831-841). The antibiotic effect of the remaining cephalosporin is assayed, for example, by looking at the kill zone of samples placed in wells cut into an agar plate seeded with an indicator organism. Alternatively, activity of transformants can be tested by growing in medium containing phenyl acetic acid and assaying for the production of cephalosporin by HPLC.

Examples

Example 1 – Hydroxylation reducing mutations in DAOCS/DACS

Three point mutations (W82S, W82A and M306I) were generated in *Cephalosporium acremonium* DAOC/DACS. Generation of the point mutations was carried out using the QuikChange® Kit. The *cefEF* gene encoding for DAOC/DACS had previously been subcloned into pET-24a by Kirsty S. Hewitson (Hewitson, K. *Part II Thesis*, University of Oxford, 1997). This was subcloned into pGEM-T using the *Nde* 1 and *BamH* 1 restriction sites, in order to aid with the mutagenesis. pGEM-T is a smaller vector than pET-24a (3000 bp *vs.* 5677 bp) leading to improved PCR efficiency. The primers used are shown in Table 1.

Table 1 – Primer used for generating point mutations in DAOC/DACS

W82S
Forward Primer
5'-GCC CTC GAG **TCG** GAG AGC ACC G-3'
Reverse Primer
5'-C GGT GCT CTC CGA CTC GAG GGC-3'
W82A
Forward Primer
5'-GCC CTC GAG **GCG** GAG AGC ACC G-3'
Reverse Primer
5'-C GGT GCT CTC **CGC** CTC GAG GGC-3'
M306I
Forward Primer
5'-C TAT GTC AAC **ATA** CGG AGG G-3'
Reverse Primer
5'-C CCT CCG **TAT** GTT GAC ATA G-3'
Bold residues denote site of mutation.

Mutagenesis was carried out by initially heat denaturating the double stranded DNA template. Mutagenic primers were then annealed and the whole

plasmid amplified using *PfuTurbo*® polymerase. This polymerase is used for whole plasmid amplification due to its proof reading ability and lack of extendase or strand displacement activity. Following amplification of the required mutated plasmid, the parental wild type plasmid was digested using *Dpn* 1. This enzyme is able to identify DNA containing methylated adenine bases and subsequently degrade it. DNA produced by any dam^+ strain such as *E. coli* is always methylated in this way, whereas DNA produced by the PCR reaction is not, so the wild type plasmid will be degraded but the required mutated plasmid will be retained. The mutated DNA is then transformed into XL1-Blue *E. coli*. Following confirmation of the presence of the desired mutation by automated DNA sequencing (Department of Biochemistry, University of Oxford), the mutated gene was subcloned into the pET-24a expression vector.

The DAOC/DACS mutants were produced using the above protocol and expression trials subsequently carried out. DAOC/DACS mutants were expressed in BL21(DE3) cells (Novagen®, VWR International) which are derived from B834 cells. The expression system used was pET (Novagen®, VWR International) which comprises the pET24a vector. Freshly transformed BL21(DE3) colonies were grown in 2TY (5 ml) supplemented with 70 μg/ml kanamycin, at 37°C overnight. Starter culture (2 ml) was used to inoculate 100 ml 2TY (supplemented with kanamycin as before) and grown at 30°C until an OD_{600} of *ca.* 0.8 was reached. The cells were then induced with IPTG (500 μM) and growth continued for 4 hours. The cells were harvested, the cell pellet resuspended in 50 mM Tris-HCl, pH 7.5 (3 ml) and the resulting suspension lysed by sonication. The soluble and whole cell extracts were analysed by SDS-PAGE. All the point mutant enzymes exhibited good levels of soluble expression. A large scale growth (6 x 700 ml 2TY) was carried out to produce *ca.* 25 g of cells of each mutant. Protein was extracted and purified using a two column protocol (DEAE anion exchange followed by S75 Superdex™ gel filtration) to give protein of *ca.* 85 % purity by SDS-PAGE analyses. Mass spectrometry data was collected to confirm the presence of the required mutation (Table 2). CD analysis was carried out to confirm no gross changes to structure had resulted from the mutations (Figure 1). The mass spectrometric data and CD spectra showed that the required mutations were present, no unwanted mutations were present, and no gross changes in secondary structure occurred.

Table 2 - Mass spectrometric data for wild type DAOC/DACS, and W82A, W82S and M306I mutants.

Enzyme	Expected mass (Da)	Observed mass (Da)
Wild Type DAOC/DACS	36348.0	36348 ± 2
W82A	36232.6	36233 ± 5
W82S	36248.6	36253 ± 6
M306I	36329.8	36334 ± 9

W82A, W82S and M306I mutants were assayed for hydroxylation activity using standard incubation conditions (Baldwin, J. E. *et al,* Biochem. J., 1987; 245: 831-841) and the conversion of DAOC to DAC monitored by HPLC (Table 3). Mutation of Trp-82 to alanine appeared to have little effect on the hydroxylation of DAOC (Table 3). However, mutation of Trp-82 to serine and Met-306 to isoleucine appears to almost completely abolished hydroxylation activity (Table 3).

Table 3 - Rate of conversion of DAOC to DAC as determined by HPLC analysis.

Enzyme	Rate of DAOC hydroxylation (nmole/min/mg)
Wild type DAOC/DACS	207.50
W82A	146.47
W82S	36.62
M306I	None Detectable

Results are based on at least duplicate reading. Standard deviations are *ca.* 10% of values.

The effect of mutation on 2-oxoglutarate conversion in the presence of DAOC was also investigated. The standard hydroxylase reaction conditions (Baldwin, J. E. *et al,* Biochem. J., 1987; 245: 831-841) were used and the effect of 2-oxoglutarate stimulation on the three mutant enzymes determined (Table 4).

Mutation of Trp-82 to alanine or serine had little effect on 2-oxoglutarate stimulation in the presence of 10 mM DAOC (Table 4). This suggests mutation of Trp-82 to serine significantly reduced oxidation of DAOC, yet the substrate was still binding in an appropriate manner to stimulate cosubstrate oxidation. Mutation of Trp-82 is sufficient to prevent complete oxidation of the respective prime substrate but substrate binding must still be occurring due to stimulation of 2-oxoglutarate. Trp-82 therefore appears to be a crucial residue in controlling penicillin/cephalosporin substrate selectivity.

Mutation of Met-306 to isoleucine appeared to abolish hydroxylation of DAOC. 2-Oxoglutarate conversion is also not stimulated in the presence of DAOC suggesting prime substrate is not binding (Table 4). This suggests that Met-306 is vital for the hydroxylase activity of the bifunctional DAOC/DACS.

Table 4 - Rate of 2-oxoglutarate conversion in the presence of 10 mM DAOC.

Enzyme	**Rate of 2-oxoglutarate conversion (nmole/min/mg)**
Wild type DAOC/DACS	165.44
W82A	180.62
W82S	148.75
M306I	5.76

Results are corrected for uncoupled turnover rates in the absence of prime substrate. Standard deviations are *ca.* 15% of values.

The three mutant enzymes were also assayed for their ability to catalyse oxidation of the two prime substrates and for the effect of mutagenesis on 2-oxoglutarate conversion in the presence of the two substrates. Mutation of 2-oxoglutarate dependent oxygenases can often cause uncoupling of 2-oxoglutarate from prime substrate oxidation. This is due to incorrect or inappropriate binding of the prime substrate causing stimulation of 2-oxoglutarate conversion but not oxidation of the prime substrate.

Assays for determining ring expansion activity were carried out using penicillin G as a substrate. This is a commercially available analogue of penicillin

N, with a phenylacetyl side chain instead of an *L*-α-aminoadipoyl side chain. Penicillin G undergoes ring expansion in the presence of DAOC/DACS to produce phenylacetyl-7-aminocephalosporinic acid (G-7-ADCA).W82A, W82S and M306I mutants were assayed by the standard HPLC conditions using penicillin G as the prime substrate (Table 5).

Mutation of Trp-82 to alanine resulted in almost complete loss of activity for penicillin conversion (Table 5). However mutation of this residue to serine had little effect on activity (Table 5). Mutation of Met-306 to isoleucine reduced activity by *ca.* 50% (Table 5).

Table 5 - Rate of conversion of penicillin G to G-7-ADCA as determined by HPLC analysis.

Enzyme	**Rate of penicillin G oxidation (nmole/min/mg)**
Wild type DAOC/DACS	50.34
W82A	2.76
W82S	22.15
M306I	29.70

Results are based on at least duplicate reading. Standard deviations are *ca.* 10% of values.

The effect of mutation upon stimulation of 2-oxoglutarate conversion was also investigated. 2-Oxoglutarate conversion was monitored using [1-^{14}C]-2-oxoglutarate. The reaction was carried out in a sealed vial and $^{14}CO_2$ emission absorbed by hyamine hydroxide. The amount of radioactivity released into the hyamine hydroxide, and hence the amount of 2-oxoglutarate converted into succinate and carbon dioxide, was determined using a scintillation counter The mutant enzymes were added to the standard radioactive 2-oxoglutarate assay mix (Lee, H. J. *et al,* J. Biochem. Biophys. Res. Commun., 2000; 267: 445-448) and the effect of addition of penicillin G to the reaction mix measured (Table 6).

Mutation of Trp-82 or Met-306 has little effect on 2-oxoglutarate activity (Table 6). The stimulation of 2-oxoglutarate upon addition of penicillin G indicated

that the prime substrate binds to all the mutant enzymes. The reduced level of penicillin oxidation observed by the HPLC assay for the W82A mutant may be indicative of incorrect substrate binding. Penicillin G can bind sufficiently for 2-oxoglutarate conversion to be stimulated, but not in the correct manner for the prime substrate itself to be oxidised, *i.e.* mutation caused uncoupling of 2-oxoglutarate conversion from that of penicillin G oxidation.

Table 6 - Rate of 2-oxoglutarate conversion in the presence of 10 mM penicillin G.

Enzyme	**Rate of 2-oxoglutarate conversion (nmole/min/mg)**
Wild type DAOC/DACS	39.88
W82A	77.83
W82S	76.38
M306I	40.87

Results are corrected for uncoupled turnover rates in the absence of prime substrate. Standard deviations are *ca.* 15% of values.

The effect of the point mutations on the activity of DAOC/DACS are summarised below in Table 7.

Table 7: Activity assay results for wild type and mutant DAOC/DACS with penicillin G and DAOC.

Enzyme	Substrate:	Penicillin G		DAOC	
	Assay:	HPLC	2-OG	HPLC	2-OG
Wild Type DAOC/DACS		100	79.2	100	79.7
W82A		5.5	154.6	70.6	87.0
W82S		44.0	151.7	17.6	71.7
M306I		59.0	81.2	0	2.8

Results are normalised to wild type activity with penicillin G or DAOC as determined by HPLC analysis (50.34 and 207.50 nmole/min/mg respectively). Assay types; HPLC, Prime substrate oxidation as determined by HPLC analysis; 2-OG, 2-oxoglutarate stimulation as determined by radioactive 2-oxoglutarate assay.

Example 2 – C terminal deletion of DAOC/DACS

C-Terminal truncation mutants were constructed using PCR. The truncated genes were amplified using the wild type DAOC/DACS gene in pGEM-T as a template. Primers were designed encoding the wild type N-terminal sequence, and C-terminal primers contained the truncated gene followed by a stop codon and appropriate restriction sites (Table 8).

Table 8 – Primer used to generate C terminal truncations of DAOC/DACS

Wild type and mutant N-terminus

5'-**CAT ATG** ACT TCC AAG GTC CCC-3'

NdeI

Wild type C-terminus (333 residues)

5'-**GGA TCC** *AAG CTT* **CTA** AGT GGC TAT AGG-3'

BamH1 *HindIII* **Stop**

Δ310 truncation C-terminus

5'-**GGA TCC** *AAG CTT* **CTA** CTT ATC CCT CCG C-3'

BamH1 *HindIII* **Stop**

Δ312 truncation C-terminus

5'-**GGA TCC** *AAG CTT* **CTA** CGC CGG CTT ATC CC-3'

BamH1 *HindIII* **Stop**

Δ317 truncation C-terminus

5'-**GGA TCC** *AAG CTT* **CTA** AGC CGC CTC CGC-3'

BamH1 *HindIII* **Stop**

Δ320 truncation C-terminus

5'-**GGA TCC** *AAG CTT* **CTA** CGC GGG GAC AGC-3'

BamH1 *HindIII* **Stop**

Δ328 truncation C-terminus

5'-**GGA TCC** *AAG CTT* **CTA** AGC TGC GGT AG-3'

BamH1 *HindIII* **Stop**

Following PCR amplification of the required truncated gene using *Vent* polymerase, addition of overhanging adenine bases onto the 3' of each strand was carried out using *Taq* polymerase. *Taq* polymerase was not used for the initial

amplification due to its high error rate, however the adenine overhanging ends are required for subcloning into the pGEM-T vector. Following confirmation of the desired sequence in the pGEM-T vector by automated DNA sequencing (Department of Biochemistry, University of Oxford), the mutant gene was treated with *Nde* 1 and *BamH* 1 restriction enzymes and subcloned into the pET-24a vector, which had been treated with the same enzymes.

Following confirmation of the required truncation, expression trials were carried out on the constructs. A freshly transformed BL21(DE3) colony was grown in 5 ml 2TY containing 70 μg/ml kanamycin at 37°C overnight. Starter culture (2 ml) was used to inoculate 100 ml 2TY containing kanamycin as before. This culture was grown at 30°C until an OD_{600} of *ca.* 0.6 was reached. The cells were induced with IPTG (500 μM) and the growth continued for 4 hours before harvesting. The cell pellet was resuspended in 50 mM Tris-HCl, pH 7.5 (3 ml), and the cells lysed by sonication. Soluble and whole cell extracts of the samples were analysed by SDS-PAGE.

The expression trials showed that the wild type and the Δ310 and Δ328 mutants gave good levels of soluble expression. Δ320 gave small amounts of soluble expression whereas Δ312 gave entirely insoluble expression and Δ317 did not express at all.

In order to increase the expression levels of the Δ312 and Δ317 mutants, a growth was carried out at a lower temperature. The overnight culture was grown in the same manner, but following inoculation of the 100 ml flask, the temperature was lowered to 17°C. The cells were induced with 250 or 500 μM IPTG to determine whether the concentration of inductant has an effect on expression levels. The growth was then carried out in the same manner and the resulting cell pellets analysed by SDS-PAGE. However, carrying out the growth at a lower temperature did not appear to aid the expression of either the Δ312 or Δ317 truncation mutants. The Δ317 mutant was also cotransformed with the *E. coli* chaperone GroESL and an expression trial carried out in the usual manner. Unfortunately the cotransformation did not appear to aid in the expression of the mutant.

A large scale growth (6 x 700 ml 2TY) of the Δ310, Δ320 and Δ328 mutants was carried out. The growth of the Δ310 and Δ328 mutants gave good expression levels and the enzymes were purified using a three step protocol: anion exchange

(DEAE followed by ResourceQ) and gel filtration (S75 Superdex™). The large scale growth of Δ320 produced only small amounts of soluble protein and attempts to purify the required protein were unsuccessful.

CD analysis (Figure 2) and mass spectrometric (Table 9) data were collected to confirm the presence of the correct mutation and that truncation had not caused any gross change in the folding of the enzyme.

Table 9 - Mass spectroscopic data for wild type, Δ310 and Δ328 DAOC/DACS mutants.

Enzyme	Expected mass (Da)	Observed mass (Da)
Wild Type DAOC/DACS	36348.0	36348 ± 2
Δ310	34447.6	34451 ± 2
Δ328	35965.3	35968 ± 8

The Δ310 and Δ328 truncation mutants were assayed for their ability to oxidise penicillin G and DAOC, the prime substrates for the two reactions carried out by DAOC/DACS. The activity of the two enzymes were analysed by HPLC (prime substrate conversion) and stimulation of the 2-oxoglutarate cosubstrate (Table 9). Standard reaction conditions were used (Lloyd, M. D. *et al,* J. Mol. Biol., 1999; 287: 943-960; Lee, H. J. *et al,* J. Biochem. Biophys. Res. Commun., 2000; 267: 445-448). Truncation of DAOC/DACS to 310 residues caused a two-fold increase in the rate of penicillin G oxidation and a two-fold decrease in DAOC hydroxylation (Table 9). Truncation to 328 residues caused a slight decrease in penicillin G oxidation and DAOC hydroxylation (Table 10).

Table 10: Rate of oxidation (nmole/min/mg) of prime substrate (HPLC) and 2-oxoglutarate stimulation (2-OG).

Enzyme	Substrate:	Penicillin G		DAOC	
	Assay:	HPLC	2-OG	HPLC	2-OG
Wild type DAOC/DACS		50.34	39.88	207.50	165.44
Δ310		102.19	53.38	103.09	139.84
Δ328		32.22	13.54	92.76	89.73

2-Oxoglutarate rates are corrected for conversion in the absence of prime substrate. Rates are based on at least duplicate readings. Standard deviations are *ca.* 15% and 10% for 2-oxoglutarate and prime substrate conversion, respectively.

Example 3 – Point mutation of methionine 306 and asparagine 34 and C terminal deletion of DAOC/DACS

Three mutants (N305L, Δ310/M306I and Δ310/M306I/N305L) mutants were constructed in the manner described previously using the primers in Table 11.

Table 11 – Primer used in generation of the N305L, Δ310/M306I and Δ310/M306I/N305L mutants

Wild type N-Terminus

5'-*CAT ATG* ACT TCC AAG GTC CCC-3'
NdeI

Δ310/M306I C-terminus

5'-*GGA TCC* AAG CTT *CTA* CTT ATC CCT CCG **TAT** GTT G-3'
BamHI HindIII *Stop*

Δ310/M306I/N305L C-terminus

5'-*GGA TCC* AAG CTT *CTA* CTT ATC CCT CCG **TAT GAG** G-3'
BamHI HindIII *Stop*

N305L

Forward primer
5'-GC GGG AAC TAT GTC **CTC** ATG CGG AGG GAT AAG CCG-3'
Reverse Primer
5'-C CGC CGG CTT ATC CCT CCG CAT **GAG** GAC ATA GTT C-3'

Bold residues denote site of mutation.

The presence of the required mutations was confirmed by automated DNA sequencing (Department of Biochemistry, University of Oxford) and expression trials carried out. Growth of a single freshly transformed BL21(DE3) colony in 5 ml 2TY supplemented with 70 μg/ml kanamycin at 37°C overnight gave a starter culture which was then used to inoculate 100 ml 2TY containing kanamycin as before. The growth was continued at 30°C until an OD_{600} of *ca.* 0.6 was reached whereupon

IPTG (500 μM) was added and the growth continued at 30°C for four hours before harvesting. Analysis of the cell pellet by SDS-PAGE showed good levels of soluble expression for all three of the enzymes. Protein purification was carried out using a two step protocol (Qseph-XL™ anion exchange and S75 Superdex™ gel filtration) to give enzyme of *ca.* 85% purity by SDS-PAGE analysis. Samples were analysed by mass spectrometry (Table 12).

Table 12 - Mass spectrometric data for the N305L, Δ310/M306I and Δ310/N305L/M306I mutants.

Enzyme	Expected mass (Da)	Observed mass (Da)
N305L	36347	36345 ± 6
Δ310/M306I	34429	34429 ± 5
Δ310/N305L/M306I	34428	34427 ± 8

The three mutants were assayed for their ability to oxidise penicillin G and hydroxylate DAOC and the effect of the substrates on 2-oxoglutarate stimulation (Table 12). As expected, mutation of Met-306 to isoleucine abolished hydroxylase activity in the Δ310/M306I and Δ310/N305L/M306I mutants (Table 13). When mutation of Met-306 was carried out in conjunction with truncation to 310 amino acids, a large increase in penicillin G oxidation activity is observed (185%). The triple mutant Δ310/N305L/M306I showed the largest activity of penicillin G oxidation with rates over 350% that of the wild type enzyme (Table 13). Mutation of Asn-305 of DAOC/DACS to leucine caused a slight increase in the rate of penicillin G oxidation relative to the wild type (Table 13). It must be noted that the N305L mutant is still a bifunctional enzyme, therefore the apparent reduction in penicillin G oxidation relative to Δ310/M306I and Δ310/N305L/M306I may be a consequence of a certain amount of deacetoxycephem product being subsequently hydroxylated and hence not appearing in the HPLC assay. This view was supported by the relatively large amounts of 2-oxoglutarate conversion compared to prime substrate oxidation (Table 13). For N305L, 2-oxoglutarate conversion appears to be occurring at a higher rate than penicillin G oxidation whereas for Δ310/M306I and

Δ310/N305L/M306I the relative rate of penicillin G oxidation is higher than that of 2-oxoglutarate.

Table 13 - Specific activity of oxidation of prime substrate (HPLC) and 2-oxoglutarate stimulation (2-OG).

Enzyme	Substrate:	Penicillin G		DAOC	
	Assay:	HPLC	2-OG	HPLC	2-OG
N305L		107	143	85	91
Δ310/M306I		185	80	3	0
Δ310/N305L/M306I		370	106	2	2

Results are normalised to wild type activity with penicillin G or DAOC as determined by HPLC analysis (50.34 and 207.50 nmole/min/mg respectively). 2-Oxoglutarate rates are corrected for conversion in the absence of prime substrate.

More detailed kinetic studies were carried out on wild type DAOC/DACS and the N305L, M306I, Δ310, Δ310/M306I and Δ310/N305L/M306I mutants. The studies were carried out using the spectrophotometric assay developed by Dr. Alain Dubus (Dubus, A. *et al*, Cell. Mol. Life Sci., 2001; 58: 835-843). This measures the increase in absorbance at 260 nm, which is a result of the formation of the double bond in the ring expanded deacetoxycephem product. The initial rate is measure in the presence of varying substrate concentration (0 – 8 mM) and the relationship between the two plotted (Figure 3). The initial rates are measured in the first few seconds of reaction so subsequent hydroxylation to the deacetylcephem product is minimised.

Kinetic analyses were carried out on the wild-type DAOC/DACS as well as the N305L, M306I, Δ310, Δ310/M306I and Δ310/N305L/M306I mutants using the spectrophotometric assay. Upon incubation of the five mutants and analysis of penicillin G conversion, the time taken for reaction to commence appeared to vary. Incubation of wild type DAOC/DACS showed a significant lag phase (Figure 4). A lag phase was also observed for the N305L mutant (Figure 5). However the mutants M306I, Δ310/M306I and Δ310/N305L/M306I all showed a drop in absorbance

before reaction commenced (Figure 6). It is unknown what is causing this drop in absorbance. The Δ310 truncation mutant appeared to exhibit a burst of activity in the first few seconds of reaction which then stopped (Figure 7). This initial burst was found to be independent of penicillin G concentration suggesting the increase in absorbance is not due to production of G-7-ADCA. Incubation of the Δ310 mutant and ascorbate produced the same burst even in the absence of penicillin G suggesting the observed rate was due to an interaction between the truncated mutant and ascorbate. Ascorbate is thought to 'activate' the enzyme, possibly by keeping the iron reduced in the ferrous state. As the concentration of ascorbate influences the observed initial rates, assays must be conducted at a defined ascorbate concentration to achieve proper comparison between the results (Dubus, A. *et al*, Cell. Mol. Life Sci., 2001; 58: 835-843). Due to this contaminating increase in absorbance it was therefore not possible to determine kinetic data for the Δ310 mutant.

The rate of penicillin G conversion for the N305L, M306I, Δ310/M306I and Δ310/N305L/M306I mutants at various penicillin G concentrations was therefore determined (Figures 8, 9, 10 and 11). Kinetic values were determined from the plots and these are summarized in Table 14. As expected, mutation M306I, which abolishes hydroxylation, decreased the rate of penicillin G oxidation relative to wild type DAOC/DACS. Truncation to 310 residues in combination with mutation of Asn-305 to leucine gave the largest increase in penicillin G conversion (Table 14). The N305L and Δ310/N305L/M306I mutants increase penicillin G oxidation by reducing the K_m value threefold. The k_{cat} values are similar to the wild type enzyme. This suggests that mutation of Asn-305 is aiding penicillin G binding to the active site rather than the actual activity of the enzyme.

Table 14 - Kinetic parameters of wild type DAOC/DACS, N305L, M306I, Δ310/M306I and Δ310/N305L/M306I enzymes using penicillin G as substrate in the presence of 100 μM 2-oxoglutarate cosubstrate as determined by the spectrophotometric assay.

Enzyme	**WT DAOC/DACS**	**Δ310/N305L/M306I**	**N305L**	**M306I**	**Δ310/M306I**
k_{cat} (s^{-1})	0.073	0.079	0.016	0.091	0.070
K_m (mM)	6.04	2.41	4.79	6.42	2.00
k_{cat}/K_m ($s^{-1}M^{-1}$)	12.0	32.8	3.3	14.2	35.0

Standard deviations are *ca.* 10% of values quoted.

CLAIMS

1. A deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) enzyme modified to reduce the hydroxylation activity of the enzyme.

2. A DAOC/DACS enzyme according to claim 1, wherein the DAOC/DACS enzyme is SEQ ID NO: 2 or a variant of SEQ ID NO: 2.

3. A DAOC/DACS enzyme according to claim 2, wherein:

(a) the methionine at position 306 of SEQ ID NO: 2 is substituted; or

(b) the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the methionine at position 306 of SEQ ID NO: 2 is substituted.

4. A DAOC/DACS enzyme according to claim 3, wherein the methionine at position 306 of SEQ ID NO: 2 or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the methionine at position 306 of SEQ ID NO: 2 is substituted by an amino acid with an aliphatic side chain.

5. A DAOC/DACS enzyme according to claim 4, wherein the amino acid with an aliphatic side chain is isoleucine.

6. A DAOC/DACS enzyme according to any one of claims 2 to 5, wherein:

(a) the tryptophan at position 82 of SEQ ID NO: 2 is substituted; or

(c) the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the tryptophan at position 82 of SEQ ID NO: 2 is substituted.

7. A DAOC/DACS enzyme according to claim 6, wherein the tryptophan at position 82 of SEQ ID NO: 2 or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the tryptophan at position 82 of SEQ ID NO: 2 is substituted by an amino acid with a non-aromatic side chain.

8. A DAOC/DACS enzyme according to claim 7, wherein the amino

acid with a non-aromatic side chain is serine.

9. A DAOC/DACS enzyme according to any one of the preceding claims, wherein the hydroxylation activity is completely abolished.

10. A DAOC/DACS enzyme according to any one of claims 2 to 9, further modified to improve the ring expanding activity.

11. A DAOC/DACS enzyme according to claim 10, wherein:

(a) the asparagine at position 305 of SEQ ID NO: 2; or

(b) the amino acid residue in the a variant of SEQ ID NO: 2 that is at an equivalent position to asparagine at position 305 of SEQ ID NO: 2 is substituted.

12. A DAOC/DACS enzyme according to claim 11, wherein the asparagine at position 305 of SEQ ID NO: 2 or the amino acid in the variant of SEQ ID NO: 2 that is at an equivalent position to the asparagine at position 305 of SEQ ID NO: 2 is substituted by a hydrophobic residue.

13. A DAOC/DACS enzyme according to claim 12, wherein the hydrophobic residue is leucine.

14. A DAOC/DACS enzyme according to any one of claims 10 to 13, wherein the enzyme comprises a deletion within the C terminus.

15. A DAOC/DACS enzyme according to claim 14, wherein from 1 to 23 amino acids have been deleted from within the C terminus.

16. A polynucleotide encoding a DAOC/DACS enzyme according to any one of the preceding claims.

17. An expression vector comprising a polynucleotide according to claim 16.

18. A host cell transformed with a polynucleotide or vector according to claim 16 or claim 17.

19. A host cell according to claim 18, wherein said host cell is a fungal host cell.

20. A host cell according to claim 19, wherein the host cell is a *Penicillium*.

21. A host cell according to any one of claims 18 to 20, wherein said host cell is capable of producing penicillin G.

22. A host cell according to claim 19, wherein the host cell is a *Cephalosporium*.

23. Use of a DAOC/DACS enzyme according to any one of claims 1 to 15 or a host cell according to any of claims 18 to 22 in a method of ring expansion.

24. A method of ring-expanding penicillin G comprising contacting penicillin G with a DAOC/DACS enzyme according to any one of claims 1 to 15.

25. A method of producing 7-aminodeacetoxycephalosporanic acid (7-ADCA) comprising culturing a host cell according to claims 20, 21 or 22 under suitable conditions such that penicillin produced by the host cell is ring-expanded to produce 7-ADCA.

26. A method of ring expanding penicillin G comprising culturing a host cell according to claim 21 under suitable conditions such that penicillin G produced by the host cell is ring-expanded to produce phenylacetyl-7-aminodeacetoxycephalosporanic acid (phenylacetyl-7-ADCA).

27. A method of producing adipyl-7-aminodeacetoxycephalosporanic acid (adipyl-7-ADCA) comprising culturing a host cell according to claim 20 or 21 under suitable conditions such that adipate feed is incorporated into the cell as adipyl-7-aminopenicillanoic acid (adipyl-6-APA) and the adipyl-6-APA is converted into adipyl-7-ADCA.

Figure 1

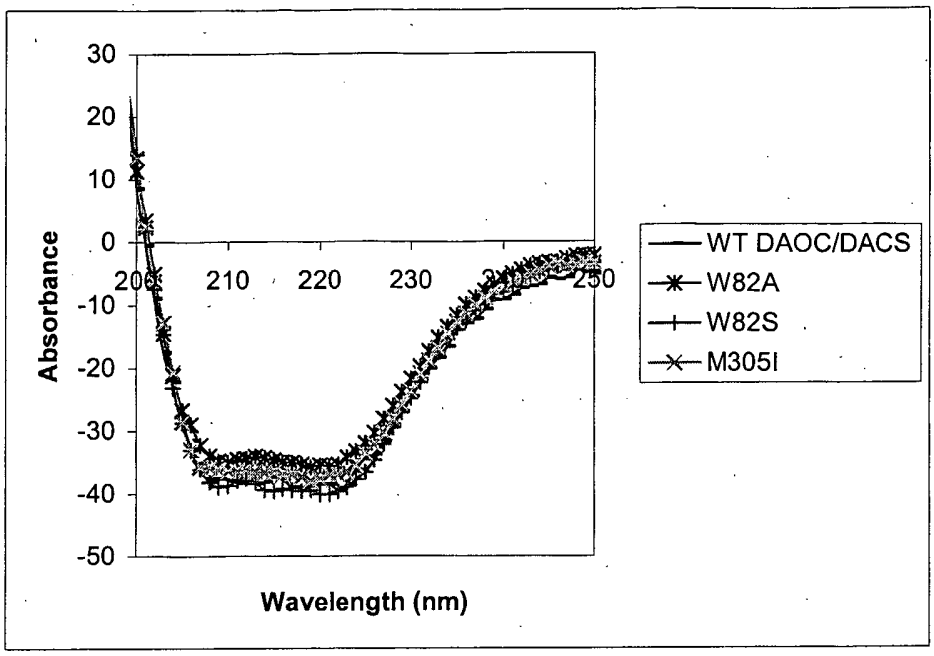


Figure 2

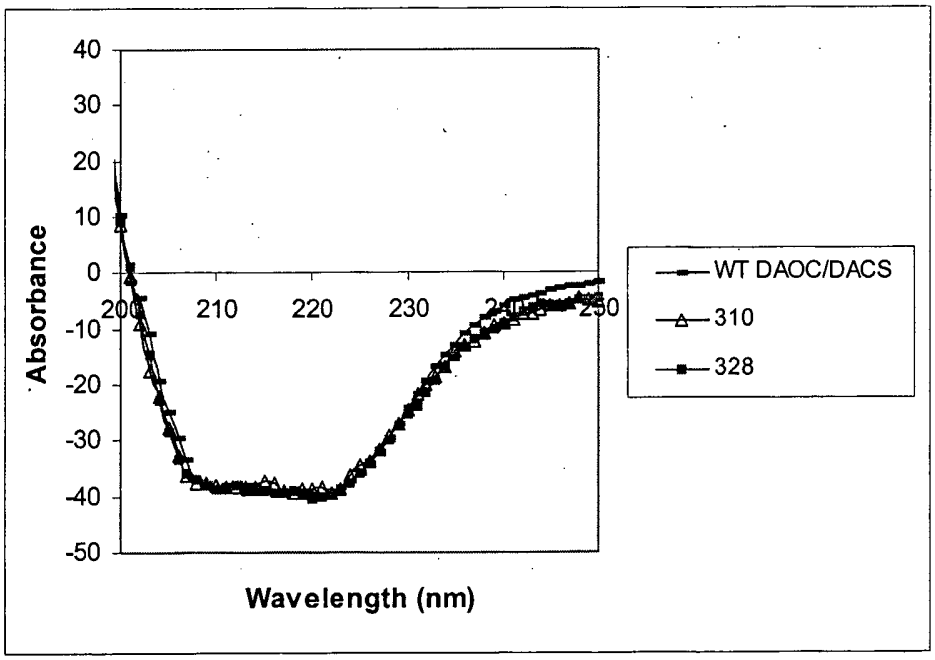


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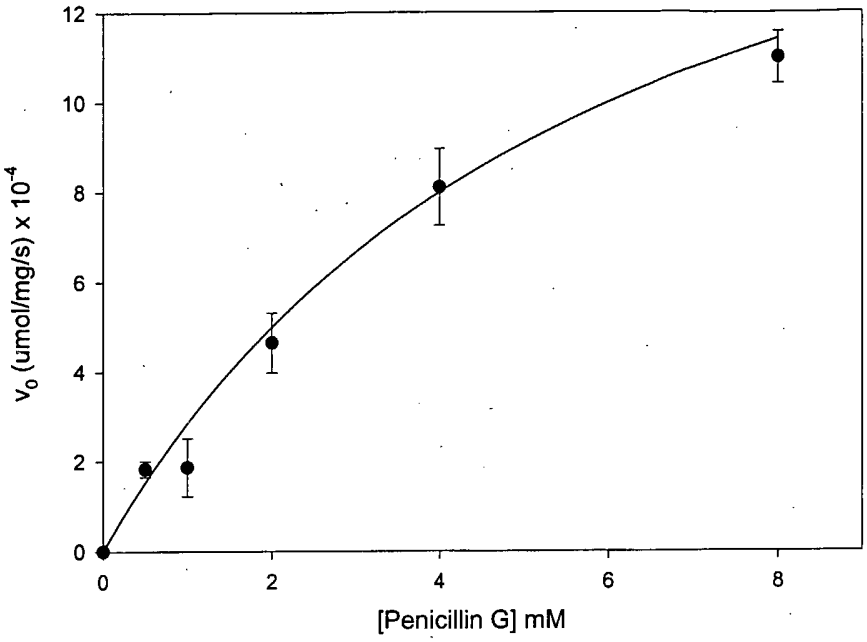


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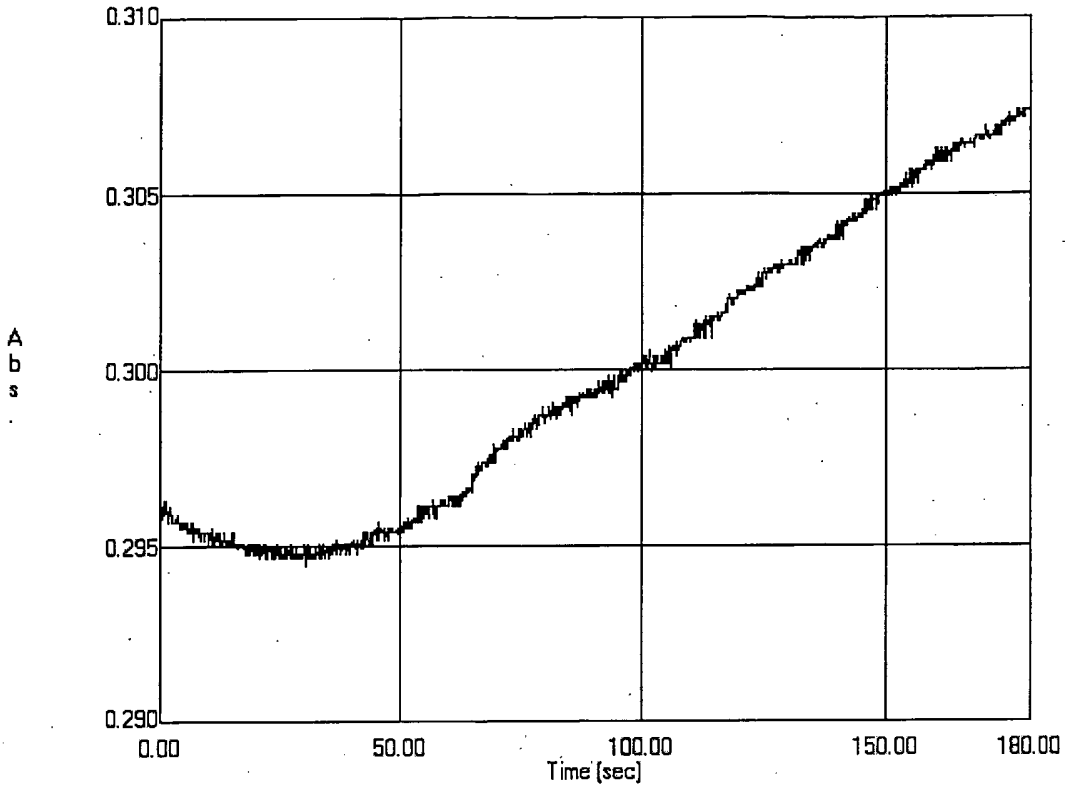

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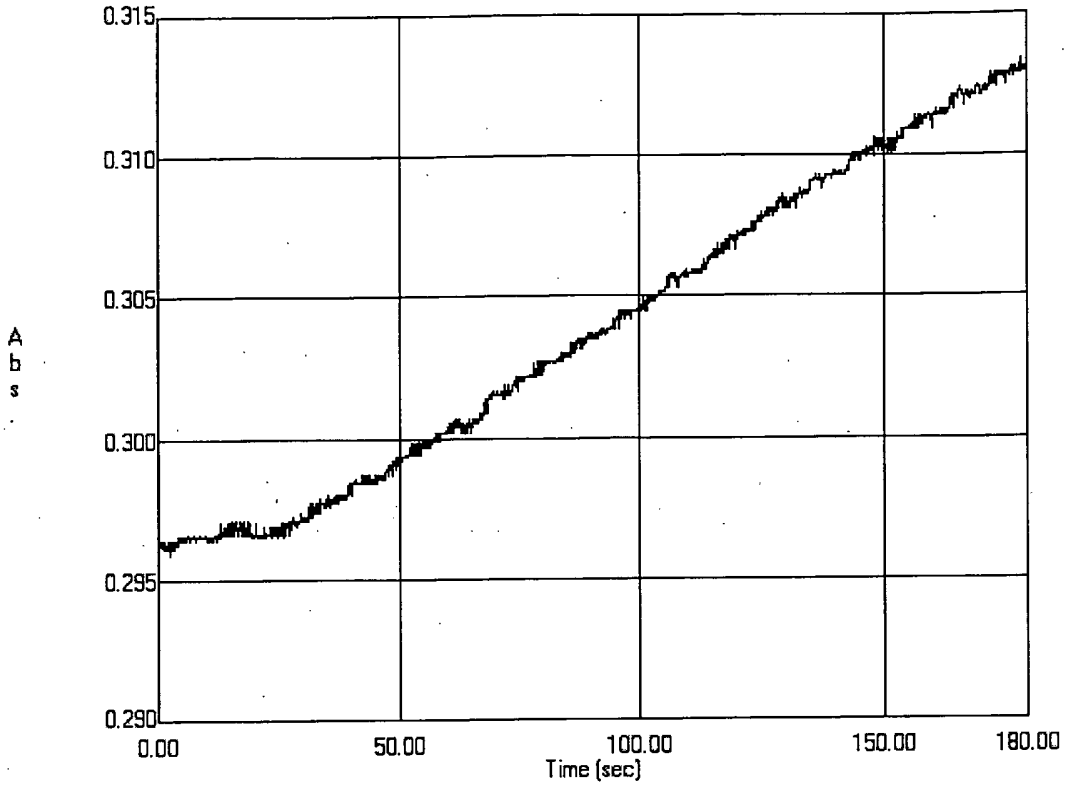

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Time (sec)

Figure 6

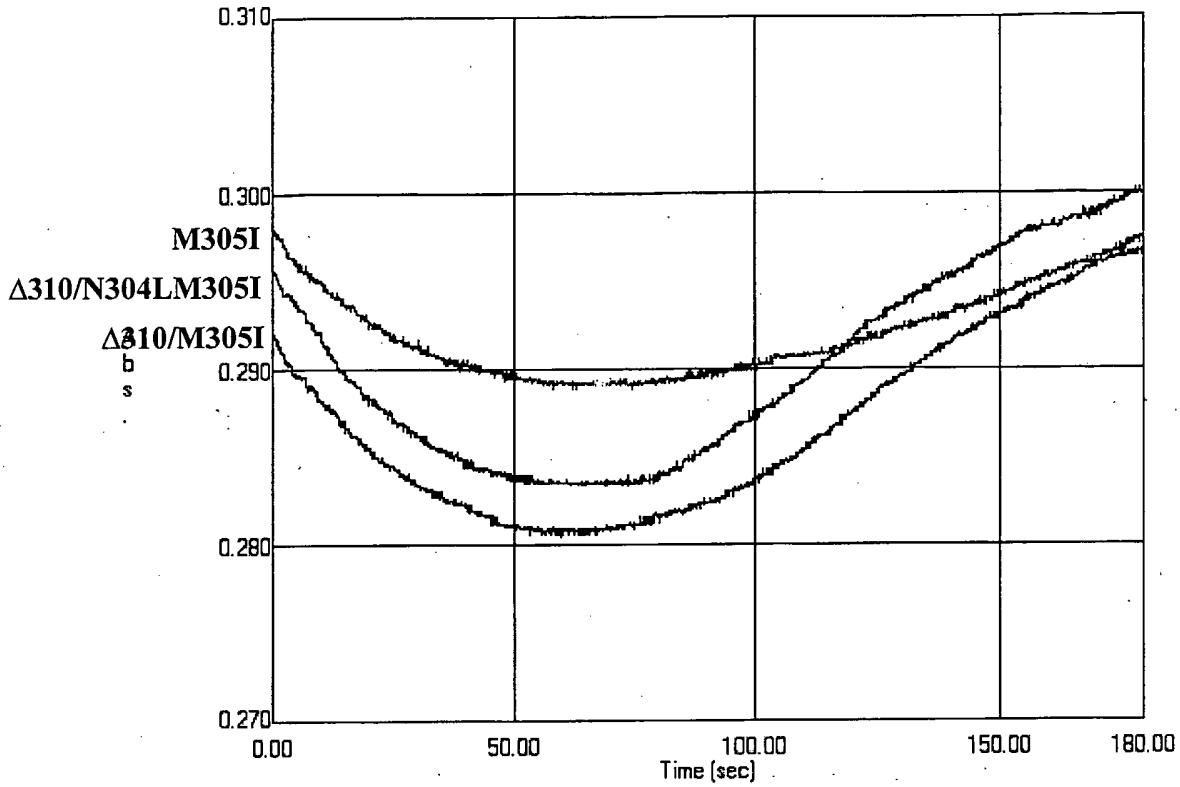

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Time (sec)

Figure 7

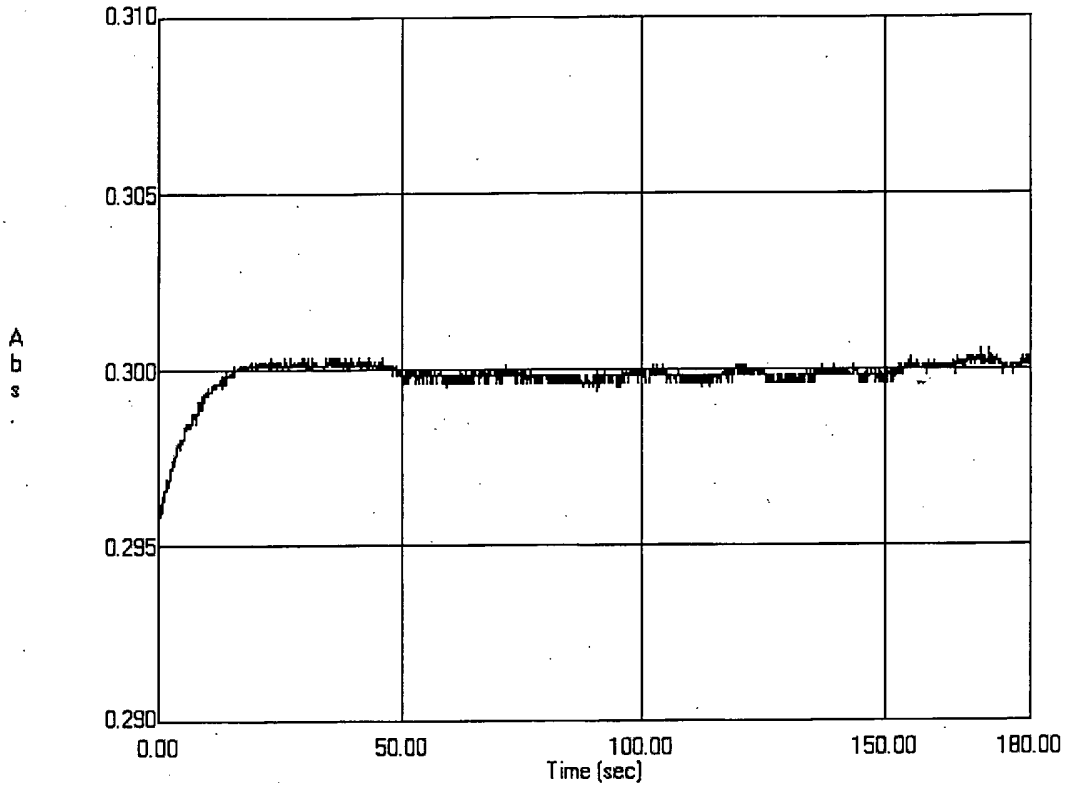

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Abs
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Time (sec)

Figure 8

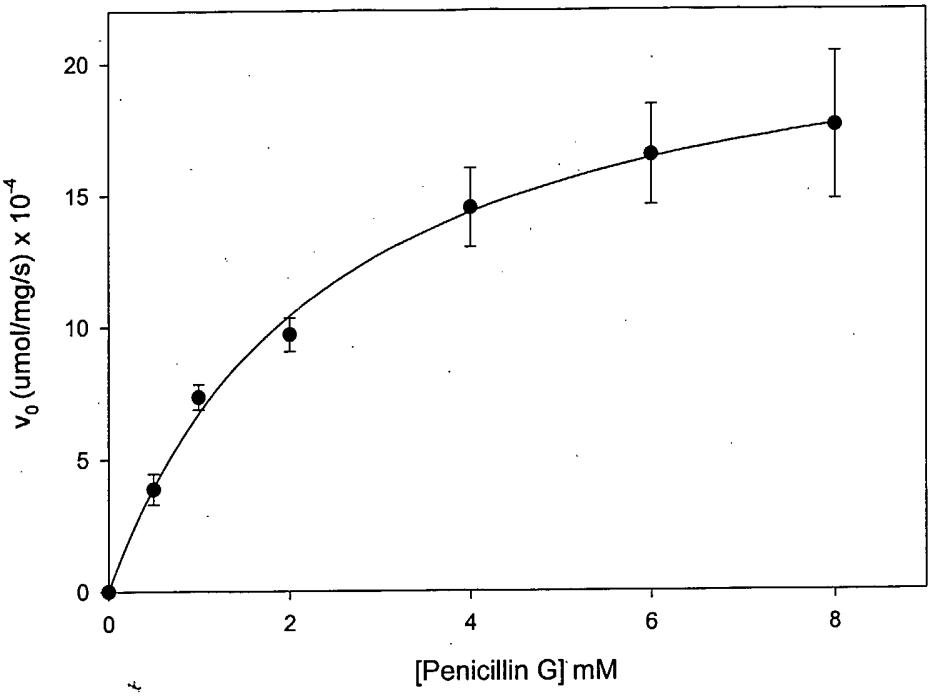


Figure 9

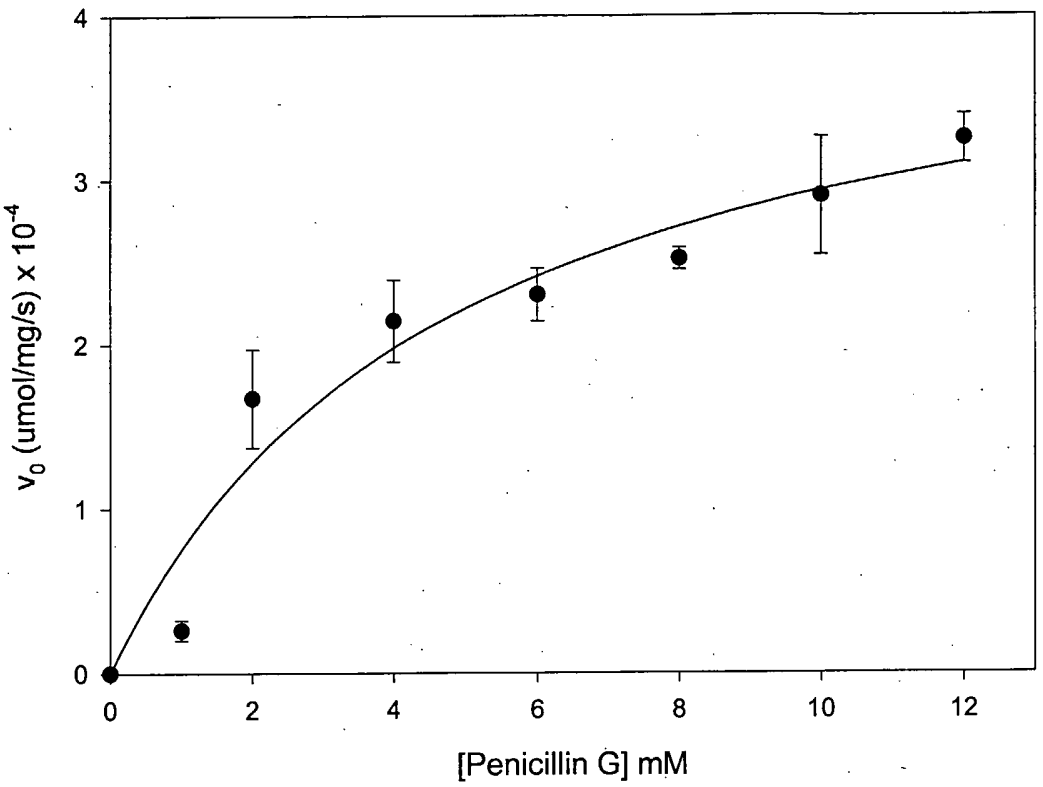


Figure 10

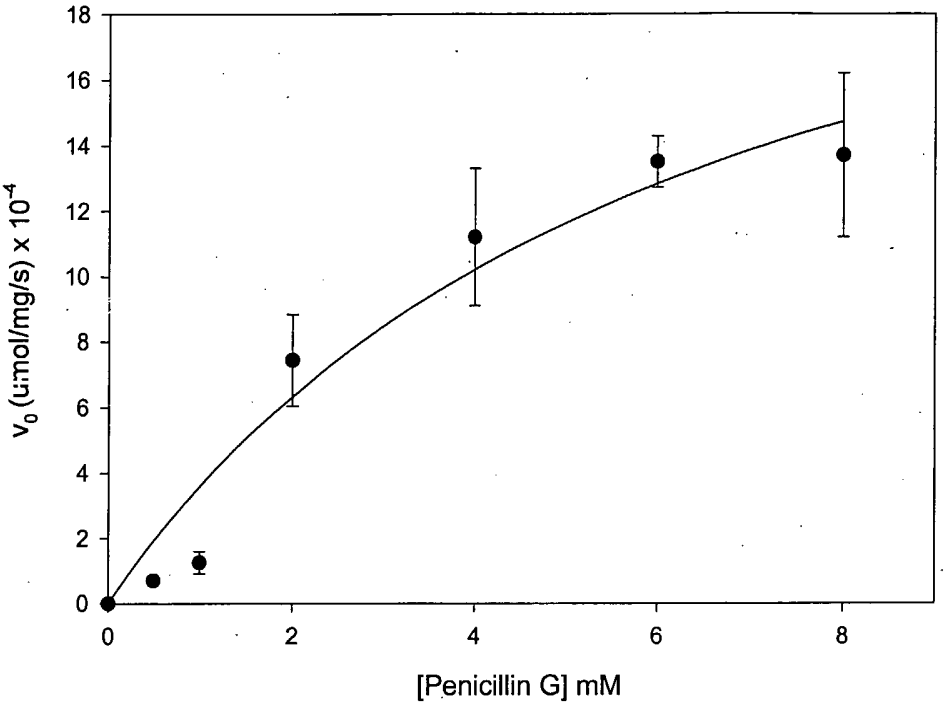
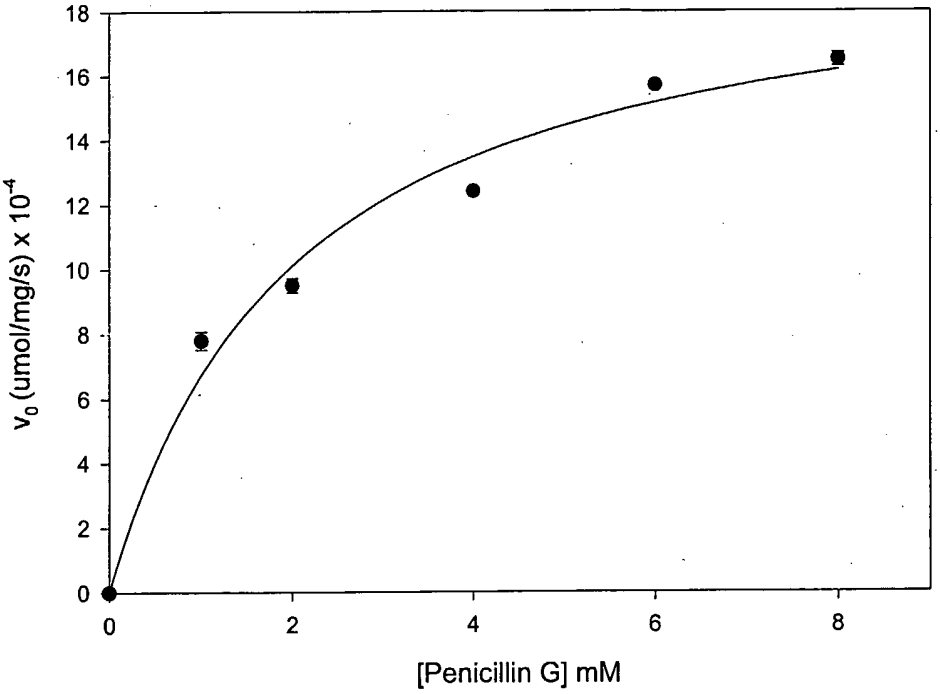


Figure 11



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cgc cta cgg tac ttc ccc gaa gtg ccg gag gac cgc gtc gcc gaa gag      528
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Glu Pro Leu Arg Met Gly Pro His Tyr Asp Leu Ser Thr Ile Thr Leu
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225                 230                 235                 240

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Ala Pro Lys His Arg Val Lys Ser Pro Gly Arg Asp Gln Arg Val Gly
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            260                 265                 270

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Pro Ser Glu Arg Thr Thr Phe Arg Glu Trp Leu Gly Gly Asn Tyr Val
    290                 295                 300

Asn Met Arg Arg Asp Lys Pro Ala Ala Ala Glu Ala Ala Val Pro Ala
305                 310                 315                 320

Ala Ala Pro Val Ser Thr Ala Ala Pro Ile Ala Thr
                325                 330
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INTERNATIONAL SEARCH REPORT

PCT/GB2004/003563

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P35/00 C12N15/53 C12N15/80

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)
IPC 7 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 practical, search terms used)

EPO-Internal, SEQUENCE SEARCH, WPI Data, BIOSIS, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UNIPROT 'Online! retrieved from NCBI Database accession no. P18548 XP002305108 the whole document ---	1-10, 14-27
X	DATABASE UNIPROT 'Online! retrieved from NCBI Database accession no. Q03047 XP002305109 the whole document ---	1-10, 14-27
X	DATABASE UNIPROT 'Online! retrieved from NCBI Database accession no. P42220 XP002305110 the whole document --- -/--	1-11, 14-27

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 2004	07/01/2005

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Keller, Y

INTERNATIONAL SEARCH REPORT

PCT/GB2004/003563

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UNIPROT retrieved from NCBI Database accession no. P42219 XP002305111 the whole document ---	1-11, 14-27
X	WO 01/85951 A (ACS DOBFAR UK LTD ; NEWBERT ROGER WILLIAM (GB); JOHNSON ROBERT IAN (GB) 15 November 2001 (2001-11-15) abstract page 1, line 1 -page 4, line 1 page 9, line 1 - line 21 page 10, line 14 -page 12, line 16 claims 1-20 figure 1 ---	1-5,9-27
P,X	BALDWIN J. ET AL: "Controlling the substrate selectivity of deacetoxycephalosporin/deacetylcephalosporin C synthase" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 279, no. 15, 9 April 2004 (2004-04-09), pages 15420-15426, XP002305107 the whole document -----	1-27

INTERNATIONAL SEARCH REPORT

PCT/GB2004/003563

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [ ] filed together with the international application in computer readable form

   [ ] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

PCT/GB2004/003563

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0185951 A	15-11-2001	AU 5243801 A	20-11-2001
		WO 0185951 A1	15-11-2001