

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 June 2008 (05.06.2008)

PCT

(10) International Publication Number
WO 2008/065367 A1

(51) International Patent Classification:

C12P 7/22 (2006.01) C07C 39/00 (2006.01)
C07C 39/24 (2006.01)

(74) Agents: MURGITROYD & COMPANY et al.; 165-169
Scotland Street, Glasgow G5 8PL (GB).

(21) International Application Number:

PCT/GB2007/004515

(22) International Filing Date:

26 November 2007 (26.11.2007)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

0623713.5 28 November 2006 (28.11.2006) GB

(71) Applicant (for all designated States except US): **THE QUEEN'S UNIVERSITY OF BELFAST** [GB/GB];
University Road, Belfast BT7 1NN (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KULAKOV, Leonid, Alekseyevich** [GB/GB]; 8 Annadale Mews, Belfast BT7 3LR (GB). **ALLEN, Christopher, Curtis, Royston** [GB/GB]; 20 Ravenhill Gardens, Belfast BT6 84P (GB).

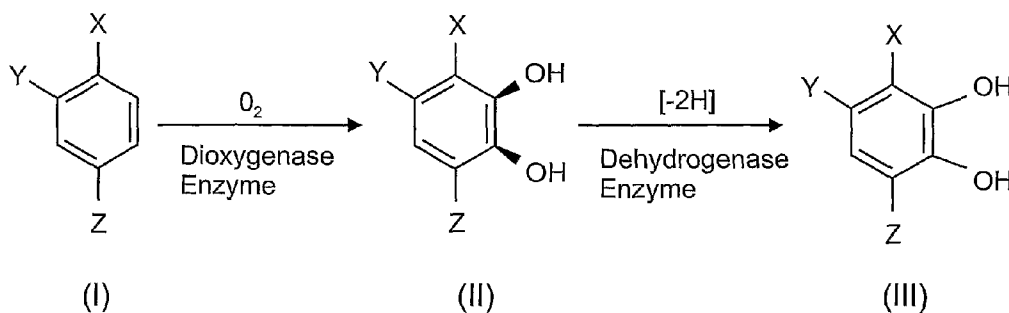
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(54) Title: MICROBIAL PREPARATION OF CATECHOLS



(57) Abstract: A process for preparing catechol derivatives comprising the steps of: i) admixing an aromatic compound with a dioxygenase enzyme to provide an intermediate *cis*-dihydrodiol aromatic compound; ii) separating the *cis*-dihydrodiol aromatic compound from the dioxygenase enzyme; and iii) directly admixing the *cis*-dihydrodiol aromatic compound with a dehydrogenase enzyme to provide a catechol.

MICROBIAL PREPARATION OF CATECHOLS

5 The present invention relates to a process of preparing catechols and novel catechols.

Catechols are vicinal di-hydroxylated aromatic compounds that may have one or more additional substituents. They may also be fused to other
10 aromatic ring systems (e.g. 1, 2-dihydroxynaphthalene). Substituted catechols are used in industry in the preparation of various pharmaceuticals and food associated products.

Complex chemical processes can provide certain catechols but not on an
15 economic scale except for very specific cases.

One known biochemical method for the preparation of substituted catechols is via *cis*-dihydrodiol intermediates. In this process two types of enzyme are involved; i) a ring hydroxylating dioxygenase enzyme
20 (sometimes called an arene dioxygenase) which converts an aromatic substrate into a *cis*-dihydrodiol, and ii) a *cis*-dihydrodiol dehydrogenase enzyme which transforms *cis*-dihydrodiols to the corresponding catechols.

An advantage of this method is the use of one general procedure to
25 produce a very wide range of different catechols, such that the process is inherently flexible. Like many biotransformation processes, these methods are performed in aqueous solution and under ambient temperature and/or neutral pH. No organic solvents other than the biotransformation substrates are necessary for these processes. This
30 means that few organic waste by-products are produced. Provided steps

are taken to ensure that all substrates are fully converted to product, these biotransformation processes are essentially 'green' processes. Little or no organic waste is produced, and relatively low energy requirements are necessary for the biotransformation unit operation(s).

5

However, this process has hitherto relied upon both enzymes being incorporated into one host strain, either a mutant or a genetic construct, which is then grown in a single fermenter (bioreactor). After completion of growth of the microbial host usually a bacterial cell in the fermenter, an aromatic substrate is presented to the organism in the same reactor, and it is then converted first to the *cis*-dihydrodiol and then into the catechol immediately i.e. both enzymatic transformations occur simultaneously. This is because many *cis*-dihydrodiols are very unstable. Therefore the *cis*-dihydrodiol does not accumulate, and the catechol accumulates over the same period that the aromatic substrate is consumed.

10

15

There are many disadvantages with this approach. The catechols ultimately produced are potent inhibitors of the ring hydroxylating dioxygenase enzymes. Therefore as the catechol accumulates in the bioreactor, there will be a gradual loss in activity of the dioxygenase, so that accumulation of the catechol product will ultimately be prevented. In addition, the two enzymatic steps cannot be independently regulated or controlled. This is because they are both expressed in the same host strain and grown or utilised under identical process conditions. This means that biotransformation processes conducted in this way can never be fully optimised.

20

25

Although it is possible to control the feed of substrate to the dioxygenase, it is not possible to then regulate the feed rate of *cis*-dihydrodiol to the dehydrogenase. This means that there is no way of controlling potential

30

substrate inhibition by the accumulation of the *cis*-dihydrodiol product either on the dioxygenase or the dehydrogenase.

5 Another disadvantage is that product recovery methods that may be employed with these procedures are usually limited as the catechol needs to be continuously removed from the bioreactor, in order to prevent toxicity to the dioxygenase.

10 Because whole cell biocatalysts need to be used in these processes, it is possible that significant loss of catalysts and therefore productivity, may be experienced due to trapping of the catalyst or other biological material (such as cellular protein) in the solid phase matrix.

15 The removal of substituted catechol product from the solid phase extraction matrix also may be problematic, as some catechols may either become unstable when bound to the solid phase matrix, or be difficult to recover. This may be more evident in the case where heavy contamination of the solid phase matrix occurs with cell debris.

20 An alternative approach to overcoming the problem of catechol toxicity has been to clone the dioxygenase and dehydrogenase enzymes into one host organism. In this case the dehydrogenase is again expressed in the same host organism as the dioxygenase, but it is independently regulated and can therefore be 'switched on' only after the *cis*-dihydrodiol has
25 accumulated in the reaction mixture. However, despite the elegance of this approach, any process that employs this catalyst system to overcome problems of catechol-induced dioxygenase toxicity will still be subject to the other disadvantages as described previously.

30 Thus, few catechols are made industrially.

The present invention provides an alternative for the production of catechols using dioxygenase and dehydrogenase enzymes.

- 5 Thus, according to the first aspect of the invention, there is provided a process for preparing a catechol comprising the steps of:
- i) admixing an aromatic compound with a dioxygenase enzyme to provide an intermediate *cis*-dihydrodiol aromatic compound;
 - 10 ii) separating the *cis*-dihydrodiol aromatic compound from the dioxygenase enzyme; and
 - iii) directly admixing the *cis*-dihydrodiol aromatic compound with a dehydrogenase enzyme to provide the catechol.

15 A major advantage of this process is that it offers complete flexibility with regards to choice of enzyme or biocatalyst employed. Therefore, the optimum combination of dioxygenase and dehydrogenase enzymes can be selected and incorporated into the process for the production of a particular catechol of interest.

20 In addition because both enzyme transformations are separate, they can be independently regulated or controlled for optimum performance in each of the fermenters. Thus, the widest range of catechol products can be produced in this process.

25 The term "directly admixing" for the purposes of this invention refers to the admixing of the *cis*-dihydrodiol aromatic compound with a dehydrogenase enzyme immediately subsequent or consecutively to the completion of step (ii), i.e. seconds or minutes. Thereafter, depending on flow and other process conditions and parameters, there is not intended to be any
30 storage or keeping of the intermediate aromatic compounds, other than

any *de minimis* requirements. Thus, the whole process can effectively be considered as a one unit operation in process design. Immediate addition of the *cis*-dihydrodiol aromatic compound to the dehydrogenase enzyme at step (iii) means that there will be no loss of any intermediate *cis*-
5 dihydrodiol production.

This provides the further advantage that many *cis*-dihydrodiol aromatic compounds are known to be unstable and cannot be stored or held over for another process for any length of time.
10

Preferably step (i) is performed in a first fermenter.

Preferably step (iii) is performed in a second fermenter.

15 Preferably the dioxygenase enzyme is produced in a first host bacterial cell.

Preferably the first host bacterial cell contains a bacterial expression vector comprising a DNA nucleotide sequence encoding the dioxygenase
20 enzyme having activity to catalyse the reaction with an aromatic compound to produce a *cis*-dihydrodiol aromatic compound.

Preferably the dioxygenase enzyme is a ring hydroxylating dioxygenase enzyme.
25

Suitably the *cis*-dihydrodiol aromatic compound is substituted at the 1,2, or 2,3 or 3,4 positions by hydroxyl groups.

Preferably step (ii) further comprises the step of separating the *cis*-
30 dihydrodiol aromatic compound from the dioxygenase enzyme by filtration.

Filtration can be carried out by a suitable filtration unit, device, membrane, etc., which may comprise centrifugation means or use of solid phase affinity columns or an ultrafiltration membrane unit to separate the enzyme
5 expressing bacteria cells and enzyme from the *cis*-dihydrodiol aromatic compound or catechol. Such means are known in the art.

In one embodiment, the filtration is an integral part of the fermenter or bioreactor. In an alternative embodiment, the filtration is separate from the
10 fermenter. For example, there may be an ultra-filtration unit which comprises an ultra-filtration loop with re-circulation of the enzyme fraction back into the first fermenter or reactor, and transfer of the enzyme-free filtrate into the second fermenter.

15 Preferably the deoxygenase enzyme is returned to step (i) and/or the first fermenter after filtration.

Preferably the *cis*-dihydrodiol aromatic compound is transferred to a second fermenter after filtration.

20

Preferably the dehydrogenase enzyme is produced in a second host bacterial cell.

25 Preferably the second host bacterial cell contains a bacterial expression vector comprising a DNA nucleotide sequence encoding the dehydrogenase enzyme having activity to catalyse the reaction between *cis*-dihydrodiol aromatic compound to produce a catechol.

Preferably the dehydrogenase enzyme is a *cis*-dihydrodiol dehydrogenase.

30

Preferably the catechol is filtered from the dehydrogenase enzyme using filtration.

5 Suitably, the dehydrogenase enzyme is returned to step (iii) and/or the second fermenter after filtration.

Each of the deoxygenase enzyme and dehydrogenase enzyme may be intracellular, so that they are effectively constrained by their host bacterial cell. Thus, as the or each host bacterial cell is separated from an
10 intermediate or catechol product stream, so is the enzyme therewith.

In one embodiment the or each bacterial expression vector is a pBADET expression vector. pBADET is a modified vector of a pBAD vector with additional restriction sites introduced.
15

pBAD vectors provide an ideal stable expression vector system for both small scale use e.g. in the laboratory, to large scale or industrial bacterial fermentation processes. Thus, confirmation of the process working for a particular catechol on a small scale can clearly confirm use of a process
20 on a large or industrial scale. pBAD vectors are ideal as they produce proteins at a level just below a variable threshold when they become insoluble and form inclusion bodies. Typically the expression vector includes a promoter which is operably linked to the sequences or gene(s) of interest. Other control elements may include one or more bacterial
25 origin(s) of replication, one or more selectable markers for example Ampicillin and a multiple cloning site. Any other suitable types of bacterial expression vectors may be used such as pUC or T7 expression vectors.

Typically the first or second host bacterial cell is an *Escherichia coli*.
30

Preferably, the *cis*-dihydrodiol aromatic compound is in an aqueous solution. Typically, such intermediate *cis*-dihydrodiol aromatic compounds are more stable when maintained in an aqueous form enabling the process of their conversion to catechols.

5

The process allows for the analysis and adjustment of temperature, pH, aeration, agitation, bacterial cell density, reactor volume levels and co-substrate feed rates to provide optimal conditions for each reaction to occur.

10

Typically, step (i) is performed in a raised oxygenated environment. In one embodiment the oxygen content is between 30 and 100% of saturated water equivalent at the particular process temperature.

15

Typically step (iii) is performed in a substantially anaerobic environment.

Preferably step (i) comprises admixing one or more further substrates or compounds therewith. One such substrate may be to help minimise any possible effect of 'catabolite repression' on catalytic gene expression in the clones or mutants. Such substrates include glycerol.

20

Each admixing may occur by batch, fed-batch or continuous means. An aromatic compound or *cis*-dihydrodiol aromatic compound can be fed into the first or second fermenter respectively by batch, fed-batch or continuous means typically by a controller mechanism in order to control the amount of substrate utilized in the reactions taking place.

25

Suitable means of admixing of any substrate may occur by stirring, shaking, rotating or any other type of agitation means known in the art of fermentation.

30

Monitoring the concentration of bacteria growth to form a fermentation mix or broth in the fermenter or bioreactor is important in determining if a suitable level of enzyme has been produced in the fermenter before a reaction begins. The timing of the addition of any substrate including the aromatic compound, *cis*-dihydrodiol aromatic compound or any further substrates such as glycerol depends on the concentration of the bacterial growth.

- 10 In one embodiment, the first and/or second bacterial cell density is monitored by the steps of;
- i) removing a sample of bacterial cells from the first and/or second fermenter
 - 15 ii) taking a optical density reading, for example using a spectrophotometer at a wavelength of 600nm i.e. OD₆₀₀.

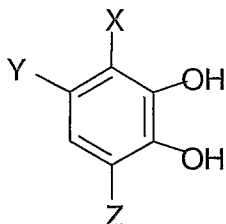
When the OD₆₀₀ is between 1.4 and 1.5, the aromatic compound and glycerol is added to the first fermenter in step (i) of the process.

- 20 When the OD₆₀₀ is between 1.8 and 2.0, the *cis*-dihydrodiol aromatic compound is added to the second fermenter in step (iii) of the process.

A non-limiting list of examples of catechol compounds which can be produced by the process of the present invention as set out in Figure 1 of the accompanying drawings, generally defined as Formula (III). The present invention extends to catechols obtainable by a process as herein described.

According to a second aspect of the present invention, there are provided a catechol of Formula (IIIa) :

(IIIa)



5

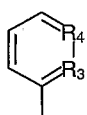
wherein: Z = H, and either

a) Y = H, and;

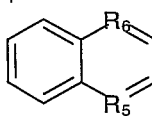
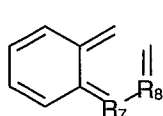
X = CR₁OOH, where R₁ = CH₃[CH₂]_{n=1-4}, CH₂=CH or CH₂[OCH₃];

10

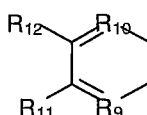
or X = SOR₂ and R₂ = CH₃[CH₂]_{n=1-4};

or X =  and R₃, R₄ = CH or N; or

15

b) X & Y =  or 

where R₅, R₆, R₇, R₈ = CH, N; or

c) X & Y = 

20

where R₉, R₁₀ = CH, N, and R₁₁, R₁₂ = H, Cl, Br, I.

This includes the chiral compounds of Formula (IIIa), as also shown in Figure 1 of the accompanying drawings.

25

The compounds of Formula (IIIa) and Formula (III) shown in Figure 1 are all sufficiently structurally similar diols that they can be provided by the skilled man using the process of the present invention.

30

The process of the present invention acts on one double bond on an aromatic ring such that substituents elsewhere on the ring as defined in Figure 1 wholly or substantially do not affect the chemistry of the present invention.

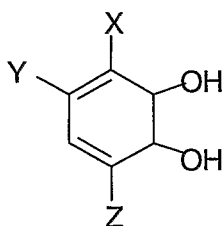
5

According to a third aspect of the invention, there is provided a process for preparing a catechol of formula (IIIa) comprising the process of the present invention as herein defined.

10

According to the fourth aspect of the invention there is provided a *cis*-dihydrodiol aromatic compound of formula (II)

(II)



15

wherein X, Y and Z are as hereinbefore defined for compounds of Formula (IIIa).

20

According to the fifth aspect of the invention, there is provided use of a catechol of Formula (IIIa) in the preparation of a pharmaceutical or a food products.

25

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*.

The present invention will now be described with embodiments with reference to the accompanying drawings in which:

30

Figure 1 shows examples of catechols which can be prepared via the process of the present invention;

5 Figure 2 shows enzymatic formation of catechols via *cis*-dihydrodiol intermediates using the process of the present invention;

Figure 3 shows a schematic diagram of the process for catechol production;

10

Figure 4 shows the biotransformation of fluorobenzene with TDO;

Figure 5 shows the biotransformation of Fluorobenzene diol with TDH – mixed reaction.

15

Referring to the drawings, Figure 1 shows examples of catechols which can be prepared via the process of the present invention.

20

Figure 2 shows a general scheme of the consecutive dual step or “tandem biotransformation” of catechols via *cis*-dihydrodiol intermediates using a ring hydroxylating dioxygenase enzyme which converts an aromatic substrate into a *cis*-dihydrodiol, and a *cis*-dihydrodiol dehydrogenase enzyme which directly transforms the separated *cis*-dihydrodiols to the corresponding catechols.

25

Figure 3 shows a general schematic diagram of the “tandem biotransformation” process of the present invention for catechol production. The process includes a dioxygenase bioreactor or first fermenter 10, to which an aromatic compound is provided along line 12 to be admixed in the first fermenter 10 with dioxygenase producing bacterial

30

cells therein to provide a intermediate *cis*-dihydrodiol aromatic compound of formula (II).

Next to the first fermentor 10 is an 'ultrafiltration loop', comprising inlet line 14, return line 16 and a first ultrafiltration unit 18 for separation of dioxygenase bacterial cells comprising the deoxygenase enzyme from the intermediate *cis*-dihydrodiol stream provided by the inlet line 14. A separated intermediate *cis*-dihydrodiol stream 20 is thus provided from the first unit 18, and passed directly to a dehydrogenase bioreactor or second fermenter 22 for the direct or consecutive formation of a catechol from the admixture of the intermediate *cis*-dihydrodiol and dihydrogenase producing bacterial cells in the second fermentor 22. The catechol can be provided as a product stream 24 from said unit 22.

The dehydrogenase fermentor 22 also has an 'ultrafiltration loop', comprising inlet line 26, return line 28 and a second ultrafiltration unit 30 for concentrating the dehydrogenase cells. There is a waste filtrate stream 32.

Each of the first and second fermentors 12, 22 may include a stirrer 34, 36 to assist admixture of the reactants therein.

Figure 3 confirms the direct admixing of the intermediate *cis*-dihydrodiol aromatic compound with a dehydrogenase enzyme immediately subsequent or consecutively to the completion of the separation in step (ii). There is not intended to be any storage or keeping of the intermediate aromatic compound, such that the whole process shown in Figure 3 can effectively be considered as a continuous one unit operation in process design.

Example 1: Production of 3-fluorocatechol from fluorobenzene.

Bacterial strains and Culture Conditions

5 *E. coli* TOPO10 (pBAD::todC1 – todA) strain containing all four genes encoding toluene dioxygenase (TDO) and *E. coli* TOPO10(pBAD::narB) strain encoding *cis*-naphthalene dihydrodiol dehydrogenase (NDD) from *Rhodococcus* NCIMB12038 were used for biotransformation (Figure 3).

10 Ampicillin (Amp) was always added to media in concentration of 100 µg/ml to ensure the propagation of plasmid. Induction of TDO and NDD genes was achieved by adding 0.02% (w/v) of arabinose (ara).

15 When culturing the strains from frozen glycerol stock, 2×YT agar medium containing (g/litre) 16 tryptone, 10 yeast extract 5 NaCl and 16 Bacto agar and Amp was used. For initial propagation 2×YT broth with Amp was used.

20 For further inoculum development and biotransformation, minimal salts medium (MSM) containing 1.9ml/L trace element solution, (g/litre) 0.96 KH₂PO₄ and 1.23 K₂HPO₄ (altogether known as Solution A), 3.00 NH₄Cl and 0.40 MgSO₄·7H₂O (altogether known as Solution B), 0.2% (w/v) glycerol, 0.15% (w/v) casein hydrolysate and Amp was used. Stocks of solutions A, B, glycerol, casein hydrolysate and Amp were sterilised separately and mixed together prior to use. For inoculum development, the
25 culture was grown aerobically on a shaker at 150 rpm at 37°C for 16h. For biotransformation, the culture was grown aerobically at 37°C in a 150L fermenter for approximately 6h until reaching the mid-log phase (OD₆₀₀ 1.4-1.5), temperature was then adjusted to 30°C, glycerol to final concentration of 0.2% (w/v) and ara to 0.02% (w/v) were added and the
30 culture was incubated for another 2h prior to the addition of substrate.

Construction of recombinant biocatalyst strains

Primers were designed for cloning of TDO genes from *Pseudomonas putida* F1 (Accession number for the DNA sequence used is J04996).

5 Forward primer (611 – 636 nt): 5'- GAG AAG CAT ATG AAT CAG ACC
GAC ACA TC –3' and Reverse primer (4199 – 4229 nt): 5' – GCG AAT
TCG CCT TCA AGT CTC AGC TTA GGT C – 3'

NdeI restriction site was introduced into 5' – part of the forward primer and
EcoRI site into reverse primer (sites are underlined). DNA fragment

10 amplified with these primers included todC1, todC2, todA and todB genes.
Primers were also designed for cloning *narB* gene from *Rhodococcus*
NCIMB12038 (Accession number AF082663).

Forward primer (6339 – 6368 nt): 5' – ACT GAT CAT ATG GGA TTT CTG
GAC GGC AAG – 3' and Reverse primer (7158 – 7186 nt): 5' – AAC GAA
15 TTC CCA CGC ACC GAC CCT CAC TG – 3'

Correspondingly NdeI and EcoRI sites were introduced into these primers.
The fragments containing tod and narB genes were amplified using
PfuTurbo polymerase obtained from Stratagene. Reaction conditions were
as follows: 95°C for 3 min and then 32 cycles of 95°C (30 s), 55°C (30 s)
20 and 72°C (4.5 min for TOD amplification and 2 min for narB amplification).
Fragments were purified using PCR DNA and Gel Band Purification kit
(Amersham) and digested with the corresponding enzymes.

pBADET DNA was restricted with both NdeI and EcoRI restriction
enzymes and then subjected to electrophoresis in 1% agarose gel.

25 Linearised plasmid DNA was isolated from gel using PCR DNA and Gel
Band Purification kit (Amersham). PCR fragments containing tod and narB
genes were ligated with pBADET DNA.

Ligated DNA was transformed into *E. coli* TOP10 cells (Invitrogen)
30 according to manufactures instruction. Transformation mixtures were

plated onto 2YT media containing ampicillin (100 µg/ml) and arabinose (0.02%). Colonies were analysed after incubation at 30°C for 24 – 30 hours.

5 *E. coli* TOP10 clones containing TDO genes were initially selected by their ability to convert indole to indigo and then activity of TDO was confirmed in biotransformation experiments. *E. coli* TOP10 clones containing narB gene were selected using restriction analysis of the plasmid DNAs; activity of TDO was then confirmed in biotransformation experiments.

10 Storage of strains

The recombinant strains were stored as frozen glycerol stocks and as freeze-dried cultures. To store bacteria in glycerol, a single colony was inoculated into 10ml of 2xYT broth with Amp and incubated overnight at 15 37°C with aeration. 500µl of this culture and 500µl of sterile 80% (w/v) glycerol were thoroughly mixed in a sterile 2-ml screw cap vial. This was immediately placed into – 20°C freezer.

To freeze-dry bacterial strains several 2xYT agar plates with Amp and ara 20 were inoculated with a single colony and incubated at 30°C for 2 days. Approximately 3 ml of sterile horse serum was poured on to one plate, biomass was suspended in the horse serum using a sterile spreader. The suspension was transferred to another plate using a sterile long Pasteur pipette to suspend more cells. Then a small amount of the suspension 25 was pipetted to a sterile glass ampoule; a sterile label and a cotton wool plug were inserted to this ampoule using a pair of sterilized tweezers. The glass ampoule was covered with a sterile hood and put in the freeze-drying machine. It was operated according to the instruction. Finally the ampoule was sealed using a burner. Ampoules containing freeze-dried 30 culture were stored at 4°C.

Growth of *E. coli*(pBAD::*tod*) and *E. coli*(pBAD::*narB*) clones in a 150L fermenter

5 i) Initial inoculum development

Two 10ml 2×YT with Amp tubes were inoculated with a single colony and incubated at 37°C for 16h with aeration. These were centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was aseptically discarded and the pellet resuspended in 20ml of sterile 0.9% (w/v) NaCl solution. The
10 suspension was used for further inoculum development.

ii) Further inoculum development

Four 2L-flasks containing 500ml of MSM with 0.2% glycerol, 0.15% casein hydrolysate and 100 µg/ml of Amp were inoculated with the above
15 suspension, 5ml each. The flasks were incubated at 37°C for 16h with aeration on a rotary shaker.

iii) Growth of *E. coli*(pBAD::*tdo*) in 150L fermenter

The 150L fermenter (New Brunswick Ltd. BIOFLO 150) containing 120L
20 medium (described above) was inoculated with the culture from four 2L-flasks. 10ml of sterilized 50% (v/v) Antifoam O-30 was added. The incubation temperature was set at 37°C throughout the experiment. At the beginning, the agitation speed was 200rpm, the airflow was 12.6SLPM and the pH was 6.9. At 7h and 9h, 20ml and 30ml of antifoam solution
25 were added respectively. OD₆₀₀ of the culture was monitored hourly in the first two hours, and then every half an hour until the OD₆₀₀ was dropped.

iv) Analysis of the clones stability

10 ml samples were taken from the fermenter to analyse the clone stability
30 during the incubation. Serial dilutions were plated onto 2×YT plates and

individual colonies analysed on the presence of the corresponding insertions. Stability of *E.coli*(pBAD::*narB*) during fermentation were assessed by the ability of TDO convert indol to indigo dye (formation of blue coloured colonies on 2xYT plates). Only one white colony was found in the sample taken at the very beginning of the biofermenter culture growth. 200 to 1000 colonies were analysed from each of the sampling points. These results show that cloned genes are stably maintained during the biotransformation process.

10 Biotransformation of fluorobenzene with *E. coli*(pBAD::tdo)

i) Growth of *E. coli*(pBAD::tdo) in 150L fermenter

The culture was grown in the 150L fermenter as described above. The OD₆₀₀ was monitored until the mid-log phase was reached, i.e. 1.4-1.5..

15

ii) Induction

The temperature was then adjusted to 30°C, glycerol to 0.2% and arabinose to 0.02% were added and incubation continued for 2h.

20 iii) Biotransformation

Substrate (fluorobenzene) and co-substrate (40% (w/v) glycerol) were slowly pumped to the fermenter. The %OD at 266nm was monitored as an indication of the accumulation of *cis*-diol in the cell-free supernatant from the reaction mixture (Figure 4). Over the course of the reaction (4 hours) a total of 250g of fluorobenzene was added in total.

25

iv) Samples analysis

Samples were taken every half an hour in the first experiment and every hour in the following experiments. A universal sample bottle was used to collect the sample from the fermenter, 3ml of the culture was pipetted to

30

5 eppendorf tubes, spun at 13,200rpm for 2min immediately. The supernatant was transferred to a labelled small plastic bottle and stored at -20°C overnight for future analysis. The samples were diluted appropriately and scanned from 200-400nm using a spectrophotometer. A
absorbance maxima would be found as the *cis*-diol accumulated at 266nm.

v) Downstream processing

10 The culture was centrifuged (35,000 rpm) and a sample of the supernatant was retained for product purification.

Growth and Biotransformation with *E. coli* (pBAD::narB)

15 Cells were grown as the biotransformation with TDO but the mid-log phase point of growth was at D_{600} 1.8-2.0 instead of 1.4-1.5 (Figures 7a & 7b). Co-substrate was not required during the addition of substrate – the culture supernatant containing fluorobenzene *cis*-diol from the first biotransformation (115L, after centrifugation).

20 The biotransformation was carried out in a 150L New Brunswick bioflo Pro fermenter. After the culture was induced for 2h, the culture was reduced to a 60L volume (half the culture was discarded) (Figure 5). One portion was pumped to another identical fermenter while the other portion was retained. Half of the supernatant of fluorobenzene biotransformation -
25 containing fluorobenzene *cis*diol - was then added to the fermenter in one step. Samples were taken hourly from both fermenters for analysis, using the same procedures as the biotransformation with TDO.

Product yields

Analysis of the 2 step fluorobenzene biotransformation was carried out after purification/isolation of the product. We observed that in 2 litre of supernatant recovered after the second transformation 4.12g of crude bioproduct was recovered. After further purification by TLC (solvent 1:1 ethyl acetate/hexane) 300mg of catechol was recovered. This is equivalent therefore to a total final concentration of 150 mg per litre, or 15g purified product. In addition 20g of purified fluorobenzene *cis*-diol was recovered. This corresponds to an overall catechol yield of 6%.

CLAIMS

1. A process for preparing catechol derivatives comprising the steps of:

- 5 i) admixing an aromatic compound with a dioxygenase enzyme to provide an intermediate *cis*-dihydrodiol aromatic compound;
 ii) separating the *cis*-dihydrodiol aromatic compound from the dioxygenase enzyme; and
 iii) directly admixing the *cis*-dihydrodiol aromatic compound with a
10 dehydrogenase enzyme to provide a catechol.

2. A process as claimed in Claim 1 where step (i) occurs in a first fermenter.

15 3. A process as claimed in Claim 1 wherein step (iii) occurs in a second fermenter.

20 4. A process as claimed in any one of Claims 1 to 3 wherein the dioxygenase enzyme is produced in a first host bacterial cell.

 5. A process as claimed in Claim 4 wherein the first host bacterial cell contains a bacterial expression vector comprising a DNA nucleotide sequence encoding the dioxygenase enzyme.

25 6. A process as claimed in any one of the preceding claims wherein the dioxygenase enzyme is a ring hydroxylating dioxygenase enzyme.

30 7. A process as claimed in any one of the preceding claims wherein a *cis*-dihydrodiol aromatic compound is substituted at the 1, 2, or 2, 3 or 3,4 positions by hydroxyl groups.

8. A process as claimed in any one of Claims 4 to 7 wherein step (ii) further comprises separating the *cis*-dihydrodiol aromatic compound from dioxygenase enzyme by filtration.

5

9. A process as claimed in Claim 8 wherein the dioxygenase enzyme is returned to step (i) after filtration.

10

10. A process as claimed in Claim 8 wherein the *cis*-dihydrodiol aromatic compound is transferred to a second fermenter after filtration.

11. A process as claimed in any one of the preceding claims wherein the dehydrogenase enzyme is produced in a second host bacterial cell.

15

12. A process as claimed in Claim 11 wherein the second host bacterial cell contains a bacterial expression vector comprising a DNA nucleotide sequence encoding the dehydrogenase enzyme.

20

13. A process as claimed in any one of the preceding claims wherein the dehydrogenase enzyme is a *cis*-dihydrodiol dehydrogenase.

14. A process as claimed in any one of the preceding claims wherein the catechol is separated from the dehydrogenase enzyme using filtration.

25

15. A process as claimed in Claim 14 wherein the dehydrogenase enzyme is returned to step (iii) after filtration.

16. A process as claimed in any one of Claims 4 to 15 wherein the first and/or second host bacterial cell is an *Escherichia coli*.

30

17. A process as claimed in any one of Claims 5 to 16 wherein at least one the bacterial expression vectors is a pBADET expression vector.

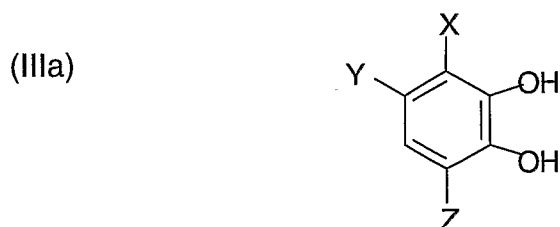
18. A process as claimed in any one of the preceding claims wherein the *cis*-dihydrodiol aromatic compound is in an aqueous solution.

19. A process as claimed in any one of the preceding claims wherein step (i) is carried out in a raised oxygenated environment.

20. A process as claimed in any one of the preceding claims wherein step (iii) is carried out in a substantially anerobic environment.

21. A process as claimed in any one of the preceding claims wherein the admixing of step (i) includes glycerol.

22. A catechol as defined in Formula (IIIa)

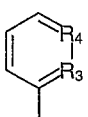


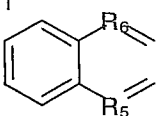
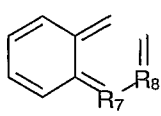
wherein: Z = H, and either

a) Y = H, and;

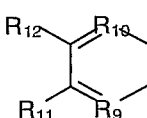
X = CR₁OOH, where R₁ = CH₃[CH₂]_{n=1-4}, CH₂=CH or CH₂[OCH₃];

or X = SOR₂ and R₂ = CH₃[CH₂]_{n=1-4};

or X =  and R₃, R₄ = CH or N; or

b) X & Y =  or 

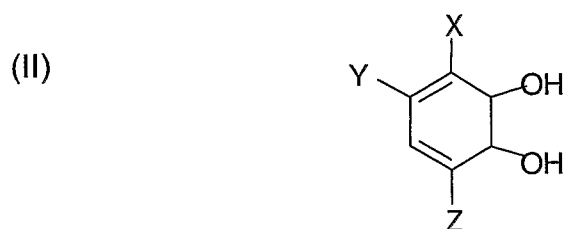
where R₅, R₆, R₇, R₈ = CH, N; or

c) X & Y = 

where R₉, R₁₀ = CH, N, and R₁₁, R₁₂ = H, Cl, Br, I.

23. A process for preparing a catechol of Formula (IIIa) as defined in Claim 22 comprising the steps as defined in any one of Claims 1 to 21.

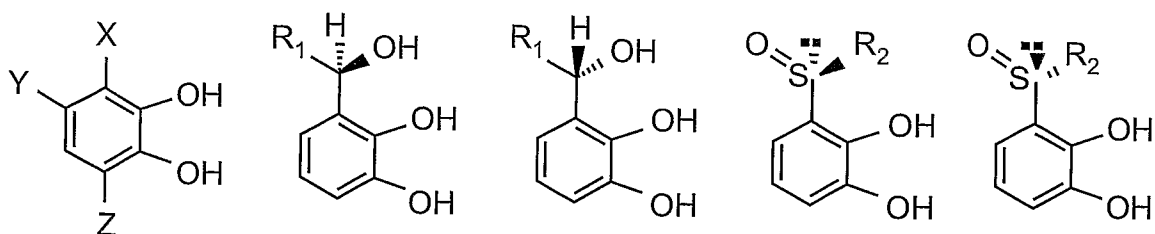
24. A *cis*-dihydrodiol aromatic compound of Formula (II):



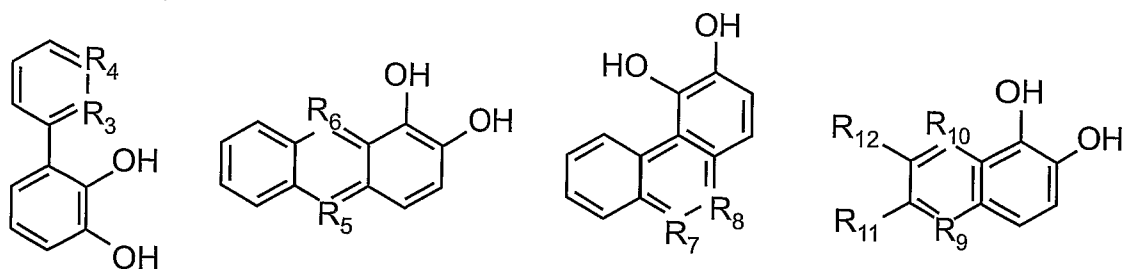
wherein X, Y and Z are as defined in Claim 22.

25. Use of a catechol as claimed in Claim 22 in the preparation of a pharmaceutical or a food product.

1 / 3



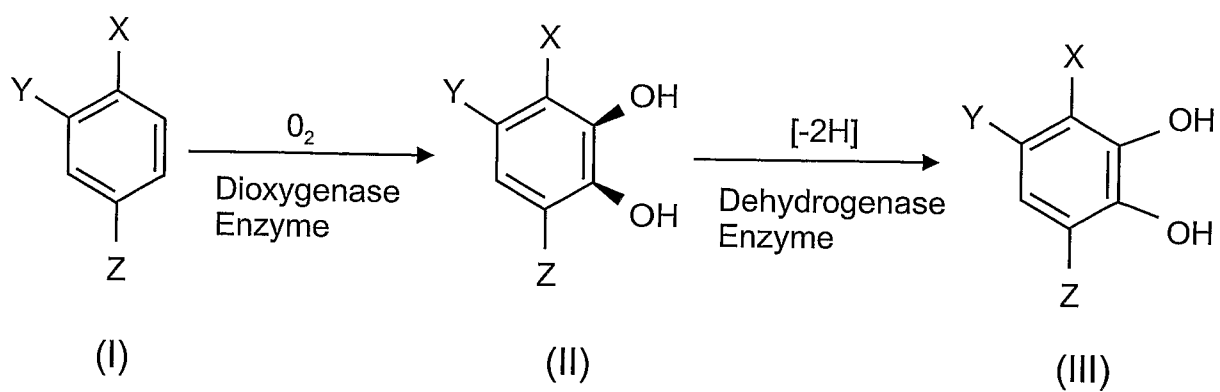
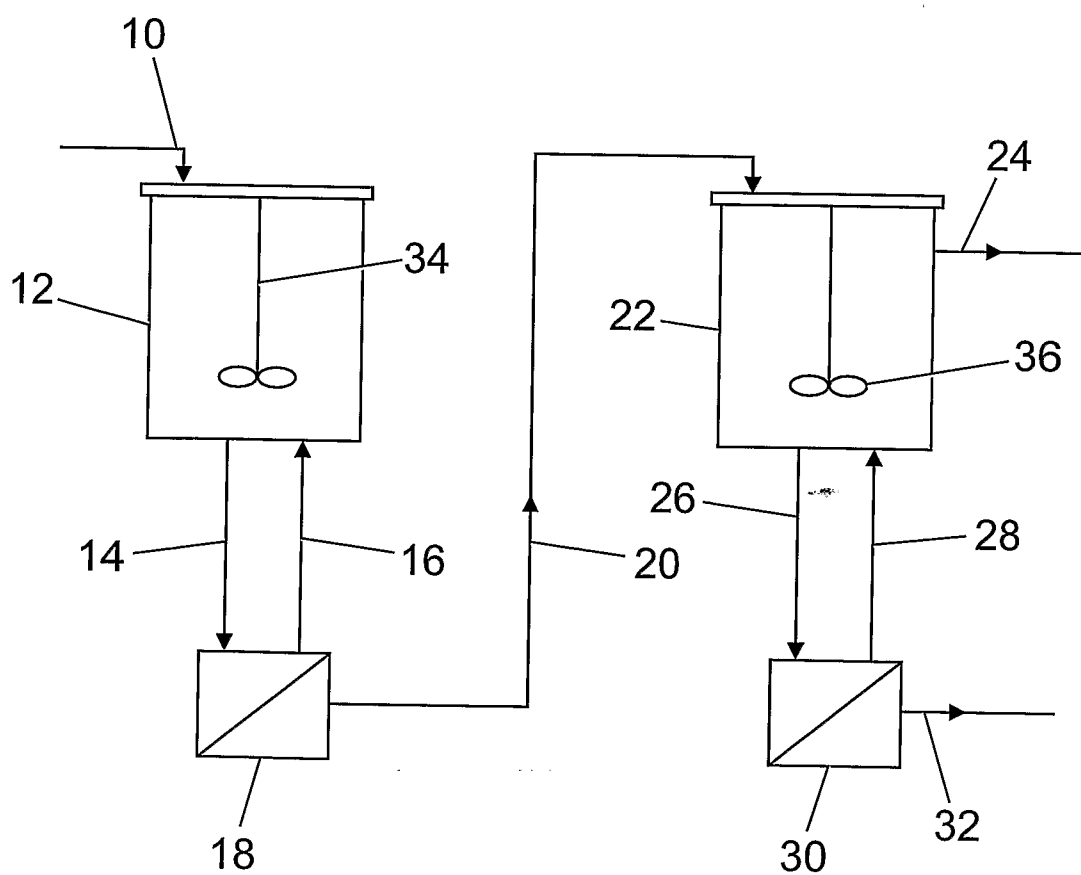
Formula (III)



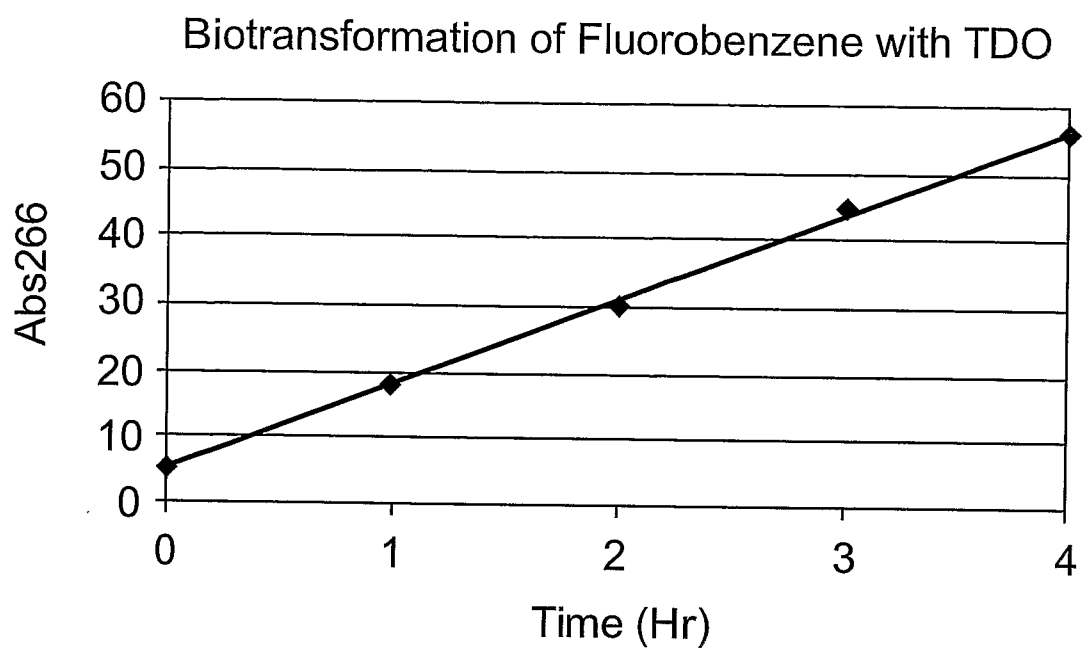
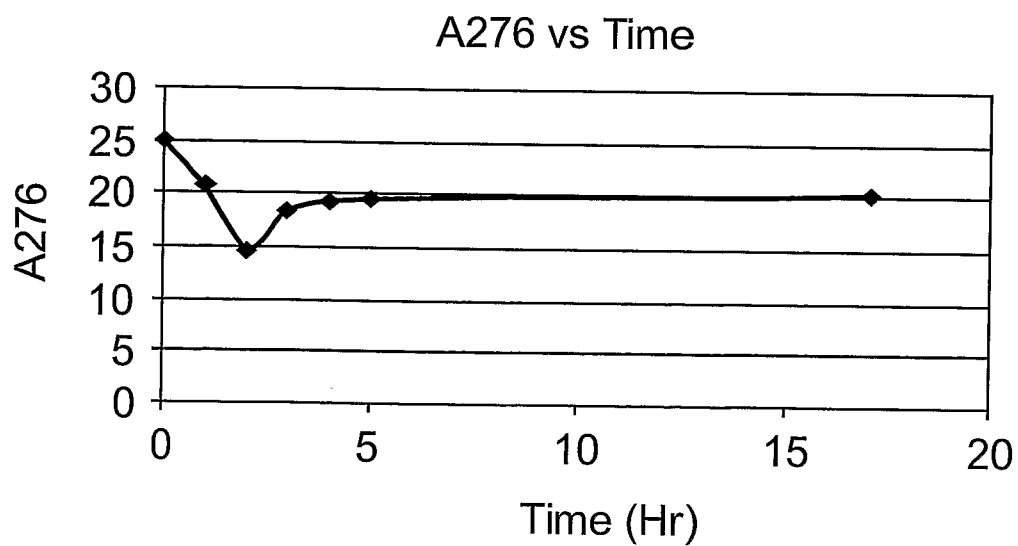
- a) Y & Z = H, X = H, CH₃-, CF₃-, CH₃[CH₂]_{n=1-4}-, [CH₃]₃C-, F-, Cl-, Br-, I-, CN-, CH(OH)CF₃-, CH₃S-, CH₂=CH₂-, CH₃CH=CH-, CH₂=CHCH₂- ; or
- b) Z = H, Y & X = CH₃-, F-, Cl-, Br-, I-, X = CH₃-, CH₃CH₂-, CH₃[CH₂]_{n=2-4}-, [CH₃]₃C-, F-, Cl-, Br-, I-, CH₃S-, CH₂=CH₂-, CH₃CH₂=CH₂- ; or
- c) Y = H, Z & X = CH₃-, F-, Cl-, Br-, I-, X = X = CH₃-, CH₃[CH₂]_{n=1-4}-, [CH₃]₃C-, F-, Cl-, Br-, I-, CH₃S-, CH₂=CH₂-, CH₃CH₂=CH₂-, CH₂=CHCH₂- ; or
- d) X & Z = H, Y = CH₃-, CH₃[CH₂]_{n=1-4}-, [CH₃]₃C-, F-, Cl-, Br-, I-, CH₃S-, CH₂=CH₂-, CH₃CH=CH-, CH₂=CHCH₂-, C₆H₆-
- e) R₁ = CH₃, CF₃-, CH₃[CH₂]_{n=1-4}-, CH₂=CH-, CH₂OH, CH₂[OCH₃]-
- f) R₂ = CH₃-, CH₃[CH₂]_{n=1-4}-
- g) R₃, R₄, R₅, R₆, R₇, R₈, R₉, R₁₀ = N-, CH-
- h) R₁₁ & R₁₂ = H-, Cl-, Br-, I-

Fig. 1

2 / 3

*Fig. 2**Fig. 3*

3 / 3

*Fig. 4**Fig. 5*

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2007/004515

A. CLASSIFICATION OF SUBJECT MATTER INV. C12P7/22 C07C39/24 ADD. C07C39/00		
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 C07C		
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, EMBASE, BIOSIS, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DI GENNARO P ET AL: "Bioconversion of Substituted Naphthalenes to the Corresponding 1,2-Dihydroxy Derivatives by Escherichia coli Recombinant Strains" TETRAHEDRON LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 38, no. 35, 1 September 1997 (1997-09-01), pages 6267-6270, XP004086753 ISSN: 0040-4039 abstract page 6268, line 3 - page 6270, line 3 figures 1,2	1-7, 11-13, 16, 18-20, 22-25
Y	----- -/--	8,14,17, 21
☒ Further documents are listed in the continuation of Box C. ☒ See patent family 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 22 February 2008		Date of mailing of the international search report 05/03/2008
Name and mailing address of the ISA/ 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		Authorized officer Schröder, Gunnar

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2007/004515

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENNARO P D ET AL: "Development of biocatalysts carrying naphthalene dioxygenase and dihydrodiol dehydrogenase genes inducible in aerobic and anaerobic conditions." RESEARCH IN MICROBIOLOGY JUN 2000, vol. 151, no. 5, June 2000 (2000-06), pages 383-391, XP002470192 ISSN: 0923-2508 page 383, column 1, paragraph 1 - page 384, column 1, paragraph 1 page 386, paragraph 2.3 page 388, paragraph 3.2 page 390, column 2, paragraphs 2,3 figure I	1-7, 11-13, 16, 18-20, 22-25
X	EP 0 268 331 A (SHELL INT RESEARCH [NL]) 25 May 1988 (1988-05-25) page 3, line 1 - page 5, line 32; compounds I-VI claims 1,7,8; examples 1-5	1-4,6,7, 11,13, 18-20, 22-25
X	BOYD DEREK R ET AL: "Tandem enzyme-catalysed oxidations of alkyl phenyl sulfides and alkyl benzenes: enantioselective routes to chiral phenols." CHEMICAL COMMUNICATIONS (CAMBRIDGE, ENGLAND) 7 SEP 2002, no. 17, 7 September 2002 (2002-09-07), pages 1914-1915, XP002470164 ISSN: 1359-7345 abstract page 1914, column 1, paragraph 1 - page 1915, column 2, paragraph 5 compounds 3,4,11 and 12 (each A-F) Scheme 1	1-4,6,7, 11-13, 16,18, 22-24
X	BOYD DEREK R ET AL: "Arene cis-dihydrodiol formation: from biology to application." ORGANIC & BIOMOLECULAR CHEMISTRY 21 JAN 2006, vol. 4, no. 2, 21 January 2006 (2006-01-21), pages 181-192, XP002470165 ISSN: 1477-0520 page 182, column 2, paragraph 3 - page 184, column 1, paragraph 2 page 185, column 2, paragraph 4 - page 186, column 1, paragraph 2 Schemes 1 and 2 compounds 1,7,11,25,26,47,48	22,24
	-/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2007/004515

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 03/102217 A (DOW CORNING [US]; GENENCOR INT [US]; MCAULIFFE JOSEPH C [US]; WHITED G) 11 December 2003 (2003-12-11) page 44, line 11 - page 46, line 23 page 1, line 19 - page 2, line 11 page 24, line 13 - page 25, line 4	8,14,21
A		5,16
Y	ZHANG Y ET AL: "A new logic for DNA engineering using recombination in Escherichia coli" NATURE GENETICS, NEW YORK, NY, US, vol. 20, no. 2, October 1998 (1998-10), pages 123-128, XP002103371 ISSN: 1061-4036 abstract page 124, column 2, paragraph 3 - page 126, column 1, paragraph 1; figure 5 page 127, column 1, paragraph 3	17
A	ZYLSTRA G J ET AL: "Toluene degradation by Pseudomonas putida F1. Nucleotide sequence of the todC1C2BADE genes and their expression in Escherichia coli." THE JOURNAL OF BIOLOGICAL CHEMISTRY 5 SEP 1989, vol. 264, no. 25, 5 September 1989 (1989-09-05), pages 14940-14946, XP002470166 ISSN: 0021-9258 abstract page 14941, column 2, paragraph 3 - page 14943, column 2, paragraph 2 figure 4; table II	5,16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2007/004515

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 0268331	A	25-05-1988	DE	3780261 D1	13-08-1992
			DE	3780261 T2	24-12-1992
			JP	63137686 A	09-06-1988
WO 03102217	A	11-12-2003	AU	2003273525 A1	19-12-2003
			CA	2485306 A1	11-12-2003
			EP	1534783 A2	01-06-2005