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(54) **NOVEL METHOD FOR PREPARING PTEROCARPAN**

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USPC **435/119**

(57) **ABSTRACT**

The present invention relates to a novel method for preparing pterocarpan from isoflavan-4-ol.

With the use of the method of the present invention, the absolute configuration of C3 and C4 positions on the B-ring of isoflavan-4-ols is maintained identically also in pterocarpans, and thus, enantiopure pterocarpans can be efficiently prepared.

Fig. 1

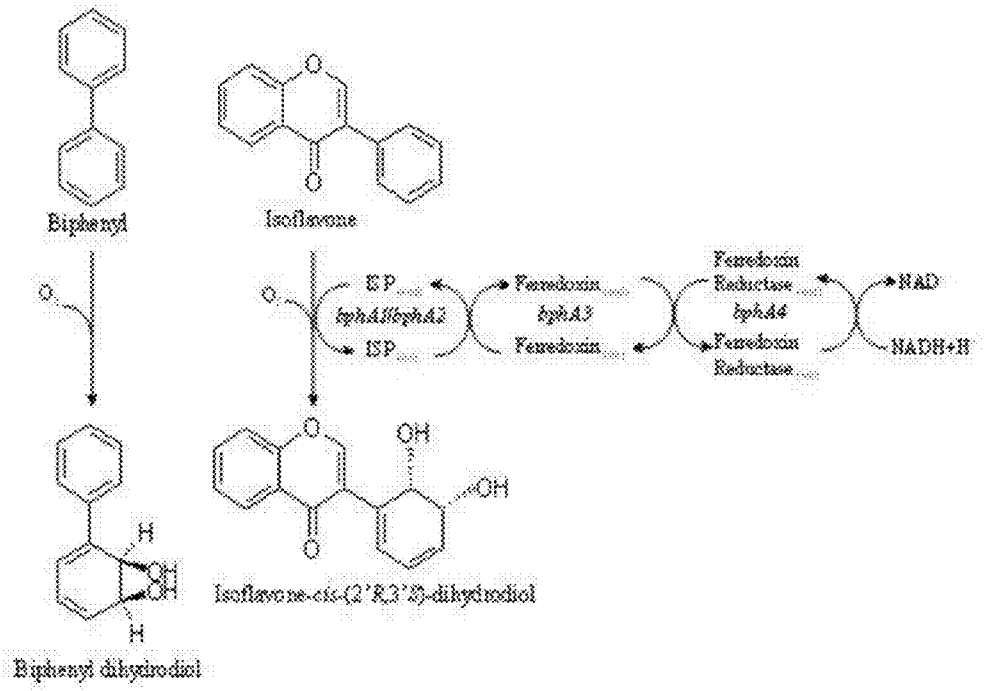

Biphenyl
Isoflavone
O
ISP
IspA4/IspA2
Ferredoxin
IspA3
Ferredoxin
Ferredoxin Reductase
IspA4
Ferredoxin Reductase
NAD
NADH+H
OH
H
Biphenyl dihydrodiol
Isoflavone-cis-(2'R,3'S)-dihydrodiol

Fig. 2

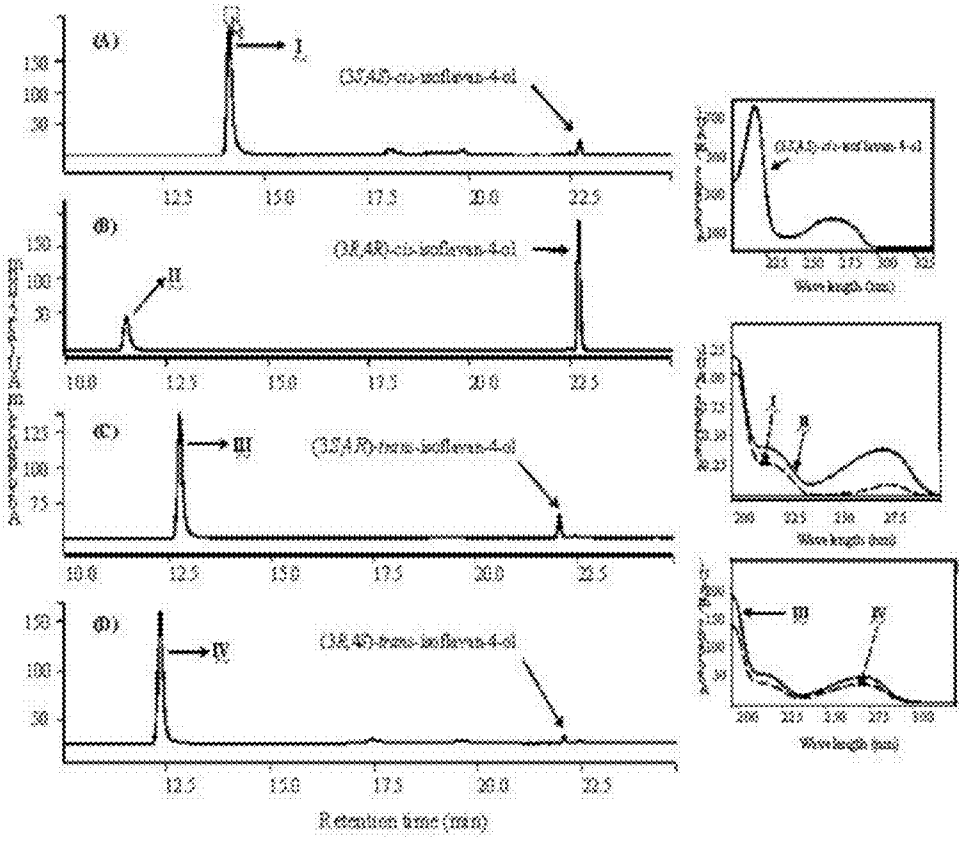

(A)
I
(3S,4S)-cis-isoflavan-4-ol
(B)
II
(3R,4R)-cis-isoflavan-4-ol
(C)
III
(3S,4R)-trans-isoflavan-4-ol
(D)
IV
(3R,4S)-trans-isoflavan-4-ol
Retention time (min)
Wavelength (nm)

Fig. 3a

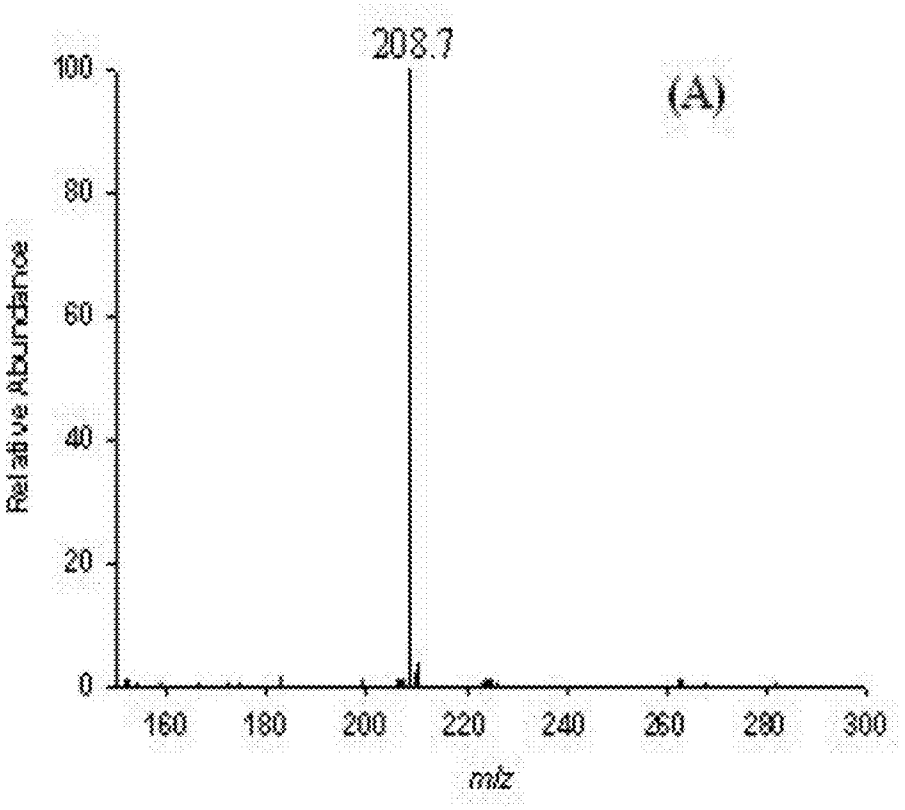

208.7
(A)
100
80
60
40
20
0
Relative Abundance
160
180
200
220
240
260
280
300
m/z

Fig. 3b

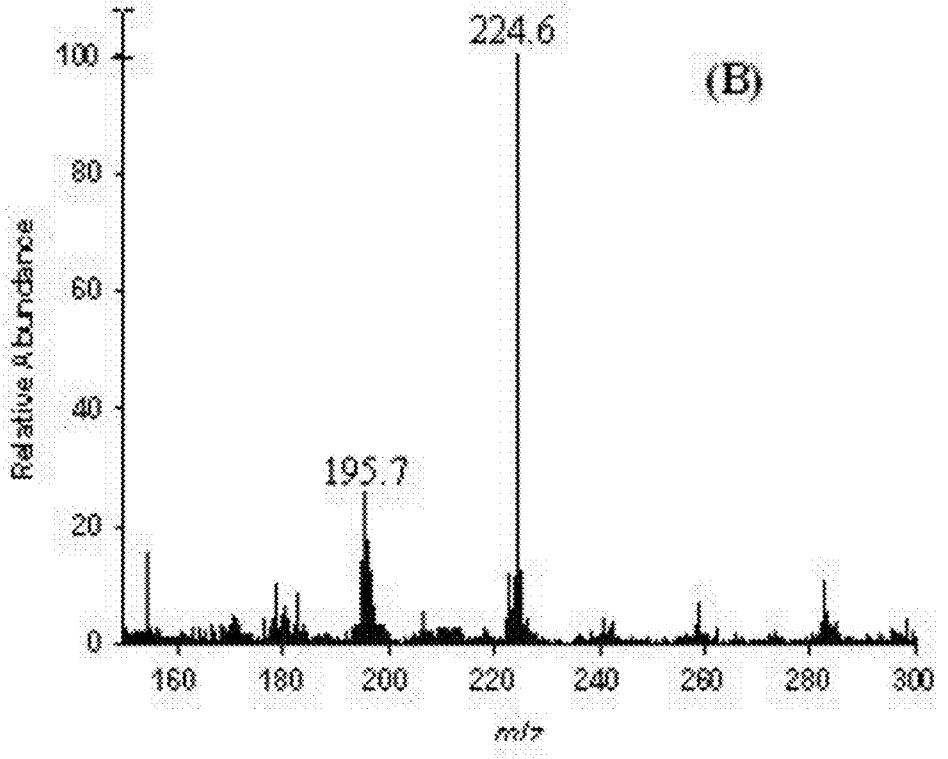

224.6
(B)
195.7
100
80
60
40
20
0
Relative Abundance
160
180
200
220
240
260
280
300
m/z

Fig. 3c

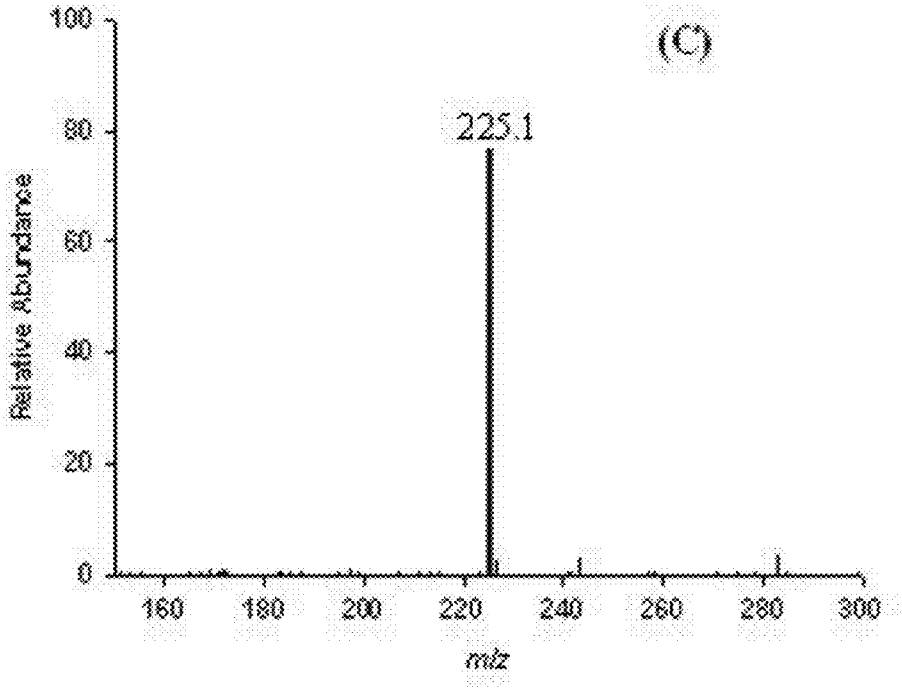

(C)
225.1
Relative Abundance
100
80
60
40
20
0
160
180
200
220
240
260
280
300
m/z

Fig. 4

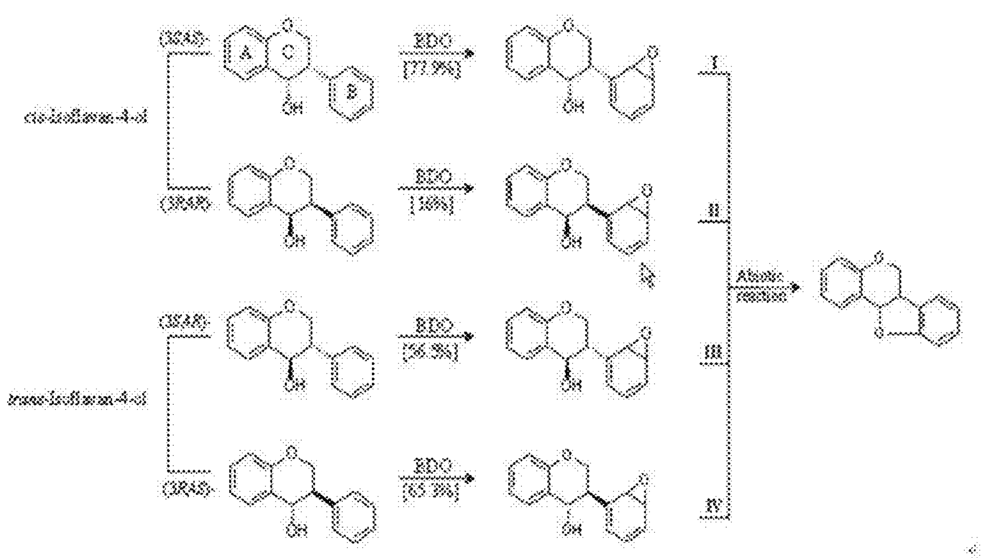


Fig. 5

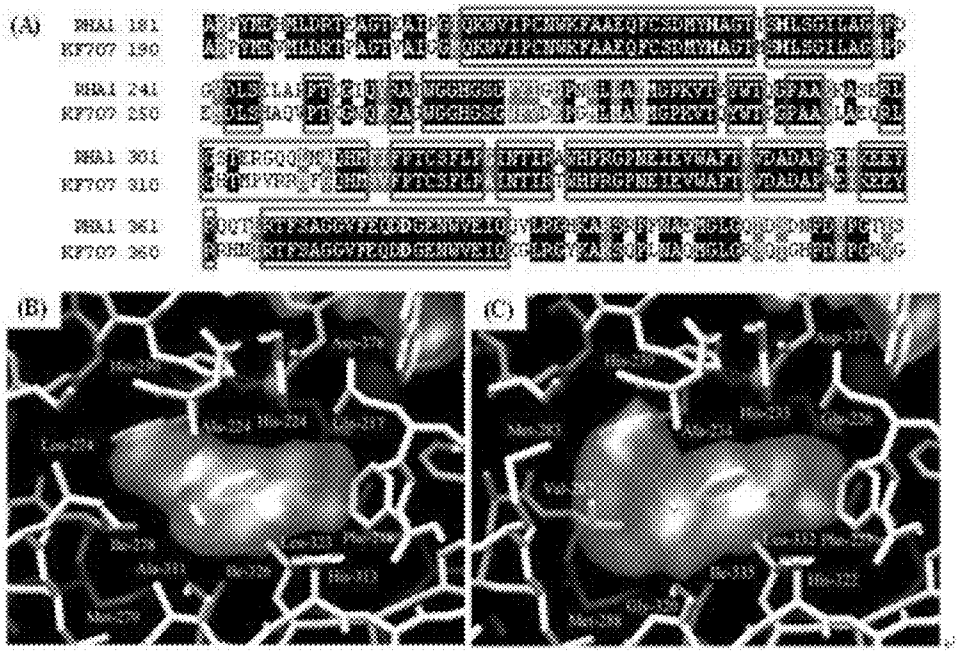


Fig. 6a

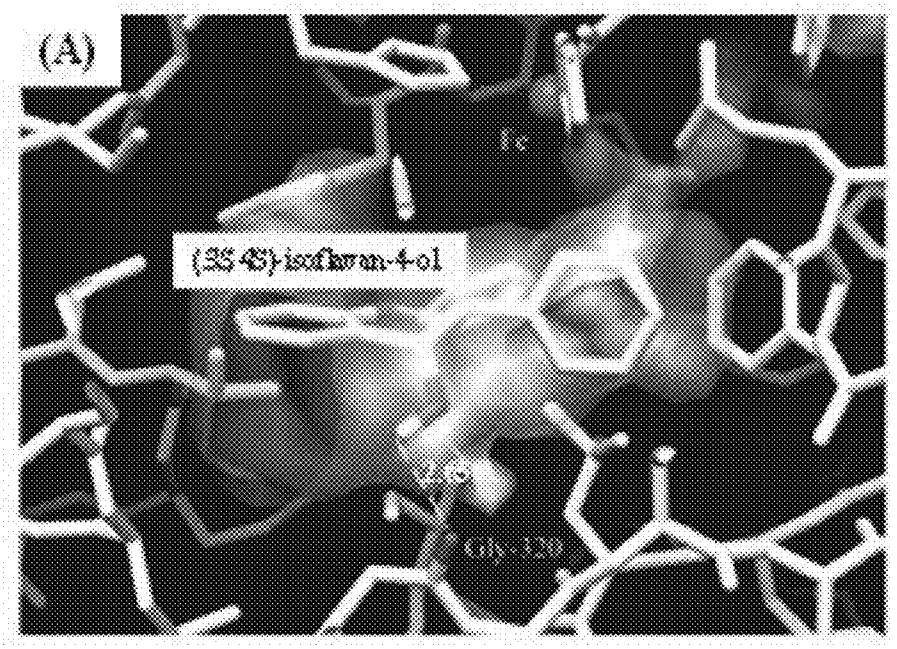


Fig. 6b

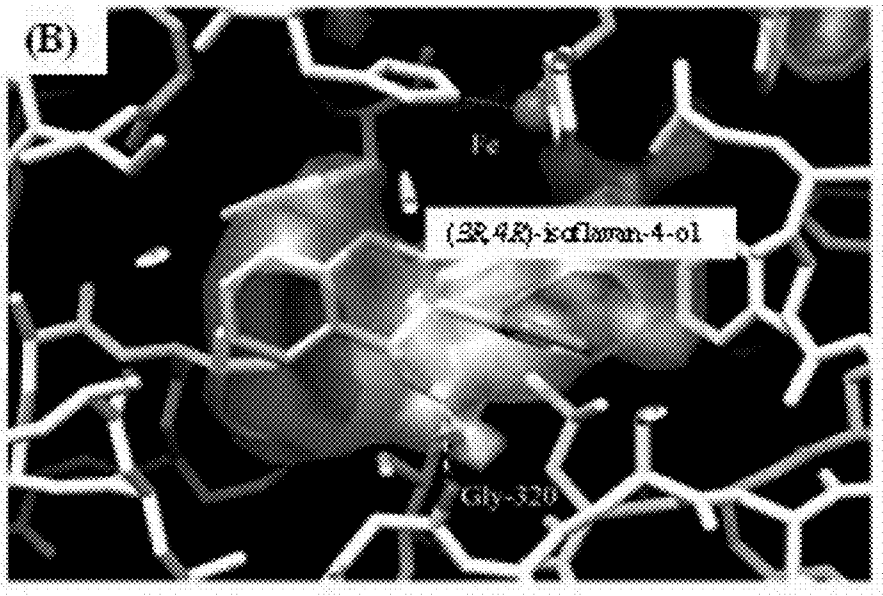


Fig. 6c

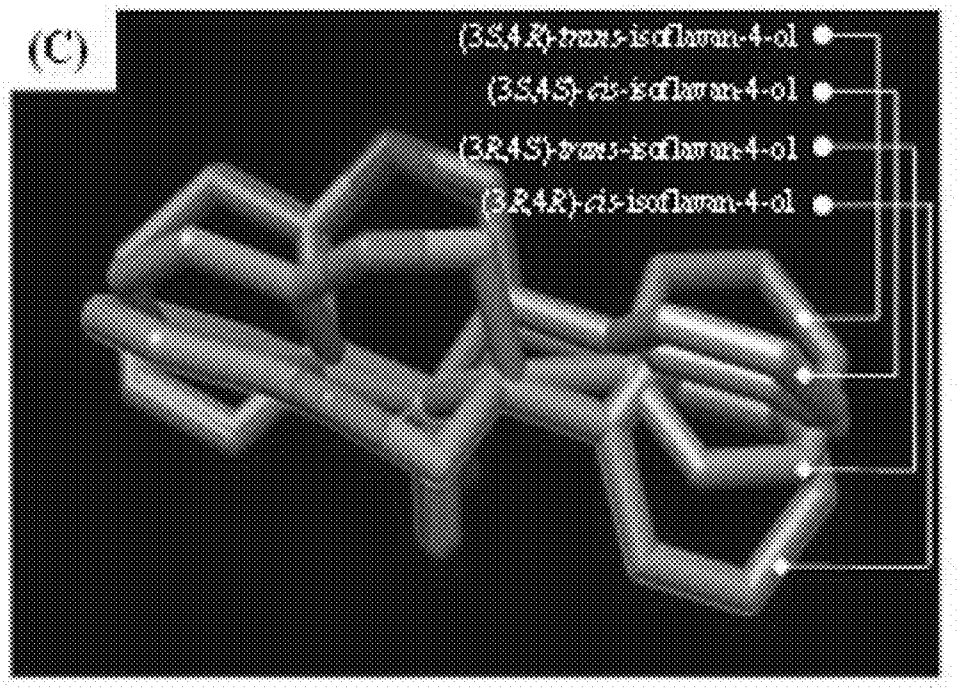

(C)
(3S,4R)-trans-isoflavan-4-ol
(3S,4S)-cis-isoflavan-4-ol
(3R,4S)-trans-isoflavan-4-ol
(3R,4R)-cis-isoflavan-4-ol

Fig. 7

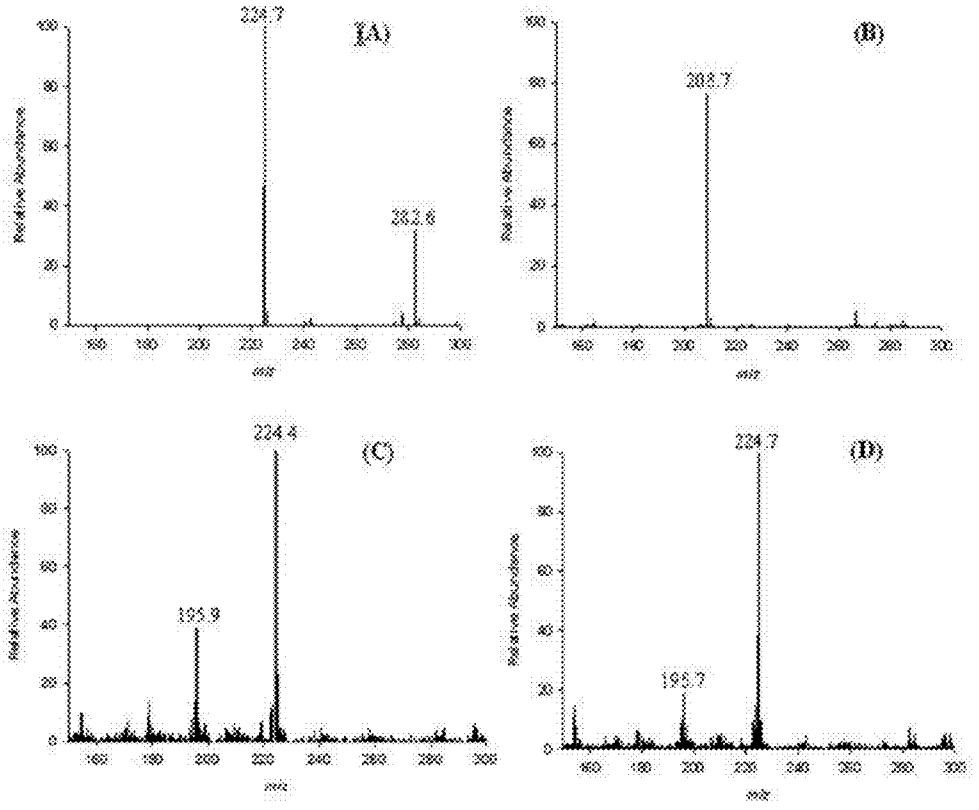


Fig. 8

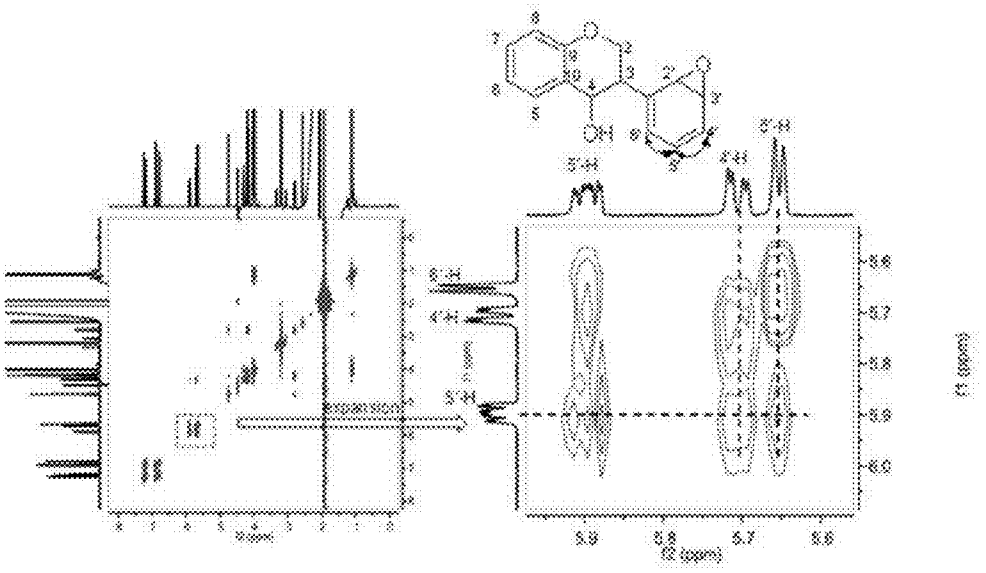


Fig. 9

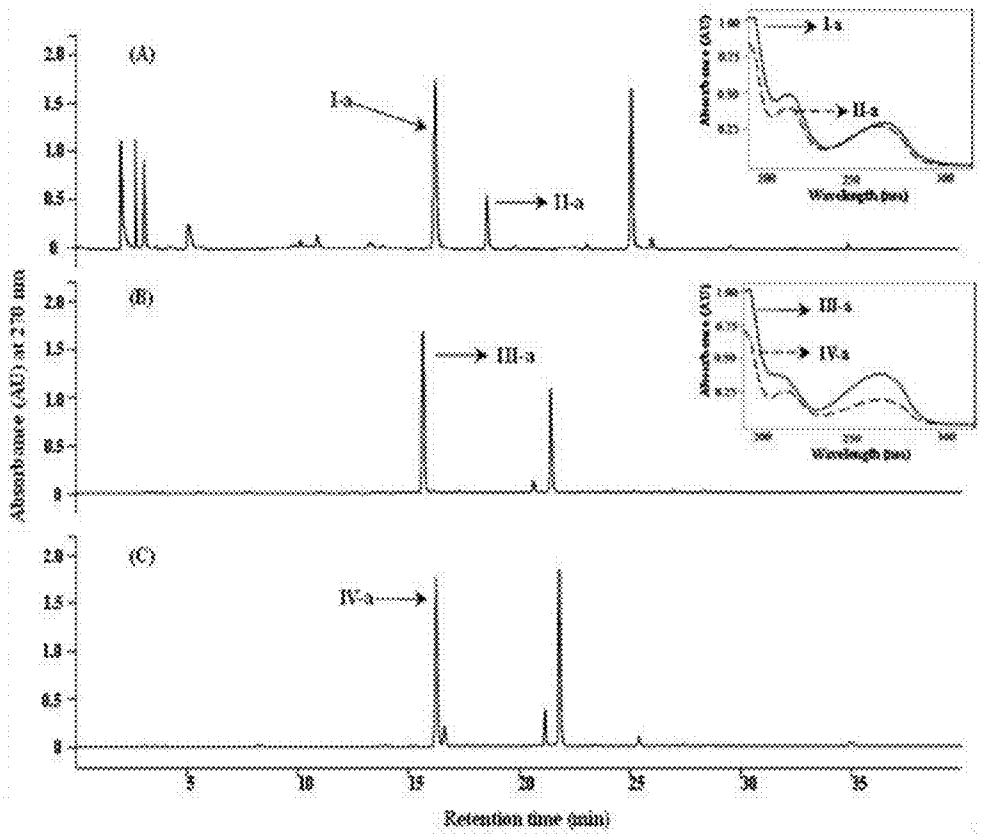

(A)
I-a
II-a
(B)
III-a
IV-a
(C)
Absorbance (AU) at 270 nm
Retention time (min)

Fig. 10a

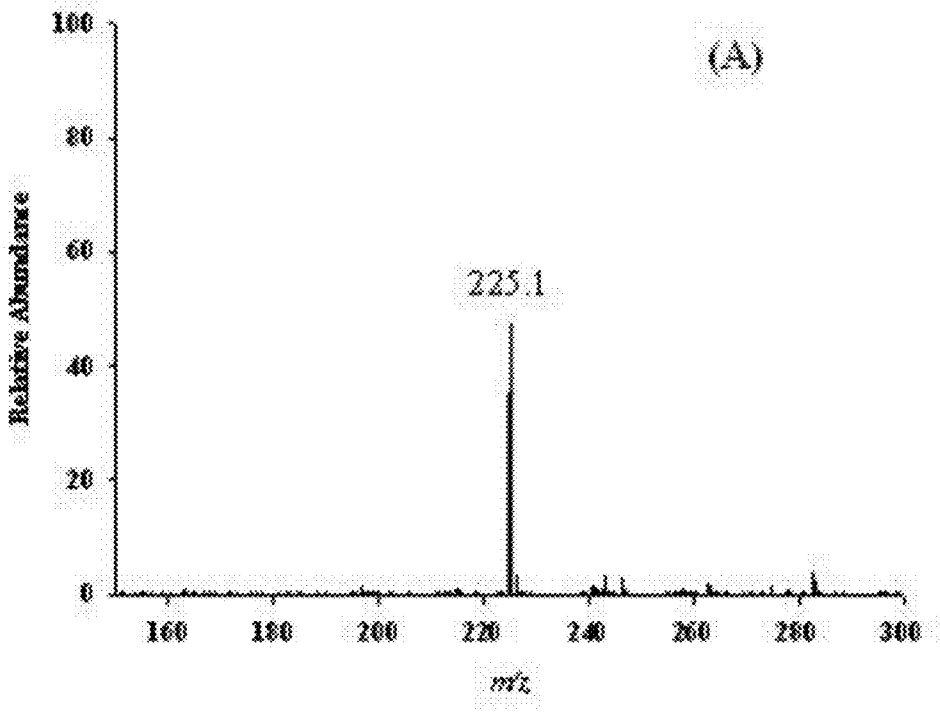

(A)
225.1
Relative Abundance
100
80
60
40
20
0
160
180
200
220
240
260
280
300
m/z

Fig. 10b

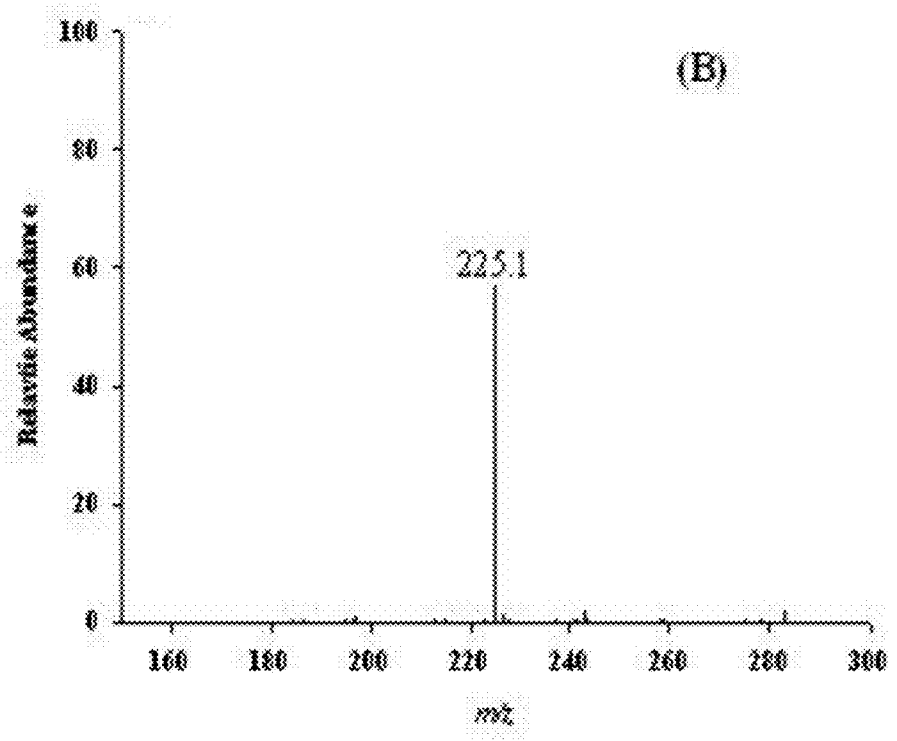


Fig. 10c

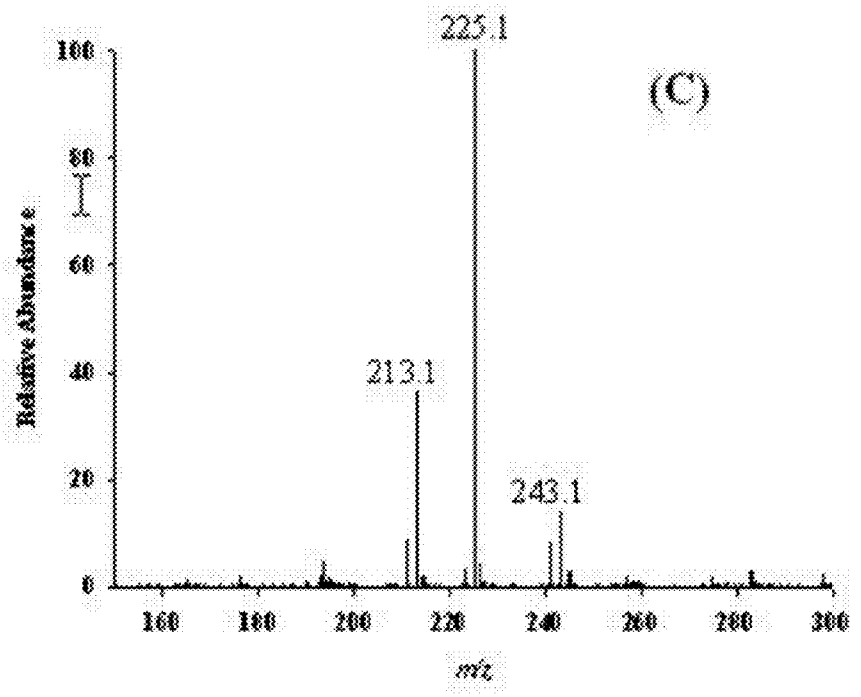

225.1
(C)
213.1
243.1
100
80
60
40
20
0
160
180
200
220
240
260
280
300
m/z

Fig. 11a

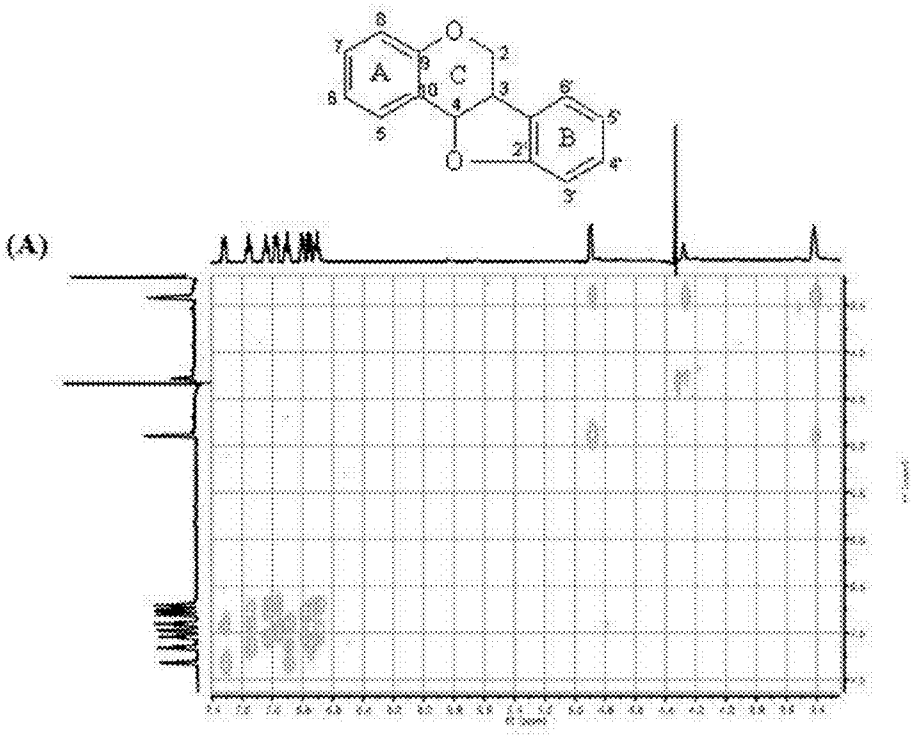
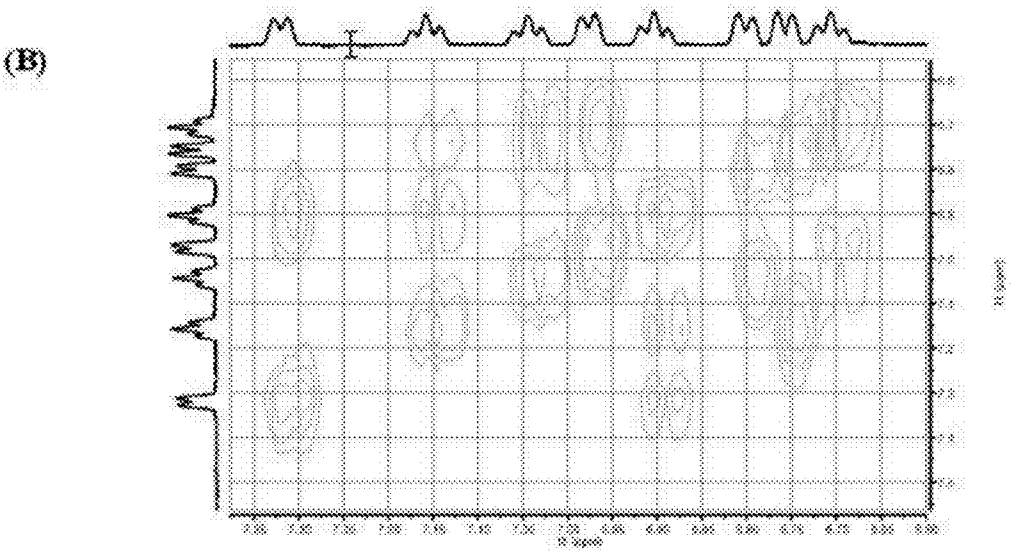


Fig. 11b



NOVEL METHOD FOR PREPARING PTEROCARPAN

TECHNICAL FIELD

[0001] The present invention disclosed herein relates to a novel method for preparing pterocarpans.

[0002] More particularly, the present invention relates to a novel method for preparing enantiopure pterocarpans.

BACKGROUND ART

[0003] Aromatic hydrocarbons, which are common environmental pollutants, may be metabolized aerobically through the initial activation of the aromatic ring initiated by the insertion of oxygen atoms by monooxygenase or dioxygenase enzymes from bacteria [9, 24]. Such aerobic biotransformations may result in the production of intermediates that contain epoxide or dihydrodiol functional groups that may be then further metabolized. It is well known that heme-type cytochrome P450 generally produce aromatic epoxides that may be further metabolized to trans-dihydrodiols [3, 25] while non-heme bacterial dioxygenases produce cis-dihydrodiols from aromatic ring structures (FIG. **1**) [28, 32].

[0004] Recently, the present inventors reported flavanone epoxide formation by the biphenyl dioxygenase (BDO) of *Pseudomonas pseudoalcaligenes* strain KF707 after expression in *Escherichia coli,* and this unique monooxygenase activity was explained based on the structural properties of the flavanone in the active site [10]. In this case, the flavanone B-ring was unable to form the biphenyl-type structure required for dihydrodiol formation due to the position of the C-3 tetrahedral center on the flavanone structure and may be compared to previous studies where flavanone substrates that possessed the same B-ring conformation as biphenyl were biotransformed to flavone cis-dihydrodiol by biphenyl dioxygenase [20, 27].

[0005] Flavonoids are a large group of natural products that have recently been garnering much attention in the disciplines of nutrition, food science, environmental science, and pharmacology due to their potential beneficial effects on health [19]. Actually, flavonoids exhibit inhibitory effect on capillary permeability, peripheral circulation improving effect, anti-inflammatory effect, anti-atherogenic effect, antioxidant effect, anti-allergic effect, and anti-cancer effect, and thus have potential uses as medicine [2, 12, 15, 34]. For example, isoflavones, such as genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone), are natural edible phytoestrogens found mainly in leguminous plants, and due to their structure similar to a mammalian estrogen [13, 17, 35], they appear to play an important role in preventing hormone-dependent diseases such as breast cancer, prostate cancer, and osteoporosis [1, 11, 14, 18].

[0006] The study of BDO biotransformation of flavonoids may aid in the production of new biologically active compounds as well as provide mechanistic insights into the functioning of BDO. The present inventors reported the absolute configurations of four isoflavan-4-ol stereoisomers in the previous study [36].

[0007] Throughout the entire specification, many papers and patent documents are referenced and their citations are represented. The disclosures of cited papers and patent documents are entirely incorporated by reference into the present specification, and the level of the technical field within which the present invention falls and details of the present invention are explained more clearly.

DISCLOSURE

Technical Problem

[0008] The present inventors have studied and tried to develop novel methods for synthesizing flavonoids that have recently been garnering much attention in the disciplines of nutrition, food science, environmental science, and pharmacology, and consequently, accomplished to synthesize enantiopure pterocarpan from isoflavan-4-ol, thereby completing the present invention.

[0009] Therefore, the present invention is to provide a novel method for preparing pterocarpan.

[0010] Other purposes and benefits of the present invention become clear by the following detailed description of invention, claims, and drawings.

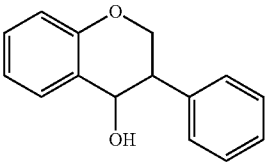
Technical Solution

[0011] In accordance with an exemplary embodiment of the present invention, a method for preparing pterocarpans includes the steps of: (a) contacting a substrate, isoflavan-4-ol, and an enzyme, biphenyl dioxygenase, to form isoflavan-4-ol-2,3-epoxide; and (b) forming pterocarpan from isoflavan-4-ol-2,3-epoxide.

[0012] The present inventors have studied and tried to develop novel methods for synthesizing flavonoids that have recently been garnering much attention in the disciplines of nutrition, food science, environmental science, and pharmacology, and consequently, accomplished to synthesize enantiopure pterocarpans from isoflavan-4-ols, thereby completing the present invention.

[0013] Isoflavan-4-ols used in the present invention are represented by the following chemical formula 1, and exist in four stereoisomers: (3S,4S)-cis-isoflavan-4-ol, (3R-4R)-cis-isoflavan-4-ol, (3R,4S)-trans-isoflavan-4-ol, and (3S,4R)-trans-isoflavan-4-ol.

Chemical formula 1



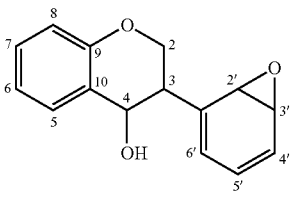
[0014] In one embodiment, isoflavan-4-ol is synthesized from the reduction of isoflavones in the presence of Pd/C and ammonium formate under nitrogen atmosphere as described in the document [36].

[0015] In the preferred embodiment, isoflavan-4-ol is transformed by biphenyl dioxygenase into an intermediate product, isoflavan-4-ol-2,3-epoxide. Biphenyl dioxygenase is regiospecific for four isoflavan-4-ol stereoisomers, and exhibits a stereospecific unique monooxygenase activity to form the corresponding epoxides.

[0016] The corresponding epoxides may be represented by chemical formula 2, and exist in four stereoisomers: (3S,4S)-cis-isoflavan-4-ol-2,3-epoxide, (3R-4R)-cis-isoflavan-4-ol-

2,3-epoxide, (3R,4S)-trans-isoflavan-4-ol-2,3-epoxide, and (3S,4R)-trans-isoflavan-4-ol-2,3-epoxide.

Chemical formula 2



[0017] When isoflavan-4-ol is transformed into isoflavan-4-ol-2,3-epoxide by using biphenyl dioxygenase in the present invention, the absolute configuration of C3 and C4 on the B-ring of isoflavan-4-ol is identically maintained also in the intermediate product, isoflavan-4-ol-2,3-epoxide. For example, when enantiopure (3S,4S)-cis-isoflavan-4-ol is used as the substrate, enantiopure (3S,4S)-cis-isoflavan-4-ol-epoxide is formed as the intermediate product.

[0018] In one embodiment, the step (a), that is, transforming isoflavan-4-ol into isoflavan-4-ol-2,3-epoxide, is carried out, by adding purified enzyme biphenyl dioxygenase to the substrate isoflavan-4-ol to yield isoflavan-4-ol-2,3-epoxide or by using bacterial cells expressing biphenyl dioxygenase to yield isoflavan-4-ol-2,3-epoxide in large quantities.

[0019] The bacteria may include *Lactobacillus, Bifidobacterium, Escherichia coli, Aquificae, Bacteroids, Chlamydia, Spirochaete,* and the like, and preferably be, but not limited to, *Escherichia coli.*

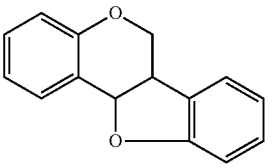
[0020] Biphenyl dioxygenase may be one purchased commercially to use, and biphenyl dioxygenase derived from any organisms may be used, but biphenyl dioxygenase derived from *Pseudomonas pseudoalcaligenes* or *Rhodococcus* species is preferable to use. Most preferably, biphenyl dioxygenase of nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:3 is used.

[0021] In one embodiment of the present invention where biphenyl dioxygenase derived from *Pseudomonas pseudoalcaligenes* was used, the relative reactivity of the stereoisomers was in the following order: (3S,4S)-cis-isoflavan-4-ol> (3R,4S)-trans-isoflavan-4-ol>(3S,4R)-trans-isoflavan-4-ol> (3R,4R)-cis-isoflavan-4-ol.

[0022] The present inventors found that isoflavan-4-ol-2,3-epoxdie formed as above is naturally transformed into pterocarpan. This transformation which occurs in step (b) in the present invention is presumed to be caused by structural instability of isoflavan-4-ol-2,3-epoxide.

[0023] Pterocarpans, the structural backbones found in plant-protective phytoalexins, such as maackiain from *Cicer arietinum* and medicarpin from *Medicago sativa,* are represented by the following chemical formula 3 and exist in four stereoisomers: (3S,4S)-cis-pterocarpan, (3R,4R)-cis-pterocarpan, (3S,4R)-trans-pterocarpan and (3R,4S)-trans-pterocarpan.

Chemical formula 3



[0024] In step (b) of the present invention, the absolute configuration of C3 and C4 on the B-ring is unchanged and identically maintained. For example, enantiopure (3S,4S)-cis-isoflavan-4-ol-2,3-epoxide is transformed into enantiopure (3S,4S)-cis-pterocarpan.

[0025] In conclusion, if, for example, enantiopure (3S,4S)-cis-isoflavan-4-ol is used as the substrate, it goes through enantiopure (3S,4S)-cis-isoflavan-4-ol-epoxide, the intermediate product, and enantiopure (3S,4S)-cis-pterocarpan is obtained. Thus, the present invention provides the novel method for preparing enantiopure pterocarpans.

Advantageous Effects

[0026] The gist of characteristic and benefit of the present invention is as follows:

[0027] (i) The present invention provides a novel method for preparing pterocarpan from isoflavan-4-ol.

[0028] (ii) With the use of the method of the present invention, the absolute configuration of C3 and C4 on the B-ring of isoflavan-4-ols is maintained identically also in pterocarpans, and thus, enantiopure pterocarpans can be efficiently prepared.

DESCRIPTION OF DRAWINGS

[0029] FIG. **1** shows reactions of biphenyl dioxygenase of *P. pseudoalcaligenes* strain KF707 with the physiological substrate biphenyl and non-physiological substrate isoflavone.

[0030] FIG. **2** shows HPLC elution profile of (3S,4S)-cis-isoflavan-4-ol (A), (3R, 4R)-cis-isoflavan-4-ol (B), (3S, 4R)-trans-isoflavan-4-ol (C) and (3R, 4S)-trans-isoflavan-4-ol (D) reaction metabolites produced by whole cells of *E. coli* expressing the biphenyl dioxygenase of *P. pseudoalcaligenes* strain KF707. Inserted drawings show UV-visible spectra of (3S,4S)-cis-isoflavan-4-ol and metabolites I, II, III, and IV.

[0031] FIG. **3** is ESI-MS spectra of cis-isoflavan-4-ol (A), its metabolite I (B), and abiotic product (C).

[0032] FIG. **4** shows a biotransformation pathway of cis-isoflavan-4-ol and trans-isoflavan-4-ol by biphenyl dioxygenase of *P. Pseudoalcaligenes* KF707. The percentage of each epoxide product is shown in brackets.

[0033] FIG. **5** shows an amino acid sequence alignment of the α-subunits of RHA1 and KF707 BDOs. (A): conserved sequences are marked with a blue box and the residues near the substrate binding site and possible substrate channel are marked with a yellow box. The Fe binding residues are shown with a bold box. Volumes of the substrate binding sites were calculated as 38.33 3 for *Rhodococcus* species (B) and 102.8 3 for *P. pseudoalcaligenes* strain KF707 (C).

[0034] FIG. **6** shows docking model of (3S,4S)-isoflavan-4-ol (A) and (3R,4R)-isoflavan-4-ol (B) in the active site of BDO KF707. The B-ring of the substrate is positioned away from the Fe center. (c) shows the overlapped structures of four isoflavan-4-ol stereoisomers.

[0035] FIG. **7**(A) is an ESI-MS spectrum of metabolite II derived from cis-isoflavan-4-ol; FIG. **7**(B) is a spectrum of trans-isoflavan-4-ol; FIG. **7**(C) is a spectrum of metabolite III of trans-isoflavan-4-ol; and FIG. **7**(D) is a spectrum of metabolite IV of trans-isoflavan-4-ol.

[0036] FIG. **8** is a WETgCOSY spectrum of metabolite III of (3S,4R)-trans-isoflavan-4-ol.

[0037] FIG. **9**(A) is an HPLC elution profile of abiotic products I-a and II-a derived from cis-isoflavan-4-ol-2',3'-epoxide; FIG. **9**(B) is a profile of III-a derived from (3S,4R)-trans-isoflavan-4-ol-2',3'-epoxide; and FIG. **9**(C) is an elution profile of IV-a derived from (3R,4S)-trans-isoflavan-4-ol-2', 3'-epoxide. Inserted drawings are UV-visible spectra of the above abiotic products.

[0038] FIG. **10** is ESI-MS spectra of abiotic products II-a derived from (3R,4R)-cis-isoflavan-4-ol-2',3'-epoxide (A), III-a derived from (3S,4R)-trans-isoflavan-4-ol-2',3'-epoxide (B), and IV-a derived from (3R,4R)-trans-isoflavan-4-ol-2', 3'-epoxide.

[0039] FIG. **11** is WETgCOSY spectra of abiotic product III-a (A), and its aromatic region (B).

BEST MODE

[0040] Hereinafter, the present invention will be described in detail through embodiments. These embodiments are just for exemplifying the present invention, and it is apparent to those skilled in the art that the scope of the present invention is not limited by these embodiments.

Embodiments

[0041] Materials and Methods

[0042] 1. Chemicals

[0043] Isoflavone was purchased from Indofine Chemical Company (Hillsborough, N.J., USA). Acetonitrile, ethyl acetate, and methanol (HPLC grade) were purchased from Fischer (Pittsburgh, Pa., USA). The four isoflavan-4-ol stereoisomers, (3S,4S)- and (3R,4R)-cis-isoflavan-4-ols, and (3S, 4R)- and (3R,4S)-trans-isoflavan-4-ols, were synthesized and purified as described in the document [36].

[0044] 2. Bacterial Strain and Culture Conditions

[0045] *Escherichia coli* JM109 (pJHF108) which carries the dioxygenase gene derived from *P. pseudoalcaligenes* strain KF707 were provided by K. Furukawa, Kyushu University, Japan. Cloning of *E. coli* JM109 (pJHF108) can be easily done by those skilled in the art according to the paper (Hirose, J., A. Suyama, T. Zaiki, S. Hayashida, and K. Furukawa, Construction of hybrid biphenyl (bph) and toluene (tod) genes for functional analysis of aromatic ring dioxygenases, Gene, 138:27-33(1994)). Sequence of dioxygenase derived from *P. pseudoalcaligenes* strain KF707 in the present embodiment is a nucleotide sequence of SEQ ID NO:1.

[0046] *E. coli* JM109 (pJHF108) was seed cultivated in 50 mL of LB medium containing ampicillin 50 mg/mL at 37° C. for 12 hours. The cultivated seed was inoculated to a 5-L fermentor (BioFlo 3000, Korea fermentor Co., Korea) containing 4.5 L of LB medium and 50 mg/mL of ampicillin, and mixed at 37° C. at 200 rpm for 8 hours. The culture broth was centrifuged at 8,000 g for 10 minutes using VS-21SMT centrifuge (Vision Scientific Co., Bucheon, Korea). Cells were washed three times with 100 mM minimal salt buffer and resuspended in the same buffer, adjusting to the optical density at 600 nm using a UV/visible spectrophotometer (Shimadzu, UV-1601PC, Kyoto, Japan).

[0047] 3. Biotransformation Reactions and Metabolite Extraction

[0048] Stock solutions (100 mM) of each purified isoflavan-4-ol stereoisomer, (3S,4S)- and (3R,4R)-cis-isoflavan-4-ols and (3S,4R)- and (3R,4S)-trans-isoflavan-4-ols were prepared in a mixture of dimethyl sulfoxide and methanol (2:1, v/v) and added to the resuspended cells at a final concentration of 100 μM. Glucose was added as a reaction energy source at a final concentration of 100 μM. The reaction mixture was incubated on a rotary shaker at 200 rpm at 37° C. for 18 hours, and then extracted with three volumes of ethyl acetate. The ethyl acetate extract was evaporated to dryness with a rotary evaporator (Eyela, Tokyo, Japan) in vacuo, and the residue was dissolved in methanol and filtered through a 0.2 μm Whatman PVDF syringe filter prior to LC-MR analysis.

[0049] 4. High-Performance Liquid Chromatography

[0050] Analytical HPLC was carried out using a Varian Prostar HPLC apparatus (Walnut Creek, Calif.) equipped with a photodiode array (PDA) detector. For the analysis of isoflavan-4-ol stereoisomers and their metabolites, a Waters Spherisorb ODS-2 column (5 mm particle size, 4.6 mm×25 cm, Milford, Mass.) was used. The mobile phase was operated as a linear gradient that made up of water-acetonitrile containing 0.1% formic acid (10% acetonitrile at 0 min, 40% acetonitrile at 15 min, 90% acetonitrile at 25 min, and 90% acetonitrile at 40 min) The flow rate was 1 mL/min and UV detection was performed at 270 nm. For the analysis of enantiomers of cis- and trans-isoflavan-4-ol, a Sumi Chiral OA-7000 column (5 mm, 4 6 mm×25 cm, Sumika Chemical Analysis Service, Ltd., Osaka, Japan) and mobile phase composed of 60:40 (v/v) of 20 mM phosphate buffer (pH 3.0) and acetonitrile were used. The flow rate was 1 mL/min and UV detection was performed at 270 nm.

[0051] 5. Liquid Chromatography/Mass Spectrometry

[0052] Liquid chromatography/mass spectrometry (LC-MS) was performed by coupling an Alliance 2695 (Waters Corporation, Milford, Mass.) LC (SunFire C18 column; 3.5 mm, 2.1×150 mm, Waters) to a Quattro LC triple quadrupole tandem mass spectrometer (Waters, Milford, Mass.) in positive electrospray ionization (ESI+) mode. For LC analysis, the mobile phase, elution program, and detection were the same as those of analytical HPLC and the flow rate was 0.2 mL/min. For MS analysis, the source temperature, desolvation temperature, electron multiplier voltage, and capillary voltage were kept at 150° C., 350° C., 700 V, and 3.0 kV, respectively. The cone voltage was 25 V. The cone gas and desolvation gas were ultra-pure nitrogen set at 30 l/h and 500 l /h, respectively.

[0053] 6. Liquid Chromatography/Nuclear Magnetic Resonance Spectroscopy

[0054] LC NMR analyses were performed under ambient conditions using a Prostar 230 ternary gradient pump, a Prostar 430 autosampler (Varian, Walnut Creek, Calif., USA), and a Prostar 335 photodiode array detector (Varian, Mulgrave, Victoria, Australia). Separation was carried out using a Hydrosphere-C18 column (3 μm particle size, 150×4.6 mm, YMC, Kyoto, Japan) and the mobile phase made up of acetonitrile and deuterated water (D2O). The gradient program was as follows: 25% acetonitrile at 0 min and 60% acetonitrile at 35 min. The flow rate was maintained at 0.4 mL/min. The chromatographic profile was recorded at 270 nm.

[0055] NMR data were acquired using a Varian NMR system 500 MHz (Varian, Palo Alto, Calif. JSA) equipped with cold probe with a 60 μL flow cell. 1H-NMR spectra were obtained in stop-flow mode. Varian WET solvent suppression was used to suppress the acetonitrile and the residual water peaks. COSY spectra were obtained using water suppression enhancement through T1 effect gradient correlation spectroscopy (WETgCOSY) pulse sequencing in which the WET element was incorporated into the gCOSY sequence.

[0056] 7. Biotransformation Kinetics of Purified (3S,4S)- and (3R,4R)-cis-isoflavan-4-ols and (3S,4R)- and (3R,45)-trans-isoflavan-4-ols

[0057] Biotransformation kinetics by whole cells of *E. coli* expressing biphenyl dioxygenase was performed as follows. The bacterial culture was prepared as described above, resuspended in 50 mL of phosphate buffer containing 1 mM glucose in a 250 mL flask, and adjusted to a final culture concentration of OD 600 nm=0.6. Then, the bacterial culture was incubated with 250 μM each of four purified isoflavan-4-ol stereoisomers ((3S,4S)- and (3R,4R)-cis-isoflavan-4-ols and (3S,4R)- and (3R,4S)-trans-isoflavan-4-ols) on a rotary shaker (200 rpm) at 37° C. The bacterial cultures (2 mL) were extracted with ethyl acetate, and the solvent was evaporated to dryness as described above. Quantification of metabolites and parent compound by using HPLC with time was monitored in triplicate experiments. Due to the instability of metabolites which were produced from parent compound, the quantity was calculated from the peak area as compared to the standard curve of the parent compound.

[0058] 8. Enzyme-Substrate Docking Study

[0059] Because the only reported X-ray protein crystallographic structure of BDO is from a *Rhodococcus* species, the present inventors changed the amino acid residues in the substrate binding site and substrate channel of *Rhodococcus* sp. strain RHA1 BDO to those of KF707 BDO. The source of the X-ray protein structure (pdb code=1ULJ) was obtained from the RCSB Protein Data Bank (http://www.pdb.org), and the corresponding amino acid residues were mutated using Swiss-PDB Viewer software. The Chimera Program was used for the enzyme-substrate docking study, including substrate volume calculation.

[0060] Results and Discussion

[0061] Purified (3S,4S)- and (3R,4R)-cis-isoflavan-4-ols and (3S,4R)- and (3R,4S)-trans-isoflavan-4-ols were used as substrates for *E. coli*-expressed biphenyl dioxygenase derived from *P. pseudoalcaligenes* strain KF707. Metabolites produced from (3S,4S)- and (3R,4R)-cis-isoflavan-4-ols and (3S,4R)- and (3R,4S)-trans-isoflavan-4-ols, were designated as I, II, III, and IV, respectively (FIG. **2**). Metabolites I, II, III, and IV were eluted at 14.6, 11.8, 12.9, and 12.4 min each, and had identical UV absorption peaks at 219 and 275 nm (FIG. **2**). Therefore, it was concluded that all four metabolites were expected to have the same valence electron energies, meaning that they included the same or closely related molecular structures and functional groups.

[0062] LC-MS analyses indicated that the isoflavan-4-ols showed a peak at m/z 208.7 corresponding to [M−(H2O)]+ (FIG. **3**A and FIG. **7**) and metabolites I, II, III, and IV showed peaks at m/z 224.6, 224.7, 224.4, and 224.7, respectively, presumably corresponding to [M−(H2O)]+species (FIG. **3**B and FIG. **7**). The 16 mass unit differences that were consistently observed between the substrates and metabolites indicated that epoxidation or monohydroxylation occurred, and it was concluded that the isoflavan-4-ol metabolites were derivatives of isoflavan-4-ol compounds that possessed either a phenol or an epoxide functional group.

[0063] The present inventors performed LC-NMR (500 MHz, D2O/acetonitrile) experiment to identify structures of metabolites I, II, III, and IV, and the resulting 1H NMR spectra were shown in the following Table 1.

TABLE 1

δ of ^{1}H					^{1}H-^{1}H	
I	II	III	III-a	IV	COSY	Assignment
4.21(dd, 10.8, 3.5)	4.20(dd, 10.8, 3.6)	4.29(dd, 10.8, 2.5)	~4.31(water overlapped)	4.21(dd, 11.0, 3.1)	2b, 3	2a
4.17(t, 10.8)	4.14(t, 10.8)	4.2(m)	~4.31(water overlapped)	4.1(m)	2a, 3	2b
2.80(dr, 10.8, 3.5)	2.86(dt, 10.8, 3.4)	2.67(m)	3.42(dt, 5.9, 4.0)	2.67(m)	2a, 2b, 4	3
4.75(d, 3.4)	4.79(d, 3.4)	4.71(5.8)	4.90(d, 5.9)	4.78(d, 6.2)	3	4
7.25(d, 7.8)	7.26(d, 7.9)	7.29(d, 7.5)	7.32(d, 7.8)	7.34(d, 7.5)	6	5
6.9(t, 7.8)	6.9(t, 7.8)	6.9(d, 7.5)	6.9(t, 7.7)	6.93(t, 7.5)	5, 7	6
7.18(t, 7.6)	7.18(t, 8.1)	7.16(d, 7.8)	7.15(t, 7.7)	7.16(t, 7.8)	6, 8	7
6.79(d, 8.0)	6.78(d, 8.0)	6.75(d, 7.9)	6.75(d, 7.8)	6.73(d, 7.8)	7	8
4.02(ethylacetate overlapped)	3.99(d, 5.69)	3.94(d, 5.4)	~	3.97(d, 5.78)	3'	2'
4.3(m)	4.32(m)	4.20(m)	6.79(d, 7.9)	4.28(m)	2', 4'	3'
5.7(dd, 9.5, 3.0)	5.68(dd, 9.5, 2.7)	5.65(dd, 9.2, 2.2)	7.04(t, 7.8)	5.65(dd, 9.7, 2.0)	3', 5'	4'
5.90(m)	5.89(m)	5.82(m)	6.70(t, 7.6)	5.78(m)	4', 6'	5'
5.65(d, 5.5)	5.74(d, 5.5)	5.85(d, 5.3)	6.98(d, 7.6)	5.57(d, 5.4)	5'	6'

[0064] As shown in Table 1, the 1H NMR spectra of metabolites I, II, III, and IV exhibited similar patterns. Four aromatic peaks at δ 6.7~7.3 were assigned to A-ring protons in isoflavan-4-ols. Three olefinic peaks at **67** 5.5~5.9 and two oxygenated aliphatic protons at δ4.0~4.3 were detected, which suggested epoxide formation on the B-ring. According to the WETgCOSY spectra obtained from metabolite III of (3S,4R)-trans-isoflavan-4-ol (FIG. **8**), the presence of three olefinic protons confirmed the epoxide formation between C2' and C3' on the B-ring of the corresponding isoflavan-4-ols [10]. These LC-NMR data were in agreement with LC-MS data, which showed the addition of an oxygen atom (m/z 208→224) in metabolites I, II, III, and IV.

[0065] The observed coupling constants of metabolites I and II (JH3,4=3.4 Hz) indicated that the H—C3-C4-H dihedral angle was close to 50°, which suggested that metabolites

I and II may be characterized by cis-isoflavan-4-ol. In metabolites III and IV, JH3,4 values were observed at 5.8 and 6.2 Hz, respectively, which indicated that metabolites III and IV were trans-isoflavan-4-ol [36]. From these results, it was concluded that the configurations of the C3 and C4 centers on the B-ring of the isoflavan-4-ol stereoisomers did not change during the BDO reaction.

[0066] From LC-NMR analyses, the epoxidation of isoflavan-4-ol by BDO was confirmed to be a regiospecific and stereospecific reaction. If the oxygen atom transfer reaction occurred on a different double bond or on a different side of the B-ring, more than one metabolite peak would have been observed from the chromatogram due to the formation of regio- or diastereomeric isomers.

[0067] The following Table 2 shows production amounts of epoxides from isoflavan-4-ols by *P. pseudoalcaligenes* KF707 BDO.

TABLE 2

Substrates[a]			Corresponding epoxides (μM)[b] produced after 5 h reaction
Cis-isoflavan-4-ol	(3S,4S)	I	194.8 ± 5.5
	(3R,3R)	II	40 ± 4.0
Trans-isoflavan-4-ol	(3S,4R)	III	141.1 ± 6.2
	(3R,4S)	IV	163.3 ± 6.8

[a]Initial concentration of substrates: 250 μM

[b]Values are means of triplicate experiments ± standard errors

[0068] From Table 2, the substrate preference of BDO between cis-enantiomers was observed in which epoxide production from (3S,4S)-cis-isoflavan-4-ol was about 4.8-fold higher than that from (3R,4R)-cis-isoflavan-4-ol after 5 h of reaction. However, such preference of BDO was not observed distinctively between two trans-enantiomers. The substrates (3S,4R)- and (3R,4S)-trans-isoflavan-4-ols were biotransformed to 141.1 and 163.3 μM of the corresponding epoxides, respectively, during the 5-h reaction. Therefore, it appears that the BDO of *P. pseudoalcaligenes* KF707 has different substrate preferences for the four stereoisomers of the isoflavan-4-ols in a regiospecific and stereospecific manner.

[0069] Interestingly, the epoxide metabolites produced from the isoflavan-4-ols were unstable and were rearranged to energetically favorable chemical structures during reaction and analyses. All four isoflavan-4-ol-2',3'-epoxides were transformed to abiotic compounds (I-a, II-a, III-a, IV-a from I, II, III, and IV, respectively) with different HPLC retention times compared to those of the epoxide metabolites (FIG. **9**).

[0070] HPLC results for four abiotic compounds I-a, II-a, III-a, and IV-a were shown in the following Table 3.

TABLE 3

	Data for abiotic products produced from each epoxide	
Metabolites	HPLC retention time (min)	Absorption maximum (nm)[a]
(3S,4S)-cis-isoflavan-4-ol-2',3'-epoxide	16.1	272
(3R,4R)-cis-isoflavan-4-ol-2',3'-epoxide	17.2	270
(3S,4R)-trans-isoflavan-4-ol-2',3'-epoxide	15.6	270
(3R,4S)-trans-isoflavan-4-ol-2',3'-epoxide	16.2	271

b Values were obtained using HPLC equipped with a photodiode array detector.

[0071] From the above Table 3, four abiotic compounds, I-a, II-a, III-a, and IV-a, were eluted at 16.1, 17.2, 15.6, and 16.2 min, respectively. LC-MS analyses in positive ion mode showed molecular ion peaks [M+H]+ at 225.1 m/z for all abiotic compounds. The result of LC-NMR analysis for III-a among four abiotic compounds showed four aromatic protons (two doublets and two triplets) at δ 6.7~7.3 (Table 1) when compared to epoxide metabolite III and these aromatic protons corresponded to protons of the B-ring of pterocarpan. According to the coupling constants and WETgCOSY (FIG. **11**), four protons of the B-ring were adjacent to each other. These NMR results together with LC-MS data, which indicated the subtraction of one oxygen and two hydrogen atoms, enabled the present inventors to identify the formation of an ether linkage between C4 and C2' to form furan.

[0072] Based on the spectroscopic characterizations of the abiotic compounds, the products were identified as pterocarpan diastereomers possessing a 6a,11a-dihydro-6H-benzofuro[3,2-c] benzopyran skeleton. These results suggest that epoxides produced from isoflavan-4-ols by strain KF707 BDO may trigger an abiotic reaction due to their instability (FIG. **4**). The formation of pterocarpan products may be explained through bond formation between C4-O and C2' combined with the loss of one molecule of H2O and the detailed mechanism is currently under study. Many pterocarpan derivatives, such as maackiain from *Cicer arietinum* and medicarpin from *Medicago sativa,* are plant-protective phytoalexins produced in response to fungal, bacterial, and viral infections [5]. Some pterocarpans have been known to exhibit not only the above phytoalexins activity, but also anti-tubercular and estrogenic activities [26] and inhibitory activities against HIV-1 in cell cultures [6, 7] and to act as antagonists against some snake venoms [23]. Because of the diverse utilities of pterocarpan derivatives, various chemical synthesis methods for pterocarpan have been introduced [21, 22, 33], and the present inventors propose that the BDO-mediated synthesis of pterocarpan may provide another novel route for the production of enantiopure four different type of pterocarpans.

[0073] To obtain more insight with regard to the unique activity of BDO toward isoflavan-4-ol stereoisomers, an enzyme-substrate docking study was performed. Since only one X-ray protein crystallographic structure of BDO is available from a Gram-positive PCB-degrading organism, *Rhodococcus* sp. strain RHA1 [8], the amino acid sequences of BDOs from *P. pseudoalcaligenes* strain KF707 were compared to those of BDO from *Rhodococcus* sp. strain RHA1 by aligning important conserved sequences (FIG. **5**A). Except for the Fe active site, the two BDOs showed many different amino acid residues inside the substrate binding sites and channel, as well as on the protein surface. In order to compare reaction specificity of enzymes, different amino acid residues between the above two BDOs associated with substrate binding and substrate channel were introduced. The important changes in the amino acid residues in the RHA1 BDO were Leu-274, Ile-278, and Ala-311 in the substrate binding site, which were matched to Met-283, Val-287, and Gly-320, respectively, in the KF707 BDO. Accordingly, the reduction of van der Waals surfaces of all three amino acid residues by one methyl group in RHA1 BDO was observed from strain KF707 BDO. Therefore, when the volumes of the substrate binding sites between the two proteins were compared, a difference of 102.8 and 38.33 3 were calculated for strain KF707 and strain RHA1, respectively (FIG. **5**B and FIG. **5**C).

Therefore, the BDO of strain KF707 may accommodate a much larger substrate when compared to strain RHA1, and this has been confirmed by other previous reports [8, 16, 27]. Interestingly, the Arg-305 residue in the BDO of strain RHA1, which is thought to be an important residue in the substrate channel, corresponds to the Pro-305 residue in the BDO of strain KF707, and it appears that this difference may alter substrate passage by allowing for a larger substrate to enter the BDO of strain KF707. However, this comparison of volumes of the substrate binding sites between the two proteins cannot fully explain the chlorinated biphenyl degrading mechanism. When compared to strain KF707 [31] which transforms double para-substituted PCB congeners favorably, strain RHA1 has high transformation activity on both ortho- and para-substituted PCB congeners [30]. *Rhodococcus* sp. strain RHA1 transformed even highly-chlorinated PCB congeners, such as heptachlorobiphenyl [29]. After validation of the new BDO structure of strain KF707 as stated above, the isoflavan-4-ol stereoisomers were replaced in the position of biphenyl, and the orientations of the isoflavan-4-ol substrates around the non-heme Fe center were adjusted to allow for epoxide formation on the B-ring. When (3S,4S)-cis-isoflavan-4-ol, which was transformed to the corresponding epoxide in the highest yield among the tested stereoisomers, was docked in the substrate binding site, the substrate fit perfectly in the substrate binding space, and hydrogen bonding formation between the 4-OH of (3S,4S)-cis-isoflavan-4-ol and the α-carbonyl of Gly-320 was observed to occur at a distance of 2.65. On the contrary, the least reactive substrate, (3R,4R)-cis-isoflavan-4-ol, did not fit well in the substrate binding site. When the hydrogen bonding formation between the 4-OH of (3R,4R)-cis-isoflavan-4-ol and the α-carbonyl of Gly-320 was allowed, the reaction moiety of the B-ring was positioned farther from the Fe center (FIG. **6**B). It was concluded that the lower reaction rate of (3R,4R)-cis-isoflavan-4-ol was due to the location of the B-ring. For epoxide formation, the B-ring of (3R,4R)-cis-isoflavan-4-ol must go through an energetically unfavorable rotation to be near to the Fe center. To explain the different reactivities of the four stereoisomers of isoflavan-4-ol, four structures were overlapped while the orientations of the 4-OH groups were maintained at the same location in the substrate binding space (FIG. **6**C). While the other three stereoisomers were relatively well overlapped with the B-rings toward the Fe reaction center, the structure of (3R,4R)-cis-isoflavan-4-ol was unable to be overlapped with the other three stereoisomers due to the different position of the B-ring compared to the others. In conclusion, the present inventors have reported the biotransformation of four isoflavan-4-ol stereoisomers, (3S,4S)- and (3R,4R)-cis-isoflavan-4-ols, and (3S,4R)- and (3R,4S)-trans-isoflavan-4-ols, to their corresponding epoxides formed between the C2' and C3' positions on the B-ring by biphenyl dioxygenase derived from *P. pseudoalcaligenes* strain KF707 in the present specification. Furthermore, the present inventors have discussed the substrate-specific differences between strains RHA1 and KF707 BDOs and further rationalized the stereoselective absolute configuration-dependent monooxygenase-like epoxide formation of the isoflavan-4-ol stereoisomers by BDO from *P. pseudoalcaligenes* KF707 through the enzyme-substrate docking model study. An understanding of the reaction mechanisms of biphenyl dioxygenase on flavonoids may provide a means for conceiving a useful method for the production of other kinds of enantiopure pterocarpans.

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SEQUENCE LISTING

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<223> OTHER INFORMATION: strain KF707, Biphenyl dioxygenase

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gccgatcaga gtctttatga gctggagctt gagcgggttt ttggtcgctc ttggctgtta 180

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gaagcctttt gcgacaagaa agaaggcgac tgcggctttg acaaggccga atggggcccg 480

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35 40 45

Glu Arg Ile Phe Gly Arg Ser Trp Leu Leu Met Gly His Glu Thr Gln
50 55 60

Ile Pro Lys Ala Gly Asp Phe Met Thr Asn Tyr Met Gly Glu Asp Pro
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Val Met Val Val Arg Gln Lys Asn Gly Glu Ile Arg Val Phe Leu Asn
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Gln Cys Arg His Arg Gly Met Arg Ile Cys Arg Ala Asp Gly Gly Asn
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Ala Lys Ser Phe Thr Cys Ser Tyr His Gly Trp Ala Tyr Asp Thr Gly
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Gly Asn Leu Val Ser Val Pro Phe Glu Glu Gln Ala Phe Pro Gly Leu
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Cys Asn Trp Lys Phe Ala Ala Glu Gln Phe Cys Ser Asp Met Tyr His
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Ala Gly Thr Thr Ser His Leu Ser Gly Ile Leu Ala Gly Leu Pro Asp
225                 230                 235                 240

Gly Val Asp Leu Ser Glu Leu Ala Pro Pro Thr Glu Gly Ile Gln Tyr
                245                 250                 255

Arg Ala Thr Trp Gly Gly His Gly Ser Gly Phe Tyr Ile Gly Asp Pro
            260                 265                 270

Asn Leu Leu Leu Ala Ile Met Gly Pro Lys Val Thr Glu Tyr Trp Thr
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Arg Gly Gln Gln Leu Met Ala Gln His Met Thr Ile Phe Pro Thr Cys
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Ser Phe Leu Pro Gly Ile Asn Thr Ile Arg Ala Trp His Pro Arg Gly
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Pro Asn Glu Ile Glu Val Trp Ala Phe Thr Val Val Asp Ala Asp Ala
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Pro Glu Glu Met Lys Glu Glu Tyr Arg Gln Gln Thr Leu Arg Thr Phe
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Ser Ala Gly Gly Val Phe Glu Gln Asp Asp Gly Glu Asn Trp Val Glu
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Ile Gln Gln Val Leu Arg Gly His Lys Ala Arg Ser Arg Pro Phe Asn
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Gly Thr Ile Ser Tyr Val Tyr Ser Glu Glu Ala Ala Arg Gly Leu Tyr
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Gly Leu Leu Asp Pro Arg Ile Tyr Ala Asp Gln Ser Leu Tyr Glu Leu
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Leu Asn Gln Cys Arg His Arg Gly Met Arg Ile Cys Arg Ser Asp Ala
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Gly Asn Ala Lys Ala Phe Thr Cys Ser Tyr His Gly Trp Ala Tyr Asp
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Ile Ala Gly Lys Leu Val Asn Val Pro Phe Glu Lys Glu Ala Phe Cys
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Asp Lys Lys Glu Gly Asp Cys Gly Phe Asp Lys Ala Glu Trp Gly Pro
145                 150                 155                 160

Leu Gln Ala Arg Val Ala Thr Tyr Lys Gly Leu Val Phe Ala Asn Trp
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Asp Val Gln Ala Pro Asp Leu Glu Thr Tyr Leu Gly Asp Ala Arg Pro
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Tyr Met Asp Val Met Leu Asp Arg Thr Pro Ala Gly Thr Val Ala Ile
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Gly Gly Met Gln Lys Trp Val Ile Pro Cys Asn Trp Lys Phe Ala Ala
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Glu Gln Phe Cys Ser Asp Met Tyr His Ala Gly Thr Thr Thr His Leu
225                 230                 235                 240

Ser Gly Ile Leu Ala Gly Ile Pro Pro Glu Met Asp Leu Ser Gln Ala
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Gln Ile Pro Thr Lys Gly Asn Gln Phe Arg Ala Ala Trp Gly Gly His
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Gly Ser Gly Trp Tyr Val Asp Glu Pro Gly Ser Leu Leu Ala Val Met
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Gly Pro Lys Val Thr Gln Tyr Trp Thr Glu Gly Pro Ala Ala Glu Leu
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Ala Glu Gln Arg Leu Gly His Thr Met Pro Val Arg Arg Met Phe Gly
305                 310                 315                 320

Gln His Met Ser Val Phe Pro Thr Cys Ser Phe Leu Pro Ala Ile Asn
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Thr Ile Arg Ile Trp His Pro Arg Gly Pro Asn Glu Ile Glu Val Trp
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Ala Phe Thr Leu Val Asp Ala Asp Ala Pro Ala Glu Ile Lys Glu Glu
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Tyr Arg Arg His Asn Ile Arg Thr Phe Ser Ala Gly Gly Val Phe Glu
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Gln Asp Asp Gly Glu Asn Trp Val Glu Ile Gln Lys Gly Leu Arg Gly
385                 390                 395                 400

Tyr Lys Ala Lys Ser Gln Pro Leu Asn Ala Gln Met Gly Leu Gly Arg
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Ser Gln Thr Gly His Pro Asp Phe Pro Gly Asn Val Gly Tyr Val Tyr
            420                 425                 430

Ala Glu Glu Ala Ala Arg Gly Met Tyr His His Trp Met Arg Met Met
        435                 440                 445

Ser Glu Pro Ser Trp Ala Thr Leu Lys Pro
    450                 455
```

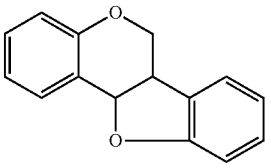
1. A method for preparing pterocarpan comprising:

(a) contacting a substrate, isoflavan-4-ol, and an enzyme, biphenyl oxygenase to form isoflavan-4-ol-2,3-epoxide; and

(b) forming pterocarpan from isoflavan-4-ol-2,3-epoxide.

2. The method of claim **1**, wherein pterocarpan is represented by the following chemical formula 3.

Chemical formula 3



3. The method of claim **1**, wherein biphenyl dioxygenase is contained in bacterial cells.

4. The method of claim **3**, wherein the bacteria are *Escherichia coli*.

5. The method of claim **3**, wherein biphenyl dioxygenase is derived from *Pseudomonas pseudoalcaligenes*.

6. The method of claim **1**, wherein the step (b) occurs naturally due to structural instability of isoflavan-4-ol-2,3-epoxide.

7. The method of claim **1**, wherein enantiopure pterocarpan is prepared.

8. The method of claim **7**, wherein the enantiopure pterocarpan is one selected from the group consisting of (3S,4S)-cis-pterocarpan, (3R,4R)-cis-pterocarpan, (3S,4R)-trans-pterocarpan, and (3R,4S)-trans-pterocarpan.

9. The method of claim **1**, wherein the absolute configuration of C3 and C4 on the B-ring of isoflavan-4-ol is identically maintained in pterocarpan.

* * * * *