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(54) **Title**: METHOD FOR PREPARING HYPOCRELLIN

(57) **Abstract**: A method of synthesizing hypocrellin B from 4,9-dihydroxy-3, 10-perylenequinones is provided. Said method consists in first oxidizing 4,9-dihydroxy-3, 10-perylenequinone, then cyclizing the oxidation product under basic conditions to provide hypocrellin B.

METHOD FOR PREPARING HYPOCRELLIN

FIELD OF INVENTION

[0001] The present invention relates to methods of preparing hypocrellin, more particularly, methods of preparing hypocrellin B from perylenequinones such as phleichrome.

BACKGROUND OF THE INVENTION

[0002] Hypocrellins are naturally occurring perylenequinones found in various parasitic fungi, including *Hypocrella bambusae* and *Shiraia bambusicola*. The photosensitizing activity and chemical structure of hypocrellin B and a related compound hypocrellin A was first described in 1992 (Nenghui et al., 1992, *J. Photochem Photobiol B* 14(3):207-17). These and other related hypocrellins (C, D) and perylenequinones have subsequently been identified in other species of fungi. Phleichrome, a perylenequinone, is a known natural product initially isolated from the mycelium of *C. phlei*.

[0003] Hypocrellins and fungal perylenequinones have been investigated for their use as photodynamic therapy (PDT) agents, antiviral, anticancer, antimicrobial and antiparasitic activities and the like (Ma et al., 2004 Antimicrobial Agents and Chemotherapy 48:4450-4452) and derivatives exhibiting enhanced absorption at specific wavelengths (for PDT applications) have been developed (see, for example, Paul et al., 2008. J. Photochem. Photobiol B 94:38-44). The availability of hypocrellins and fungal perylenequinones for these uses however, is limited by extraction from the fungi that produce it, or complex multi-step synthetic methods from precursors (PCT Publication WO 98/33470, US 6,936,571, Hauser et al., 1994 J. Org Chem 59:1697-1969). Diwu et al., 1992 (Tetrahedron 48:45-54) describes a one-step coupling reaction to produce perylenequinone from 1,2 naphthoquinone.

[0004] *H. bambusae* has not been reported as successfully grown in culture, thus its role as a source of hypocrellin is limited to its harvest in China. Other fungi that produce perylenequinones have been successfully grown in culture. For example, Seto et al (2005, *Biosci. Biotechnol. Biochem* 69(8):1515-1519; which is incorporated herein by reference) observed that Timothy plants infected by the fungus *Epichloe typhina* are resistant to disease caused by the *C. phlei* fungus, and identified metabolites (*cyclo*-(L-Pro-L-Leu) and *cyclo*-(L-Pro-L-Phe) that stimulate *C. phlei* to produce phleichrome. A publication by Lee et al., (2007, Biotechnology and Bioprocess Engineering 12:505-518; which is incorporated herein by

reference) describes culture conditions and extraction of phleichrome from *C. phlei*. A recent publication by Kim et al. (2009, Plant Pathol J. 25:179-183; which is incorporated herein by reference) has described a transformation protocol for genetic manipulation of *C. phlei*, which may be useful for development of fungal strains that are enhanced for production of phleichrome or other perylenequinones.

[0005] Synthesis of hypocrellin B from precursors is complicated as illustrated by PCT publication WO 98/33470; and extraction from natural sources may not provide sufficient product for widespread use in pharmaceuticals. A more expedient method of obtaining hypocrellin B would be advantageous.

SUMMARY OF THE INVENTION

[0006] The present invention relates to methods of preparing hypocrellin, more particularly, methods of preparing hypocrellin B from perylenequinones such as phleichrome.

[0007] It is an object of the invention to provide an improved method for preparing hypocrellin.

[0008] The present invention provides for a method of preparing hypocrellin B comprising: combining a 4,9-dihydroxy-3,10-perylenequinone with an oxidation reagent to provide an oxidation product of phleichrome; and cyclization of the oxidation product to provide hypocrellin B.

[0009] The 4,9-dihydroxy-3,10-perylenequinone may be phleichrome.

[0010] The oxidation reagent may comprise DMSO and acetic anhydride. The cyclization step may occur under basic reaction conditions. In some embodiments, the cyclization step comprises combining the oxidation product with an alkali hydroxide; in some embodiments, the alkali hydroxide is LiOH.

[0011] It is therefore an advantage of some aspects of the present invention to provide a one-step method of preparing hypocrellin B from phleichrome. Such a method represents an improvement over obtaining hypocrellin B from the tree fungus *H. bambusae*, which has limited geographical and seasonal availability. Such a method also represents an improvement over a multi-step synthesis of hypocrellin B from precursors by reducing the number of steps and quantity of reagents consumed.

[0012] This summary of the invention does not necessarily describe all features of the invention. Other aspects, features and advantages of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention.

DETAILED DESCRIPTION

[0013] The present invention relates to methods of preparing hypocrellin, more particularly, methods of preparing hypocrellin B from perylenequinones such as phleichrome.

[0014] As an example, a method of preparing hypocrellin B from 4,9-dihydroxy-3,10-perylenequinone is provided.

[0015] Examples of 4,9-dihydroxy-3,10-perylenequinones include phleichromes, cercosporin, fagopyrin, altertoxin, hypericin, elsinochromes and the like. These compounds are produced by various fungi or plants – generally the compound is named after the fungi or plant from which it was isolated and studied. For example, cercosporin may be obtained from an extraction of *Cercospora spp.*; phleichrome may be obtained from an extraction of *Cladosporium phlei.*; altertoxin may be obtained from an extraction of *Alternaria laternata*; elsinochrome may be obtained from an extraction of *Elsinone spp.*; hypericin may be obtained from an extraction of *Hypericum perforatum*; fagopyrin may be obtained from an extraction of *Fagopyrum sagittatum.* See, for example, Daub et al. 2000 Annu. Rev Phytopathol 38:461-90; Kim et al., supra; Li et al., 2001 J. Food Prot 64 :567-71; Ma et al., 2003. World J Gastroenterol 9 :485-90; Hinneburg et al. 2005. 53 :3-7; Poutaraud et al., 2001 Phytochem Anal 12:355-62.

[0016] *C. phlei* may be cultured by known methods and the phleichrome pigment obtained by extraction of the mycelial mass, culture medium or both the mycelial mass and culture medium, according to known methods (see, for example Lee et al., 2007, Biotechnology and Bioprocess Engineering 12:505-518; which is incorporated herein by reference). The *C. phlei* fungus may also be genetically manipulated to alter natural pigment production (see, for example, Kim et al., 2009, Plant Pathol J. 25:179-183; which is incorporated herein by reference). Alternately, phleichrome may be obtained by synthesis, as described for example the method of Broka, 1991 (Tetrahedron Letters 32:859-862, which is incorporated herein by reference) or of Coleman and Grant 1995. (J.AmChem Soc 117:10889-10904, which is incorporated herein by reference).

[0017] Phleichrome may be purified to the desired degree of purity, for example from about 30 to about 99% purity, or any amount therebetween, using known purification techniques.

Oxidation of phleichrome and cyclization

[0018] Once phleichrome is obtained, the secondary alcohol groups of phleichrome (1; see structure below) may be oxidized to yield the corresponding ketone (2). Examples of oxidizing reagents include DMSO, Collins' reagent, Corey's reagent, pyridinum dichromate,$Na_2Cr_2O_7$ in water, $K_2Cr_2O_7$ in DMF (with heat), CrO_3 on a silica support (e.g. silica gel) or the like. Such reagents and conditions for their use are described in, for example, *March's Advanced Organic Chemistry* (MB Smith and J March, eds.Wiley & Sons, 5th edition 2001 chapter 19-3; which is incorporated herein by reference) and references therein.

[0019] In some embodiments, oxidation of a secondary alcohol may be performed using DMSO in combination with DCC and anhydrous phosphoric acid, oxalyl chloride, acetic anhydride, methanesulfonic anhydride, tosyl chloride, P_2O_5-Et_3N, trichloromethyl chloroformate, KI and $NaHCO_3$, and the like.

[0020] In some embodiments, the oxidation of phleichrome is performed using DMSO in combination with acetic anhydride (Albright-Goldman oxidation) to produce compound (2). Such an oxidation is exemplified in step 1 of Scheme 1 (outlined in Example 1). Acetic anhydride may be used with about 3 to 6 equivalents of acetic anhydride, or about 4, or about 5 equivalents. In some embodiments, acetic anhydride may be used in a significantly greater excess, from about 6 to about 10, to about 20 or more equivalents; for example about 6, 7, 8, 9, 10,11, 12, 13, 11, 15, 16, 17, 18, 19, 20 equivalents. The oxidation may be performed at ambient (room) temperature, or heat may be applied and the oxidation performed at about 20, 25, 30, 25, 40, 45, or 50 degrees Celsius, or any temperature therebetween.

[0021] The compound (2) is subsequently cyclized to yield the product hypocrellin B (3). In some embodiments this may be via an intramolecular Aldol condensation. The cyclization reaction may comprise combining compound (2) with either LiOH in methanol-water, or 10% NaOH at ambient temperature, for 1-6 hours.

[0022] In some embodiments, the reaction of step 1 is conducted under conditions sufficient to permit the cyclization to occur spontaneously (see, for example Scheme 2).

Uses of hypocrellin B

[0023] Hypocrellin B may be used in various applications. For example, it may be used as a starting compound for synthesis of other perylenequinones (WO 98/44470). Derivatives of hypocrellin B, including monomer ruthenium complexes, may be produced for use as photodynamic therapy agents (Paul et al., 2009. J. Photochem Photobiol B 94::38-44; Zhou et al., 2005. Bioorganic and Medicinal Chemistry Letters 15:3067-3070).

[0024] Photodynamic agents such as hypocrellin B or a derivative thereof may be coupled to an antibody or another binding agent that specifically binds a tumor or microbe (for example, WO 2001/012217, WO 2004/044191). Hypocrellin B or a derivative thereof may be useful as an as a sonosensitizer and/or a photosensitizer (for example WO 2002/060483, WO 2003/063901), and, when activated by light, modulate the activity of an immunotherapeutic agent (for example, WO 2002/060482).

[0025] Uses and derivatives reference herein are for example purposes - other uses for hypocrellin B and/or derivatives thereof will further be apparent to those of skill in the relevant art.

[0026] The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Example 1: Two step chemical conversion of Phleichrome to Hypocrellin B

[0027] Oxidation by DMSO/acetic anhydride (Albright-Goldman oxidation) followed by cyclization with LiOH or NaOH (Scheme 1)

[0028] Phleichrome (1) was produced and extracted from *C. phlei* mycelial mass as described by Lee et al. (2007, Biotechnology and Bioprocess Engineering 12:505-518). Biomass was extracted with ethyl acetate or dicholoromethane. The dark colored residue left upon evaporation of organic solvents was purified on silica gel using 1-5% MeOH-dichloromethane as eluent.

[0029] A solution of Phleichrome (200 mg) in DMSO (4 mL) and acetic anhydride (2 mL) was heated at 40 °C for 24 h. The reaction mixture was diluted by dichloromethane, washed with water and evaporated to produce a crude product (2) (1,12-Bisacetonyl-2,6,7,11-tetramethoxy-

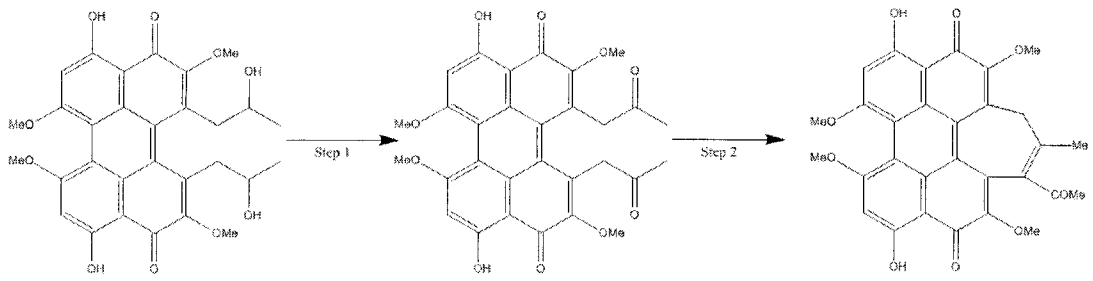
4,9-dihydroxy-3,10-perylenedione). The crude product (2) was used in the following cyclization reaction without further purification.

1. cyclization with LiOH.

[0030] A solution of the crude product (2) in methanol (10 mL) was cooled to 0°C, and LiOH (700 mg) in water (2 mL) was added. The mixture was stirred at room temperature for 4 h and acidified with 8% HCl to a pH of approximately 4. The mixture was extracted with dichloromethane, and the solvent evaporated. The extracted product was purified on silica gel using methanol-dichloromethane 1-5% as eluent to obtain hypocrellin B (product (3); 81 mg, ~40%, two steps) as a dark purple solid. The thin layer chromatographic profile (methanol-dichloromethane 5%) and ^{1}H-NMR was identical to that of Hypocrellin B extracted from *H. bambusae*. ^{1}H NMR ($CDCl_3$):δ 6.44, 6.42 (2s, 2H, ArH), 4.15, 4.09, 4.05 (4s, each 3H, 4 OCH_3), 4.04, 3.22 (dd, 2H, J_{AB} = 11.5 Hz, CH_2), 2.37, 1.85 (2s, each 3H, $2CH_3$). LC-MS: 529 (M + H).

2. cyclization with NaOH.

[0031] The crude product (2) was dissolved in 10% sodium hydroxide (10 mL) and stirred for 1 h. The aqueous solution was extracted with dichloromethane and then acidified with 2M HCl to a pH of approximately 4. The mixture was then extracted with dichloromethane and extract purified on silica gel column (methanol-dichloromethane 1-5%) to provide Hypocrellin B (product (3); 70 mg, ~35%). Thin-layer chromatography results and ^{1}H-NMR were identical to that obtained from naturally occurring and extracted Hypocrellin B from *H. bambusae*, and as obtained by cyclization product using LiOH as outlined above.

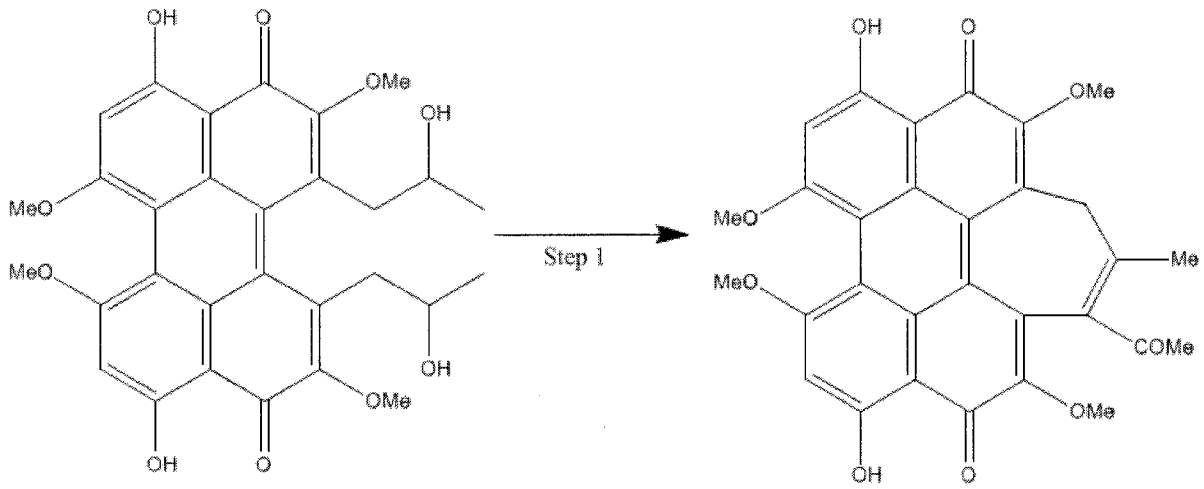


1 2 3

[0032] **Scheme 1: preparation of hypocrellin B (3).** Step 1 DMSO/acetic anhydride, 40°C, 24h. Step 2 LiOH in water, RT 4h, followed by acidification (Lown cyclization) or 10%NaOH, RT 1h, followed by acidification

Example 2: One step chemical conversion of Phleichrome to Hypocrellin B (Scheme 2)

[0033] A solution of Phleichrome (100 mg) in DMSO (2 mL) and acetic anhydride (1 mL) was heated at 40 °C for 24 h. The reaction mixture was diluted by dichloromethane (15 mL), washed with water and evaporated. The residue was chromatographed on two silica gel columns (1-5% MeOH-dichloromethane) to provide Hypocrellin B (25 mg, ~25%). Thin-layer chromatography results and ^{1}H-NMR were identical to that obtained from naturally occurring and extracted Hypocrellin B from *H. bambusae*, and as obtained by the two-step chemical conversion (Scheme 2).



1 3

Scheme 2: preparation of hypocrellin B (3). Step 1 DMSO/acetic anhydride, 40°C, 24h.

Example 3: Oxidation of phleichrome with PDC

[0034] Pyridinium dichromate (PDC) (27 mg, 2 eq) was added to a solution of Phleichrome (20mg) in DMF (1 mL) with stirring. The stirring was continued overnight and the reaction mixture was diluted with dichloromethane, washed with water and concentrated under vacuum. The residue left was purified on silica gel column using methanol in dichloromethane (0.5% to 10%). No product was obtained.

Example 4: Oxidation of Phleichrome by DMSO/Oxalyl chloride (Swern Oxidation) followed by cyclization with LiOH

[0035] DMSO (0.10 mL) was added to a stirred solution of oxalyl chloride in dichloromethane (0.477 mL, 1.9 M) at -60°C. After 5 min of stirring, the solution was added to phleichrome (50 mg) in dichloromethane (0.5 mL) at 0°C. Stirring was continued for 2 h until tlc (10% MeOH in dichloromethane) showed all starting material disappeared. Triethylamine (0.5 mL) was added and the reaction mixture was concentrated to remove solvent.

[0036] The remaining residue was dissolved in MeOH (5 mL) and to this mixture was added LiOH (600 mg) in water (6 mL) at 0°C. Stirring was continued for 3 h at room temperature and acidified with 8% HCl. Extraction with dichloromethane and evaporation of the solvent left a dark residue which was purified on silica gel using methanol-dichloromethane 1-5% as eluent. A product was obtained, however ^{1}H-NMR analysis demonstrated that the product was not Hypocrellin B.

Example 5: Oxidation by DMSO/methanesulfonic anhydride followed by cyclization with LiOH

[0037] A solution of Phleichrome (50 mg) in DMSO (1 mL) and methanesulfonic anhydride (160 mg) was stirred for 24 h. The reaction mixture was diluted by dichloromethane, washed with water and evaporated. The crude product was used in the following cyclization reaction without further purification.

[0038] To a solution of the above crude product in methanol (2 mL) cooled at 0°C was added LiOH (150 mg) in water (0.15 mL). The mixture was stirred at room temperature for 4 h and acidified with 8% HCl. Extraction with dichloromethane and evaporation of the solvent left the crude product which was purified on silica gel using methanol-dichloromethane 1-5% as eluent. No product (Hypocrellin B) was obtained (verified by 1H NMR and thin layer chromatography).

[0039] All citations are herein incorporated by reference, as if each individual publication was specifically and individually indicated to be incorporated by reference herein and as though it were fully set forth herein. Citation of references herein is not to be construed nor considered as an admission that such references are prior art to the present invention.

[0040] One or more currently preferred embodiments of the invention have been described by way of example. The invention includes all embodiments, modifications and variations substantially as hereinbefore described and with reference to the examples and figures. It will be apparent to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims. Examples of such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way.

WHAT IS CLAIMED IS:

1. A method of preparing hypocrellin (B) comprising:

obtaining a 4,9-dihydroxy-3,10-perylenequinone;

combining the 4,9-dihydroxy-3,10-perylenequinone with an oxidation reagent to provide an oxidation product of the 4,9-dihydroxy-3,10-perylenequinone; and

cyclization of the oxidation product to provide hypocrellin B.

2. The method of claim 1 wherein the 4,9-dihydroxy-3,10-perylenequinone is phleichrome.

3. The method of claim 1 wherein the oxidation reagent comprises DMSO and acetic anhydride.

4. The method of claim 1 wherein the cyclization step occurs under basic reaction conditions.

5. The method of claim 1 wherein the cyclization step comprises combining the oxidation product with an alkali hydroxide.

6. The method of claim 5 wherein the alkali hydroxide is LiOH or NaOH.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/001595

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07C 45/66*** (2006.01) , ***C07C 45/29*** (2006.01) , *C07C 49/755* (2006.01)
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)
C07C 45/66 (2006.01) , ***C07C 45/29*** (2006.01) , *C07C 49/755* (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, West, Scopus, TotalPatent, STN, Google Scholar, Espacenet (hypocrellin, synthesis of, preparation of, phleichrome, etc.)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/33470 (ALTAREX CORP, CA) 06 August 1998 (06-08-1998) pages 18 and 21, figure 15	1-6
X	LOWN J.W. "1996 Hoffman-LaRoche Award Lecture. Photochemistry and photobiology of perylenequinones" Can.J. Chem. 1997, vol. 75, pages 99-119 ISSN: 0008-4042 Fig. 6	1-6
X	O'BRIEN E.M. ET AL. "Perylequinone Natural Products: Total Synthesis of Hypocrellin A" J. Org. Chem. 2010, vol. 75, pages 57-68 published on web 09-11-2009 eISSN: 1520-6904 page 60, Scheme 4	1-6

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

[X] 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 application or patent but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 December 2010 (07-12-2010)	13 January 2011 (13-01-2011)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Alessandra Mezzetti (819) 934-6736

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2010/001595

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO9833470A2	06 August 1998 (06-08-1998)	AU6017198A	25 August 1998 (25-08-1998)
		CA2277362A1	06 August 1998 (06-08-1998)
		EP1015033A2	05 July 2000 (05-07-2000)
		EP1015033A4	05 July 2000 (05-07-2000)
		JP2001508071	19 June 2001 (19-06-2001)
		WO9833470A3	29 October 1998 (29-10-1998)