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5-DIHYDROCORIOLIN C

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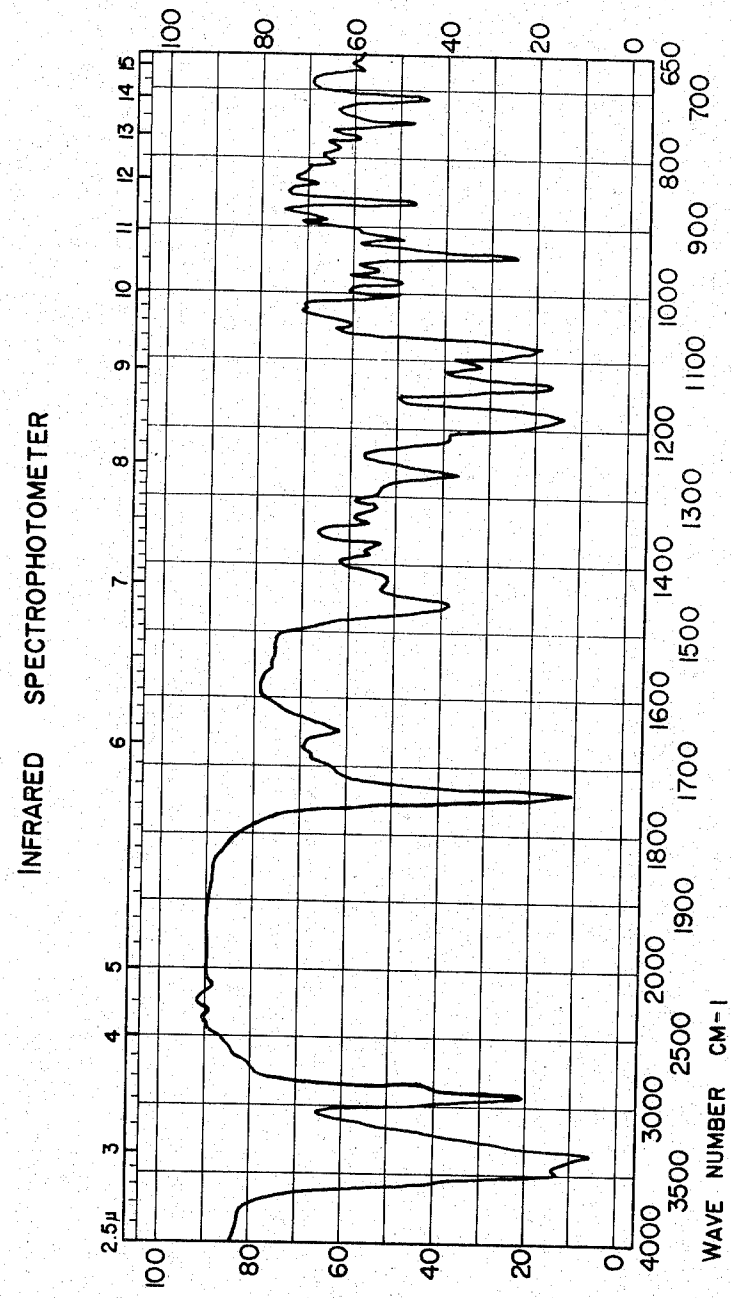

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5-DIHYDROCORIOLIN C

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ABSTRACT OF THE DISCLOSURE

5-dihydrocoriolin C is obtained by cultivating microorganisms belonging to basidiomycetes and being capable of producing 5-dihydrocoriolin C in a medium appropriate for the production of 5-dihydrocoriolin C under aerobic conditions thereby to produce 5-dihydrocoriolin C in the medium and recovering it from the medium. 5-dihydrocoriolin C is a novel substance, but has no antibacterial or antitumor effect by itself, but coriolin C and 2'-dehydrocoriolin C obtained by oxidation of 5-dihydrocoriolin C have an antibacterial effect upon *Staphylococcus aureus, Bacillus subtilis,* etc., and also has a life-prolonging effect upon Ehrlich ascites tumor on mouse or mouse leukemia L–1210.

This invention relates to 5-dihydrocoriolin C and a process for producing 5-dihydrocoriolin C, and more particularly to 5-dihydrocoriolin C obtained by cultivating microorganisms belonging to basidiomycetes and being capable of producing 5-dihydrocoriolin C in a medium appropriate for the production of 5-dihydrocoriolin C under aerobic conditions, thereby to produce 5-dihydrocoriolin C in the medium, and the recovering 5-dihydrocoriolin C from the resulting culture broth, and a process for producing the same.

An object of the present invention is to provide a novel substance, 5-dihydrocoriolin C.

Another object of the present invention is to provide 5-dihydrocoriolin C capable of readily forming coriolin C and 2'-dehydrocoriolin C having an antibacterial and antitumor effect by oxidation.

Still another object of the present invention is to provide a process for producing 5-dihydrocoriolin C.

Further objects of the present invention will be apparent from the following description and claims.

The accompanying drawing shows an infrared absorption spectrum determined in the form of tablet of 5-dihydrocoriolin C of the present invention together with potassium bromide.

5-dihydrocoriolin C obtained in the present invention is recovered in a crude state and crystalline state, and has no antibacterial or antitumor effect by itself, but coriolin C and 2'-dehydrocoriolin C obtained by oxidizing the 5-dihydrocoriolin C with an oxidizing agent have an antibacterial effect upon *Staphylococcus aureus, Bacillus subtilis,* etc. and a life-prolonging effect upon Ehrlich ascites tumor on mouse and mouse leukemia L–1210, as shown in the following test result. (For coriolin C; see Umezawa et al.: Tetrahedron letters, No. 19, pp. 1637–1639 (1970) 2'-dehydrocoriolin C is a novel compound.)

The result of tests on antibacterial effects of coriolin C and 2'-dehydrocoriolin C by way of agar dilution method is given in Table 1:

TABLE 1.—ANTIBACTERIAL SPECTRA OF CORIOLIN C AND 2'-DEHYDROCORIOLIN C

Test organism	Coriolin C	2'-dehydrocoriolin C
St. aureus:		
FDA 209–P	[1] 1.56	[1] 1.56
Tera ima	1.56	1.56
Smith	1.56	1.56
M. flavus FDA–16	<0.78	1.56
B. anthracis	<0.78	<0.78
B. subtilis NRR B–558	<0.78	1.56
E. coli NIHJ	25.0	100.0
S. typhi–T63	>100.0	>100.0
S. enteritis	12.5	12.5
Ps. aeruginosa AS	>100.0	100.0

[1] Minimal inhibitory concentration (mcg./ml).

These two compounds have an action to inhibit growth of gram positive microorganisms, and almost equal effects can be attained with these two compounds.

Actions of coriolin C and 2'-dehydrocoriolin C upon Ehrlich ascites tumor on mouse, mouse leukemia L–1210 and growth of Yoshida sarcoma cells in tissue culture are given below:

Curing test on Ehrlich ascites tumor on mouse was carried out with daily dosage of 0.19 to 12.5 mg./kg. of each of said two compounds for 10 days. As a result, all the control mice were killed within 23 days after the inoculation of tumor cells, but mice to which 12.5, 6.25, 3.12, 1.56 and 0.678 mg./kg. of coriolin C or 2'-dehydrocoriolin C were administered into their abdominal cavities could survived 90%, 80%, 65%, 63% and 60% for the former for more than 50 days and 80%, 75%, 60%, 60% and 50% for the latter for more than 50 days, correspondingly, and no ascites retention was observed.

Curing test on mouse leukaemia L–1210 was carried out with daily dosage of 6–100 mg./kg. of coriolin C or 2'-dehydrocoriolin C for 10 days, and the mice, to which 6, 12.5, 25, 50 and 100 mg./kg. of coriolin C or 2'-dehydrocoriolin C were administered, were survived with a life-prolonging effect of 119%, 131%, 140%, 150%, and 175% for the former, and 115%, 120%, 125%, 135% and 160% for the latter, correspondingly, on the basis of surviving days of control mice as 100.

In the growth inhibition test using tissue culture of Yoshida sarcoma cells on rats, 60% propagation inhibition was attained with 0.75 mcg./mg. of each of coriolin C and 2'-dehydrocoriolin C.

As to acute toxicity of coriolin C and 2'-dehydrocoriolin C on mouse, LD_{50} at the abdominal cavity administration was more than 50 mg./kg., and LD_{50} at the subcutaneous administration was more than 90 mg./kg. Further, in the use of continuous administration into the abdominal cavity for 10 days, total mice tested were survived for more than 50 days, even when total dosage reached 100 mg./kg., and it was found that the toxicity was low.

As described above, coriolin C and 2'-dehydrocoriolin C have an excellent antibacterial and antitumor effect.

Coriolin C can be also produced by fermentation, but its yield is so small that its process by fermentation has a very low productivity and cannot be carried out industrially. On the other hand, in the present invention, 5-dihydrocoriolin C can be obtained in high yield, and the 5-dihydrocoriolin C thus obtained can be readily converted

to coriolin C with an oxidizing agent. Therefore, the production of coriolin C is carried out more economically through the oxidation of 5-dihydrcoriolin C than through direct fermentation route. The 5-dihydrocoriolin C obtained in the present invention as very useful as a raw material for producing said coriolin C and its related substance 2'-dihydrocoriolin C.

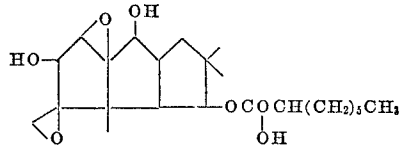
The 5-dihydrocoriolin C obtained according to the present invention has the following properties:

5-dihydrocoriolin C is obtained in colorless crystals, melts at 183° C. and is soluble in methanol, ethanol, ethyl acetate, acetone, chloroform and benzene, but sparingly soluble or insoluble in water and petroleum ether. 5-dihydrocoriolin C does not show other ultraviolet absorption than end absorption. Infrared absorption spectrum of 5-dihydrocoriolin C taken in potassium bromide tablet is as shown in FIG. 1, and the main absorption wave numbers ($cm.^{-1}$) thereof are 3490; 3360; 2950; 1741; 1649; 1460; 1419; 1389; 1370; 1340; 1315; 1270; 1184; 1139; 1109; 1081; 1032; 1003; 983; 965; 949; 921; 891; 868; 841; 809; 788; 781; 751; 716; 672.

5-dihydrocoriolin C has a molecular weight of 424, as measured by mass spectrometry, and shows elemental analysis values of C: 65.07% and H: 8.55% and contains no nitrogen.

From the above-mentioned elemental analysis values and molecular weight measured by mass spectrometry, $C_{23}H_{36}O_7$ can be derived as the molecular formula (molecular weight: 424).

As a result of analysis of nuclear magnetic resonance spectrum of 5-dihydrocoriolin C and studies on its behaviors towards reagents, it has been found that 5-dihydrocoriolin C has the following structural formula:



As a result of systematic studies on the substances produced by the basidiomycetes, the present inventors have found 5-dihydrocoriolin C. 5-dihydrocoriolin C is distingiushed in physical and chemical properties, molecular formula and structural formula from the well known coriolin, coriolin B, coriolin C, 5-ketocoriolin B or hirustic acid C, and is a novel substance.

Microorganism capable of producing 5-dihydrocoriolin C used in the present invention is *Coriolus consors (Berk) Imaz* ATCC No. 20305, which is broadly distributed from Honshu to Kyushu, Japan and is described in detail in Rokuya Imazeki and Jiro Hongo: Zoku Genshoku Nippon Kinrui Zukan (Full color Japan Bacteria Picture Book, continued), pp. 141–142, published in 1968 by Hoikusha Publishing Co., Japan.

When microorganism capable of producing 5-dihydrocoriolin C are inoculated in an appropriate medium, and cultivated under aerobic conditions, a culture liquor containing 5-dihydrocoriolin C can be obtained. It is possible to apply a solid cultivation method to the cultivation, but a liquid cultivation method is more preferable in mass production. Any cultivation temperature can be employed, so long as the microorganisms capable of producing 5-dihydrocoriolin C can grow at that temperature, but 15° to 30° C. is usually preferable, but 25° to 28° C. is particularly preferable for the cultivation.

As the medium for producing 5-dihydrocoriolin C, a medium containing a carbon source, nitrogen source, inorganic salts, production promoters, etc. can be used.

As the carbon source, commercially available sugars, oils and fat, etc. can be used. For example, glucose, glycerine, starch, dextrin, maltose, lactose, saccharose, oil and fat, molasses, etc. can be used in a pure or crude state.

As the nitrogen source, soybean powders, meat extract, peptone, dried beer yeast, yeast extract, corn steep liquor, casein, cotton seed oil, fish meal, nitrates, ammonium salts, urea, amino acids such as glutamic acid, etc. can be used.

As inorganic salts, sodium chloride, potassium chloride, magnesium sulfate, calcium carbonate, phosphates, etc. or a very small amount of salts of heavy metal such as copper, manganese, iron, zinc, etc. can be used. Further, addition, particularly divisional addition, of acetic acid or mevalonic acid increase the production of 5-dihydrocoriolin C at the cultivation. One example of the medium appropriate for the production of 5-dihydrocoriolin C is given below:

Composition I (seed medium):	Percent
Glucose	5.0
Peptone	0.2
Dried beer yeast	0.3
Potassium dihydrogen phosphate	0.3
Magnesium sulfate	0.1
Polyoxyethylenic surfactant	0.01

Composition II (production medium):	Percent
Glucose	5.0
Peptone	0.2
Dried beer yeast	0.3
Potassium dihydrogen phosphate	0.3
Magnesium sulfate	0.1
Polyoxyethylenic surfactant	0.01
Calcium carbonate*	1.6

*Sterilized separately and added to the medium at the start of cultivation.

In carrying out the present invention, a seed liquor of microorganisms capable of producing 5-dihydrocoriolin C cultivated in the medium of Composition I is cultivated with shaking, at first, in the medium of Composition II at 15° to 30° C. for a few days. In the case of large scale cultivation using a fermentation tank, the seed cultivation is carried out at two stages. According to the ordinary procedure, the first seed liquor obtained by cultivation in a seed medium in a shaking flask is, at first, transfered into a fermentation tank provided with the seed medium in advance, and subjected to cultivation. Finally, the second seed liquor is inoculated in the production medium in another fermentation tank and subjected to cultivation. However, it is possible to omit the second seed cultivation.

Through these cultivations, coriolin, coriolin B, 5-ketocoriolin B and 5-dihydrocoriolin C are produced, and since coriolin exists mainly in a liquid portion, and coriolin B, 5-ketocoriolin B and 5-hydrocoriolin C exist mainly in mycelium, the mycelium portion containing coriolin B, 5-ketocoriolin B and 5-dihydrocoriolin C in the culture is separated by the well known method such as filtration, centrifugal separation, etc. When the mycelium portion is subjected to extraction with an organic solvent such as methanol, acetone, ethyl acetate, butyl acetate, methylisobutylketone, etc., 5-dihydrocoriolin C is extracted and transferred to the organic solvent layer.

When a water-miscible organic solvent, for example, acetone, methanol, ethanol, etc. is used as an extracting agent at the extraction, the extract liquid is concentrated under a reduced pressure, and then the concentrated liquid is subjected to reextraction with a nonhydrophilic, organic solvent, for example, ethyl acetate, methylisobutylketone, etc., water-soluble impurities can be removed.

In the extract liquor, there are coriolin B, 5-ketocoriolin B, etc. in addition to the desired 5-dihydrocoriolin C, but since coriolin B is most sparingly soluble in ethyl acetate, among these byproducts, coriolin B is deposited, at first, by concentrating the extract liquid, and therefore it can be removed by filtration.

5-dihydrocoriolin C is more crystallizable than 5-ketocoriolin B in the mother liquor, and therefore by concentrating the mother liquor freed from coriolin B and leaving the concentrated mother liquor standing, 5-dihydrocoriolin C can be deposited as needle-like crystals and recovered.

However, when there are much impurities in the extract liquid, it is hard to deposit crystals of 5-dihydrocoriolin C, and therefore 5-dihydrocoriolin C is separated from 5-ketocoriolin B usually by chromatographic method using silica gel as a carrier and a solution mixture of methanol-chloroform, chloroform, benzene, etc. as an eluting agent. At the elution, 5-ketocoriolin B is eluted at first, and then 5-dihydrocoriolin C. Their separation is satisfactory, because the numbers of hydroxyl groups in the molecules are differed by two between these two compounds.

Crude powders obtained by concentrating the elution fraction of 5-dihydrocoriolin C to dryness are dissolved in a small amount of an organic solvent, and 5-dihydrocoriolin C can be deposited therefrom as needle-like crystals by adding to the resulting solution, the solvent in which 5-dihydrocoriolin C is sparingly soluble, for example, n-hexane, water, etc.

Further, as a process for producing coriolin C and 2'-dehydrocoriolin C from 5-dihydrocoriolin C by oxidation, there are available various processes, such as (1) oxidation by chromic acid in pyridine, (2) oxidation by manganese dioxide in a neutral solution, (3) oxidation by a dimethylsulfoxide solution of dicyclohexyl carbodiimide, etc., but said two processes (1) and (2) are particularly convenient for obtaining said two compounds.

Now, the present invention will be explained in detail, referring to Examples.

EXAMPLE 1

25 g. of wood shavings (20 to 50 meshes) of *Magnolia hypoleuca* was placed in a 1-l. capacity conical flask, and 170 ml. of a liquid culture medium containing 2% glucose and 0.5% dried beer yeast (which will be hereinafter referred to as "GY medium") was added thereto. Then, the flask was sterilized in an autoclave at 120° C. for 20 minutes, and then microorganism capable of producing 5-dihydrocoriolin C, (ATCC, No. 20305), was inoculated onto the medium from an agar slant, and cultivated at 25° to 27° C. for 10 to 14 days in a stationary condition. The resulting culture was used as wood shaving seed.

Then, the wood shaving seed was added to 500 ml. of the sterilized GY medium to prepare a microorganism suspension, and 100 ml. each of the microorganism suspension was inoculated into each of five 5-l. capacity conical flasks containing 1 l. of the medium of the following Composition I each, and cultivated at 27° C. for 72 hours on a rotary shaking cultivater with 190 r.p.m.

Composition I (seed medium):	Percent
Glucose	5.0
Peptone	0.2
Dried beer yeast	0.3
Potassium dihydrogen phosphate	0.3
Magnesium sulfate	0.1
Polyoxyethylenic surfactant	0.01

The entire amount of this first seed liquor was inoculated in a 200-l. capacity stainless steel fermentation tank containing 120 l. of the medium having the same composition as above, and cultivated at 27° C. for 72 hours under a stirring condition of 200 r.p.m. and an aeration rate of 120 l./min.

Then, 12 l. of the resulting second seed liquor was inoculated in a 200-l. capacity stainless steel fermentation tank containing 120 l. of a medium having the following Composition II, and cultivated for 168 hours under the same cultivating conditions as above.

Composition II (production medium):	Percent
Glucose	5.0
Peptone	0.2
Dried beer yeast	0.3
Potassium dihydrogen phosphate	0.3
Magnesium sulfate	0.1
Polyoxyethylenic surfactant	0.01
Calcium carbonate*	1.6

*Sterilized separately and added to the medium at the start of cultivation.

After the completion of cultivation, the resulting culture broth was filtered, whereby 5.5 kg. of wet myceliums was obtained. The myceliums are transferred to a vessel, and 9 l. of acetone was added thereto. After stirring, the solution was filtered. The myceliums are further washed with 3 l. of acetone, and the washing liquor and filtrate were joined together, and concentrated. The resulting concentrated liquor was admixed with 5 l. of ethyl acetate, stirred and extracted.

A solvent layer was separated, and concentrated to 350 ml. under a reduced pressure, and left standing, whereby by-produced coriolin B crystals were deposited. The deposited crystals were filtered, and washed with a small amount of benzene. The washing liquor and filtrate were joined together, and further concentrated to 150 ml., whereby 5-dihydrocoriolin C precipitate was deposited. The precipitate was filtered, washed with benzene, and dried, whereby 8.59 g. of crude crystals was obtained.

The crude crystals were recrystallized in an aqueous 80% methanol solution, whereby 5.19 g. of pure 5-dihydrocoriolin C (melting point: 183° C.) was obtained.

EXAMPLE 2

10 ml. each of the second seed liquor obtained in the same manner as in Example 1 was added to each of 150, 500-ml.-capacity, shaking flasks containing 125 ml. of the medium of Composition II each, and cultivated at 27° C. for 7 days on a reciprocating shaker with 130 reciprocations per minute. 15.5 l. of the resulting culture broth was filtered, whereby 550 g. of wet myceliums were obtained.

The wet myceliums were admixed with 2 l. of acetone, stirred, extracted, filtered and washed with 1 l. of acetone. The washing liquor and filtrate were joined together, and concentrated under a reduced pressure. 600 ml. of the concentrated liquor was admixed with 1.2 l. of ethyl acetate, and extracted. Then, the resulting solvent layer separated, concentrated to about 50 ml. under a reduced pressure, and left standing, whereby crystals of by-produced coriolin B were deposited. The crystals were filtered and washed with benzene.

The filtrate and washing liquor were joined together, and concentrated to dryness under a reduced pressure, whereby 5.6 g. of oily matter containing the desired 5-dihydrocoriolin C was obtained.

The oily matter was dissolved in a small amount of chloroform, and poured through a column packed with 250 ml. of silica gel suspended in chloroform, for adsorption. Then, a 1% methanol-chloroform solvent mixture in a volume almost equal to that of the column was passed through the column, whereby 5-ketocoriolin B was eluted at first, and then bypassing further said solvent mixture in about two times the volume of the column through the column, the desired 5-dihydrocoriolin C was eluted. The 5-dihydrocoriolin C fraction was collected, concentrated to dryness under a reduced pressure, and recrystallized in an aqueous 80% methanol solution, whereby 2.34 g. of pure 5-dihydrocoriolin C (melting point: 183° C.) was obtained.

REFERENCE EXAMPLE 1

3 g. of 5-dihydrocoriolin C was dissolved in 30 ml. of pyridine, and admixed with a pyridine suspension of chromic acid-pyridine complex prepared by adding 2.5 g.

of chromic acid to 40 ml. of pyridine, with stirring, and subjected to reaction at 3° to 5° C. for 6 days.

After the reaction, 350 ml. of ethyl acetate was added thereto, and the deposited impurities were filtered. Filtrate was washed with total 500 ml. of water divisionally, and an upper layer was separated and concentrated to dryness under a reduced pressure, whereby 2.53 g. of crude product was obtained.

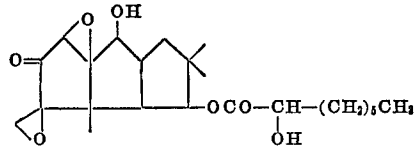
The crude product was dissolved in 10 ml. of ethanol, and adsorbed in a column filled with 100 ml. of Sephadex LH 20 (trade name for dextran derivatives used for gelfiltrant in organic solvent, manufactured by Pharmacia Fine Chemicals, Inc.). The column was eluted with total 85 ml. of ethanol. Later 25 ml. of eluted liquid was collected, and concentrated to dryness under a reduced pressure. The concentrated residue was dissolved in 5 ml. of chloroform, and adsorbed in a column filled with 50 ml. of silica gel. The column was eluted with 100 ml. of 1% methanol-chloroform solvent mixture, whereby eluted fractions of 2'-dehydrocoriolin C was obtained.

Then, the column was eluted with 100 ml. of 2% methanol-chloroform solvent mixture, whereby eluted fraction of coriolin C was obtained.

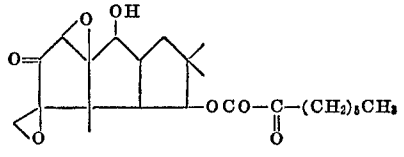
Each of these fractions was concentrated to dryness under a reduced pressure, whereby 267 mg. of powdery 2'-dehydrocoriolin C (melting point: 51° to 55° C.) and 851 mg. of crude crystals of coriolin C were obtained.

Crude crystals of coriolin C was recrystallized in n-hexane, whereby 722 mg. of coriolin C (melting point: 73° to 75° C.) was obtained.

Coriolin C obtained in the present example was the well known substance having the following structural formula:



On the other hand, 2'-dehydrocoriolin C was a novel compound and has the following properties: colorless powders: melting point: 51° to 55° C.; $[\alpha]_D^{25}$: −19.0° (C=1%, chloroform); soluble in methanol, ethanol, ethyl acetate, acetone, chloroform, benzene, and carbon tetrachloride, but sparingly soluble or insoluble in water, n-hexane, and petroleum ether. Further, the elemental analysis of 2'-dehydrocoriolin C reveals 65.69% carbon and 7.67% hydrogen, and the molecular weight determined by mass spectrometry reveals $C_{23}H_{32}O_7$ as molecular formula. Further, as a result of analysis of nuclear magnetic resonance spectrum, it has been found that 2'-dehydrocoriolin C has the following structural formula.



REFERENCE EXAMPLE 2

1 g. of 5-dihydrocoriolin C was dissolved in 100 ml. of anhydrous benzene, admixed with 8 g. of manganese dioxide with stirring, and subjected to reaction at room temperature for 32 hours. The reaction mixture was filtered, and the precipitate was washed with anhydrous benzene. The filtrate and washing liquor were joined together, and concentrated to dryness under a reduced pressure, whereby 610 mg. of solid matter was obtained.

The solid matter was dissolved in 3 ml. of ethanol, and adsorbed in a column filled with 25 ml. of Sephadex® LH 20. The column was eluted with total 25 ml. of ethanol. Later 9 ml. of eluted liquid fractions was collected, and concentrated to dryness under a reduced pressure.

The concentrated residue was dissolved in 1.2 ml. of chloroform, and adsorbed in a column filled with 15 ml. silica gel. The column was eluted with 30 ml. of 1% methanol-chloroform solvent mixture, whereby an eluted fraction of 2'-dehydrocoriolin C was obtained. Then, by eluting the column with 30 ml. of 2% methanol-chloroform solvent mixture, an eluted fraction of coriolin C was obtained. Each of these fractions was concentrated to dryness under a reduced pressure, whereby 62 mg. of powdery 2'-dehydrocoriolin C (melting point: 51° to 55° C.) and 180 mg. of crude crystals of coriolin C were obtained. The latter was recrystallized in n-hexane, whereby 132 mg. of coriolin C crystals (melting point: 73° to 75° C.) were obtained.

REFERENCE EXAMPLE 3

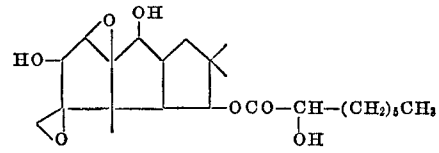
200 mg. of 5-dihydrocoriolin C was dissolved in a solution mixture of 1.2 ml. of anhydrous dimethyl sulfoxide, 0.7 ml. of benzene, 0.077 ml. of pyridine and 0.038 ml. of trifluoroacetic acid, and admixed with 300 mg. of dicyclohexyl carbodiimide with stirring. The solution was left standing at the normal temperature for one hour, and further subjected to reaction at 3° to 4° C. for 15 hours.

After the completion of reaction, 12 ml. of ether and 130 mg. of oxalic acid (as a 10% methanol solution) were added thereto, and after 30 minutes, 12 ml. of water was added thereto. The resulting precipitates was filtered and washed with ether.

An ethereal layer was separated from the filtrate, and washed with an aqueous 5% sodium bicarbonate solution, and then with water, and concentrated under a reduced pressure. The concentrate was separated by silica gel chromatography in the same manner as in Reference Example 1, whereby 42 mg. of crystals of coriolin C (melting point: 73° to 75° C.) and 13 mg. of powdery 2'-dehydrocoriolin C (melting point: 51° to 55° C.) were obtained.

What is claimed is:

1. 5-dihydrocoriolin C having the formula:



No references cited.

NORMA S. MILESTONE, Primary Examiner

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424—278; 195—80