Inhibitors of Melanogenesis from the Roots of *Peucedanum praeruptorum*

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Summary

A chemical investigation of *Peucedanum praeruptorum* has resulted in the isolation of 3 khellactone derivatives, which have inhibitory effects on melanogenesis in B16 mouse melanoma cell lines. The khellactone derivatives were isolated from the crude extract of the roots of *Pecedanum praeruptorum* by a combination of adsorption chromatography and HPLC. The structures of isolated compounds were identified as 3',4'-diangeloyl-*cis*-khellactone, 3'-angeloyl-4'-senecioyl-*cis*-khellactone and, 3',4'-disenecioyl-*cis*-khellactone by ^{1}H NMR, ^{13}C NMR and mass spectral studies and by comparisons of spectral data with reported literatures.

These khellactone derivatives can be a good candidate for new skin whitening agent due to its strong inhibitory activity and safety.

Introduction

The ambient levels of harmful ultraviolet radiation emitted by the sun are increasing due to destruction of the ozone layer. Skin is exposed to sunlight and often suffers from various harmful effects of ultraviolet rays. Melanin in human skin which is synthesized within the melanocyte on the melanosome by the action of tyrosinase, which converts tyrosine to L-dopa, dopaquinone and subsequent autopolymerization. [1,2] Melanin is a major defense mechanism against ultraviolet light of the sun, but abnormal hyperpigmentation such as freckles or brown spots could be a serious aesthetic problem. Therefore, potent active agents for the improvement of freckles or brown spots are sought for their cosmetic use.

To develop such an active agent, we examined the inhibitory activities of plant extracts on melanogenesis in B16 F1 melanoma cells. We found that 3 khellactone derivatives showed the potent inhibition of melanogenesis, which were isolated from the methanolic extract of *Peucedanum praeruptorum* Dunn. and were respectively elucidated as compound **I**(3',4'-diangeloyl-*cis*-khellactone [4,5]), compound **II**(3'-angeloyl-4'-senecioyl-*cis*-khellactone [3,4,6]) and compound **III**(3',4'-disenecioyl-*cis*-khellactone [3,4,5,7])

Materials and Methods

MATERIAL

Peucedanum praeruptorum Dunn was purchased at Kyeongdong market in Seoul.

CHEMICALS AND INSTRUMENTS

Silica gel 60(230-400mesh, Merck) and Sephadex LH-20(20-100μ, Pharmacia) for column chromatography, silica gel $60F_{254}$(Merck Art.5715) for thin layer chromatography and YMC pack-sil column for HPLC were used., AutoSpec mass spectrometer(Micromass, Manchester, UK), Bruker DMX-600 FT-NMR spectrometer, Shimadzu IR-435 for structure elucidation were used.

DMEM(Dulbecco's modified Eagle's medium), FBS(fetal bovine serum), antibiotics and Trypsin(2.5%) for B16 F1 melanoma culture were purchased from Sigma, and other chemicals

were used as special grade.

ISOLATION OF COMPOUND I, II and III

The root (2 kg) of *P. praeruptorum* were extracted with CH_3OH three times at room temperature. The concentrated CH_3OH extract (180 g) was added to water and then partitioned successively with n-hexane and AcOEt. The AcOEt layer(95g), which showed significant melanogenic inhibitory effect, was fractionated using silica gel column chromatography with n-hexane-AcOEt stepwise elution. Compound I,II and III were eluted with n-hexane-AcOEt (10:1 and 1:1) and were further purified by HPLC with n-hexane-AcOEt(5:1) as eluting solvent.

B16 F1 MOUSE MELANOMA CULTURE

B16 F1 mouse melanoma cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics in humidified incubator at 37℃ under 5% CO_2.

EFFECT OF COMPOUND I,II and III ON MELANIZATION OF B16 F1 MOUSE MELANOMA

Cells were seeded into 60㎜ petridishes at a density of $5x10^5$ cells per dish. After cells were attached, medium was replaced with fresh medium(0.5% DMSO) containing various concentrations of compound I,II and III. Then cells were cultured for 2 days and the medium was replaced with fresh medium, further incubated for a day. Then cells were detached with cell scrapper, harvested, and counted with haemacytometer. Melanin was extracted and measured according to the method of Lotan with some modifications [8]. Briefly, cell pellets were resuspended in 1㎖ of distilled water and freezed at -20℃ and thawed at 37℃. This freezing-thawing process was performed for three times. Perchlonic acid was added to the cell suspensions at a final concentration of 0.5N. The tubes were set on ice for 10 min and centrifuged at 15,000g for 5 min. The pellets were extracted with 0.5N perchloric acid for 2 times, with cold ethanol/ether (3:1) for 2 times, and once with ether. The resulting pellets were dried in air and 1㎖ of 1N NaOH was added to each tube. The tubes were incubated in a boiling water bath for 10 min to dissolve the pellets. Melanin contents were measured by reading the absorbances at 400 ㎚ and expressed as $A_{400}/10^6$ cells. [9]

Results and discussion

STRUCTURE ELUCIDATION OF COMPOUND I,II and III

The structure of these compounds was determined by their NMR spectroscopy and the results are given in the Table 1 & 2 and Figure 1. The assignments of the NMR spectra were confirmed by the 2D experiments such as COSY, HSQC [10,11], and HMBC. [12]

NMR in CDCl3 (Table 1 & 2) showed H-3 and H-4 as AB quartets with the coupling constant J_{AB} = 9.5 Hz. H-4 appeared as a doublet centered between δ7.59 and 7.58 and H-3 ranged from δ6.22 to 6.20. H-5 and H-6 were found between δ7.36 and 7.34 and between δ6.82 and 6.80, respectively. These values for the coumarin and benzene protons are those proper to khellactone derivatives and to simple coumarins with an alkyl chain at C-8 and an alkyloxy one at C- 7. [13,14'15'16]

The relative *trans* configuration of compounds **4** and the *cis* configuration of **1, 2, 3** were assigned on the basis of the coupling constant $J_{3'4'}$ which was 3.4Hz for the *trans* compounds and 4.9Hz for the *cis* compounds. [13,14'15'16]

Assignments of the protonated carbons were made using HSQC experiment with the $^1J_{CH}$ couplings. The position of H-3' and 4' were detrmined from HMBC experiment with evaluation of the $^3J_{CH}$

couplings between the *gem*-dimethyl singlets(H-2'-methyl) and H-3' or 4' carbons. Also the positions of the esters(angelate and senecioate) moieties attached to khellactones were determined from the HMBC experiment with couplings between an oxymethine proton(H-3' and H-4') and ester carbonyl carbon, and couplings between the double bond proton(H-2" for senecioyl, H-3" for angeloyl, Me-2" for acetate) and ester carbonyl carbon.

The angeloyl and senecioyl moieties attached to the khellactones were identified by NMR, the spectra coinciding with those already reported. [13,14'15'16]

Compound Ⅰ was obtained as white powder. The EIMS showed an $[M]^+$ at m/z 426.47. The ^{1}H-NMR spectrum(400MHz ; $CDCl_3$) of Compound Ⅰ shows singlet *gem*-dimethyl protons at δ1.46 and 1.49, Two complex signals at δ1.84(Me-2",6H,1.5Hz) and 1.97(Me-3",6H,1.5Hz) of methyls

attached to a double bond were assigned to two angeloyl protons. Two multiplet at 6.02 and 6.12 were assigned to methine protons attached to double bonds of the angelate. In the aromatic proton region two pairs of doublets at δ6.21 and 7.59 (J_{34} = 9.5Hz) and at δ6.81 and 7.35 (J_{56} = 8.6Hz) were attributed to the protons at C-3, C-4, C-6 and C-5, respectively. Two doublets at δ5.45 and 6.70 were assigned to the protons at C-3' and C-4' of *cis*-khellactone diester which showed a characteristic splitting pattern($J_{3'4'}$ = 4.9Hz). Two angelates were placed on the C-3' and C-4' positions based on the HMBC correlation between oxymethines(H-3' and H-4') and carbonyl carbons(δ166.3 and 166.5).

Compound Ⅱ was obtained as white powder. The EIMS showed an $[M]^+$ at m/z 426.47. The ^{1}H-NMR spectrum(400MHz ; $CDCl_3$) of Compound Ⅱ shows singlet *gem*-dimethyl protons at δ1.46 and 1.49, two complex signals at δ1.86(Me-2",3H,1.5Hz) and 1.96(Me-3",3H,1.5Hz) of methyls attached to a double bond were assigned to two angeloyl protons, and two doublets at δ1.87(*cis*-Me -3", 3H,1.2Hz) and 2.19(*trans*-Me-3",3H,1.2Hz) of methyls attached to a double bond to senecioyl protons. A multiplet at δ5.62 was assigned to methine protons attached to double bonds of the senecioate. Another multiplet at δ6.10 was assigned to methine protons attached to double bonds bonds of the angelate. In the aromatic proton region two pairs of doublets at δ6.21 and 7.58 (J_{34} = 9.5Hz) and at δ6.80 and 7.35 (J_{56} = 8.6Hz) were attributed to the protons at C-3, C-4, C-6 and C-5, respectively. Two doublets at δ5.40 and 6.65 were assigned to the protons at C-3' and C-4' of *cis*-khellactone diester which showed a characteristic splitting pattern($J_{3'4'}$ = 4.9Hz). The angelate was placed on the C-3' positions and the senecioate on the C-4' based on the HMBC correlation between oxymethines(H-3' and H-4') and carbonyl carbons(δ166.3 and 165.0)., respectively.

Compound Ⅲ was obtained as white powder. The EIMS showed an $[M]^+$ at m/z 426.47. The ^{1}H-NMR spectrum(400MHz ; $CDCl_3$) of Compound Ⅲ shows singlet *gem*-dimethyl protons at δ1.42 and 1.46, two doublets at δ1.88(*cis*-Me-3", 6H,1.2Hz) and two doublets at δ2.17(*trans*-Me-3",6H, 1.2Hz) of methyls attached to double bonds were assigned to senecioyl protons. Two multiplets at

δ5.63 and 5.67 were assigned to methine protons attached to double bonds of the senecioate. In the aromatic proton region two pairs of doublets at δ6.20 and 7.58 (J_{34} = 9.5Hz) and at δ6.80 and 7.34 (J_{56} = 8.6Hz) were attributed to the protons at C-3, C-4, C-6 and C-5, respectively. Two doublets at δ5.36 and 6.62 were assigned to the protons at C-3' and C-4' of *cis*-khellactone diester which showed a characteristic splitting pattern($J_{3'4'}$ = 4.9Hz). Two senecioates were placed on the C-3' and C-4' positions based on the HMBC correlation between oxymethines(H-3' and H-4') and carbonyl carbons(δ165.1 and 165.2).

EFFECT OF COMPOUND I,II and III ON MELANIZATION OF B16 F1 MOUSE MELANOMA

When cell pellets treated with 0.3% DMSO (A:control), 1mmol Arbutin (B:positive control), 0.01 mmol compound I (C), II (D) and III(E) were determined by visual assessment, the color of pellet B, C, D and E were similar. It means that compound I, II and III inhibited melanogenesis at 100 times lower concentration compared to arbutin in B16 mouse melanoma.(Fig.2-I) when melanin contents per 10^6 cells of C, D and E were measured by reading the absorbances at 400 ㎚, there were 47%, 48% and 47% decreases compared to A, respectively. These are very significant decreases in melanine contents compared with arbutinin, wihich showed 48% decrease at 1 mmol concentration.(Fig.2-II)

Conclusion

Based on B16 F1 melanoma assay, compound I, II and III, with melanization inhibitory effect, were isolated and identified as 3',4'-diangeloyl-*cis*-khellactone, 3'-angeloyl-4'-senecioyl-*cis*-khellactone and 3',4'-disenecioyl-*cis*-khellactone from its various spectra.

These compounds potently inhibited the melanogenesis in B16 F1 mouse melanoma(IC_{50}<5μg/ml)

In preliminary studies, compound I, II and III were found safe in the tests of cytotoxity and allergenicity. (data not shown)

The mechanism through which compound I,II and III inhibits melanization is under

investigation and compound I, II and III can be a good candidate for new skin whitening agent due to its strong inhibitory activity and safety.

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Table 1. ^{1}H NMR spectral data of 3 pyranocoumarins (ppm from TMS in $CDCl_3$, coupling constants (J, Hz))

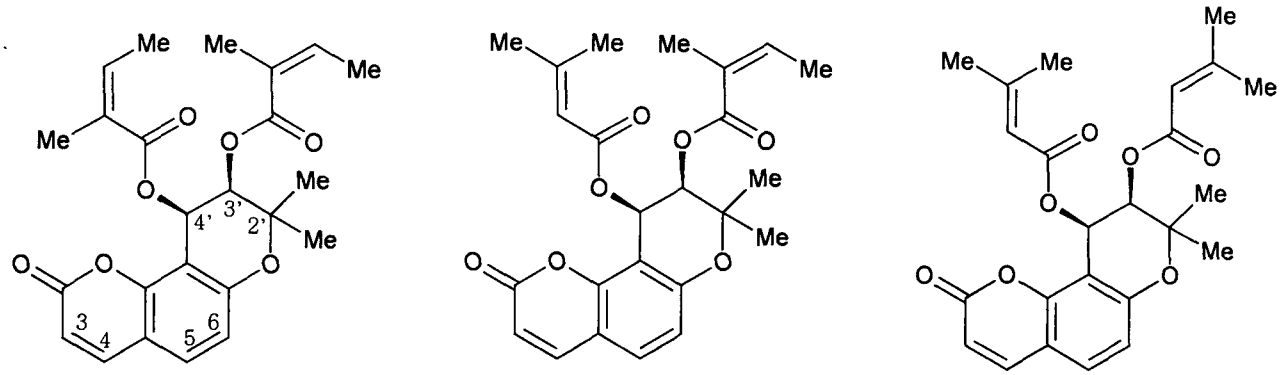
H position	Compound I	Compound II	Compound III
H-3	6.21 (9.5)	6.21 (9.5)	6.20 (9.5)
H-4	7.59 (9.5)	7.58 (9.5)	7.58 (9.5)
H-5	7.35 (8.6)	7.35 (8.6)	7.34 (8.6)
H-6	6.81 (8.6)	6.80 (8.6)	6.80 (8.6)
H-3'	5.45 (4.9)	5.40 (4.9)	5.36 (4.9)
H-4'	6.70 (4.9)	6.65 (4.9)	6.62 (4.9)
Me-2'	1.49 1.46	1.49 1.46	1.46 1.42
H-2" senecioyl		5.62	5.67
H-3" angeloyl	6.12 6.02	6.10	
Me-2"(Se)	1.86 1.83	1.86	
Me-3"(An)	1.98/1.96	1.96	
Me-3"(Se)		2.19/1.87	2.19/2.15,1.89/1.88

Table 2. ^{13}C NMR spectral data of 3 pyranocoumarins (ppm from TMS in $CDCl_3$)

C position	Compound I	Compound II	Compound III
C-2	159.7	159.8	159.9
C-3	113.3	113.3	113.2
C-4	143.2	143.1	143.2
C-5	129.2	129.1	129.0
C-6	114.4	114.3	114.3
C-7	156.7	156.7	156.8
C-8	107.6	107.7	107.6
C-9	154.1	154.0	154.1
C-10	112.5	112.5	112.5
C-2'	77.5	77.5	77.7
C-3'	70.2	70.3	69.4
C-4'	60.2	59.5	59.8
Me-2'	25.4	25.5	25.1
	22.5	22.5	22.7
C-1"(An)	166.5/166.3	166.3	
C-1"(Se)		165.0	165.2/165.1
C-2"(An)	127.4/127.0	127.2	
C-2"(Se)		115.1	115.3(*2)
C-3"(An)	139.8/138.4	139.4	
C-3"(Se)		157.9	158.2/157.5
Me-2"	20.4	20.4[a]	
Angeloyl	20.3		
Me-3"(An)	15.8/15.6	15.7	

Me-3"(Se)	27.4/20.3[a]	27.5/20.4, 27.4/20.3

[a] interchangeable



Compound I Compound II Compound

Figure 1. Structures of compound I, II and III from *Peucedanum praeruptorum*

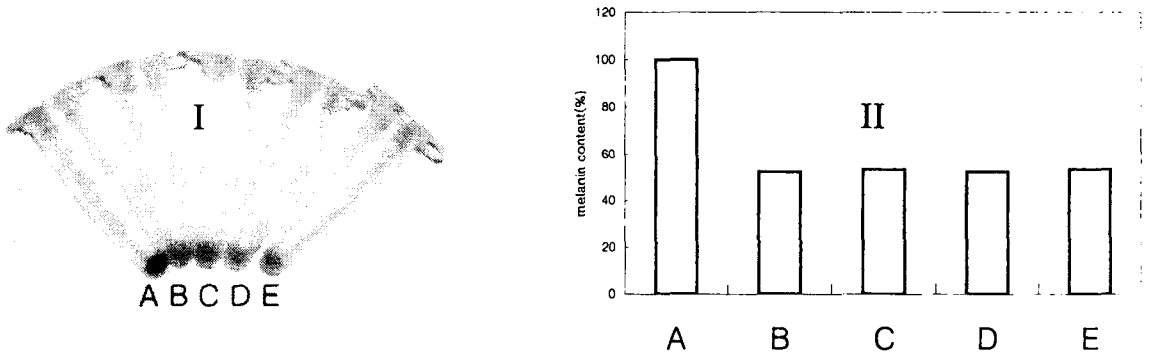


Figure 2. I. B16 melanoma cell pellets treated for 3 days with 0.3% DMSO (A:control), 1mmol Arbutin (B:positive control), 0.01 mmol compound I (C), II (D) and III (E). II. Melanin contents in 10^6 cells in samples in I (n = 3)