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Three Melanogenesis Inhibitors from the Roots of *Veratrum nigrum*

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여로의 멜라닌 생성 억제 물질

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Abstract – Three melanogenesis inhibitors were isolated from the roots and rhizomes of *Veratrum nigrum* L. and were identified as (3S,20S,25S)-22,26-iminocholesta-5,22(N)-dien-3-ol (verazine), (3S,20R,25S)-22,26-iminocholesta-5,22(N)-dien-3-ol (epi-verazine) and (3R,23R)-14,15,16,17-tetradehydroveratraman-3,23-diol (veratramine) on the basis of their spectroscopic data. It was turned out that these compounds did not directly inhibit tyrosinase activity, the key enzyme responsible for the formation of melanin pigment, while these compounds showed strong inhibition on the melanogenesis in B16 F1 mouse melanoma (IC_{50} < 1 μg/ml). Due to the strong inhibitory activity and safety compared to current whitening agents such as arbutin, kojic acid and AHA, the compound can be a good candidate for new skin whitening agents.

Key words – Epi-verazine, Melanogenesis, Skin whitening, Veratramine, *Veratrum nigrum*, Verazine

Skin is exposed to sunlight and often suffers from various harmful effects of ultraviolet rays. Melanin in human skin is synthesized within the melanocyte on the melanosome by the action of tyrosinase, which converts tyrosine to L-dopa, dopaquinone and subsequent autopolymerization.[1)] 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 effect of plant extracts on the inhibition of melanogenesis in B16 F1 melanoma cells. We found that (3S,20S,25S)-22,26-iminocholesta-5,22(N)-dien-3-ol (verazine), (3S,20R,25S)-22,26-iminocholesta-5,22(N)-dien-3-ol (epi-Verazine) and (3R,23R)-14,15,16,17-tetradehydroveratraman-3,23-diol (veratramine), which were isolated from *Veratrum nigrum* roots showed remarkable effects on melanin biosynthesis of B16 F1 mouse melanoma.

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Veratrum nigrum is one of the member of the Chinese crude drug "Yeo-Ro", which is used for treating dysentery, jaundice, headache, scabies, chronic malaria and hypertension.[2)] Yeo-Ro is prepared from dried roots and rhizomes of several *Veratrum* species (Liliaceae) such as *V. nigrum* L., *V. nigrum* L. var. *ussuriense* Loes., *V. maackii* Reg., and *V. puberulum* Loes. F.

MATERIALS AND METHODS

Chemicals and Instruments

Silica gel 60 (230-400 mesh, Merck) for column chromatography, silica gel $60F_{254}$ (Merck Art.5715) for thin layer chromatography, and YMC pack-sil column and CombiHT SB-C18 column for HPLC were used. AutoSpec mass spectrometer (Micromass, Manchester, UK), Bruker DMX-600 FT-NMR spectrometer, and Shimadzu IR-435 for structure elucidation were used.

DMEM (Dulbeccos modified Eagles medium), FBS (fetal bovine serum), antibiotics and Trypsin (2.5%) for B16 F1 melanoma culture and MCDB 153, phorbol-12-myristate-13-

acetate, insulin, hydrocortisone, basic human fibroblast growth factor, alpha-melanocyte stimulating hormone, bovine transferrin for normal human melanocyte culture were purchased from Sigma, and other chemicals were used as special grade.

Isolation of compounds I, II, and III

Veratrum nigrum root was collected from Mt. Paekdu in northern region of Korea. At the sametime, voucher specimen was made and deposited at herbarium of Chonbuk National University (JNU). The crushed root (10 kg) of *Veratrum nigrum* was extracted with CH_3OH three times at room temperature. The concentrated CH_3OH extract (540.0 g) was dissolved in water and partitioned successively with *n*-hexane, chloroform and butanol. The butanol layer (238 g), which showed significant melanogenic inhibitory activiy, was fractionated using silica gel column chromatography with acetonitrile-methanol stepwise elution(20:1 →1:1). Compounds I, II, and III ware eluted with methanol-acetonitrile (10:1, fraction III). This fraction was subjected to silica gel column chromatography with n-hexane-acetone-methanol (70:40:5). Compounds I, II, and III were further purified by HPLC with methanol-0.2%TFA (56:44) as eluting solvents (Fig. 1).

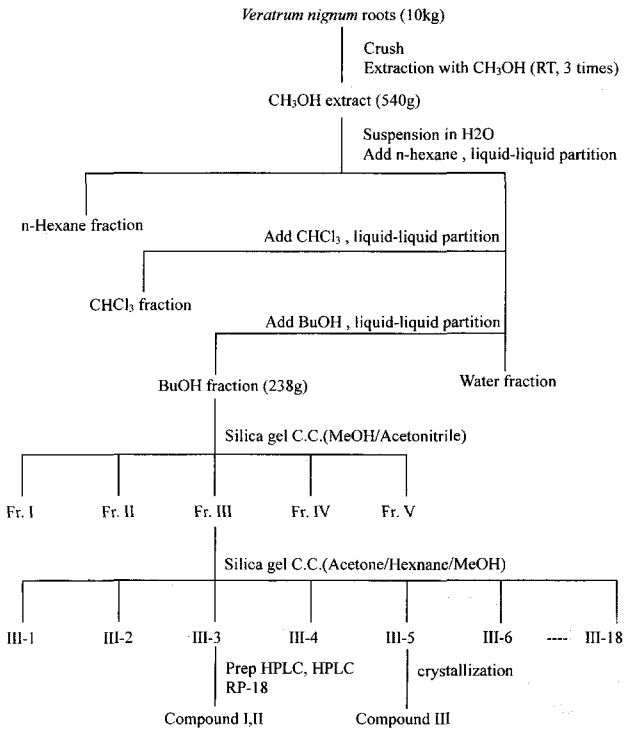

Fig. 1. Extraction and isolation of compounds I, II, and III from *Veratrum nigrum* roots.

Melanization inhibition assay on B16F1 mouse melanoma

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

Cells were seeded into 60 mm petridishes at a density of 5×10^5 cells per dish. After cells were attached, medium was replaced with fresh medium (0.5% DMSO) containing various concentrations test of compounds. Cells were cultured for 2 days, and the medium was replaced with fresh medium, and further incubated for a day. Cells were harvested and counted with haemacytometer. Melanin was extracted and measured according to the method of Lotan with some modifications.[3] Briefly, cell pellets were resuspended in 1 m*l* of distilled water and freezed at −20°C and thawed at 37°C. This freezing-thawing process was repeated for three times. Perchlonic acid was added to the cell suspensions at a final concentration of 0.5 N. The tubes were set on ice for 10 min and centrifuged at 15,000 g for 5 min. The pellets were extracted with 0.5 N 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 m*l* of 1 N 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 nm and expressed as $A_{400}/10^6$ cells.[4]

Safety assessment

The cytotoxicity of each compound was evaluated using the MTT test. Phosphate buffer (pH 7.4) containing 5 ug/ml of MTT was added to human fibroblast cultures in 96-well plates, and the plate was incubated at 37°C for 4 hr. Precipitated formazan dye was solubilized in isopropanol containing 0.04 M HCl for 30 min, and the absorbance was measured at 570 nm using 655 nm as a reference. Cell survival was calculated from the absorbance.

The allergenicity of each compound was evaluated by Local Lymph Node Assay (LLNA).[5] Twenty five microliter of test solution or vehicle were applied to both ears of mice daily for 3 days. On next day after final application, draining (auricular) lymph nodes were excised and pooled for each experimental group. A single cell suspension of lymph node cells (LNCs) was prepared by mechanical disaggregation through sterile 200-mesh gauze. The density of LNCs suspension was adjusted to 1×10^7 cells/ml in RPMI-FCS. LNCs suspensions were seeded into 96-well plates and cultured for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air with 2 μCi of ^{3}H-TdR (Amersham International, Amersham, U.K.). The

culture was terminated by automated cell harvesting and ^{3}H-TdR incorporation was determined by β-scintillation counting.

RESULTS AND DISCUSSION

Structure elucidation of compounds I, II, and III

Verazine – Colorless needles, mp 175-177°C, UV (MeOH) λ_{max} 206 nm. The molecular formula was determined as $C_{27}H_{43}NO$ (MW 397.65) on the basis of the high resolution ESI mass spectrum. The IR (film) spectrum exhibited signals for λ_{max} 3333 (OH), 2931, 1659 (C=N), 1567, 1413, 1370, 1118 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.69 (3 H, s, CH_3-18), 0.88 (3 H, d, *J*=6.6 Hz, CH_3-27), 0.99 (3 H, s, CH_3-19), 1.07 (3 H, d, *J*=6.9 Hz, CH_3-21), 2.96 (1 H, m, H-26$_{ax}$), 3.65 (1 H, dd, *J*= 6.7, 16.6 Hz, H-26$_{eq}$), 3.05 (1 H, m, H-3), 5.32 (1H, br d, *J*= 5.0 Hz, H-6); ^{13}C NMR, see Table I.

From ^{1}H-NMR,^{13}C-NMR, DEPT spectra data and those of Maged S. Abdel-Kader *et al.*[6] compound I was identified as (3S,20S,25S)-22,26-iminocholesta-5,22 (N)-dien-3-ol (Fig. 2).[7,8]

Table I. ^{13}C-NMR data of verazine and epi-verazine ($CDCl_3$)

Position	Compound I	Compound II
1	37.3	37.3
2	31.6	31.6
3	71.6	71.6
4	42.3	42.3
5	140.9	140.9
6	121.5	121.5
7	31.8	31.8
8	31.8	31.8
9	50.1	50.1
10	36.5	36.5
11	21.0	21.1
12	39.7	39.1
13	42.4	42.2
14	56.4	56.3
15	24.3	24.0
16	27.7	26.4
17	53.1	53.6
18	12.0	11.8
19	19.3	19.4
20	47.0	46.6
21	18.3	18.1
22	175.3	174.5
23	26.5	27.3
24	27.2	27.8
25	27.4	27.6
26	56.4	56.9
27	19.1	19.5

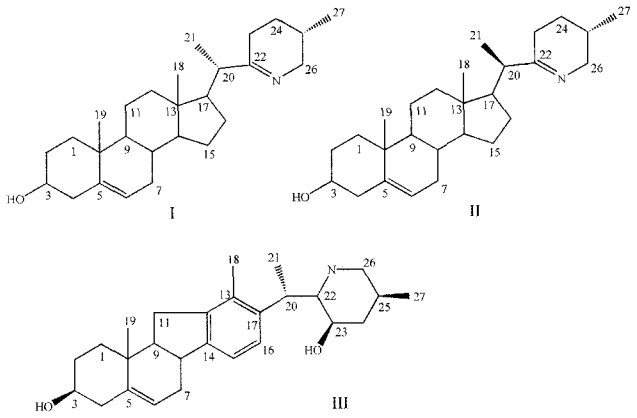

Fig. 2. Structures of verazine (I), epi-verazine (II), and veratramine (III) from *Veratrum nigrum* roots.

Epi-verazine – The molecular formula was determined as $C_{27}H_{43}NO$ (MW 397.65) on the basis of the high resolution ESI mass spectrum. The IR (film) spectrum exhibited signals for λ_{max} 3328 (OH), 2936, 1659 (C=N), 1569, 1413, 1370, 1118 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.70 (3 H, s, CH_3-18), 0.90 (3 H, d, *J*=6.6 Hz, CH_3-27), 0.98 (3 H, s, CH_3-19), 0.97 (3 H, d, *J*=7.8 Hz, CH_3-21), 2.93 (1 H, m, H-26$_{ax}$), 3.69 (1 H, m, H-26$_{eq}$), 3.48 (1 H, m, H-3), 5.32 (1 H, br d, *J*=5.0 Hz, H-6); ^{13}C NMR, see Table I.

From ^{1}H-NMR,^{13}C-NMR, DEPT spectra data and those of Maged S. Abdel-Kader *et al.*[6] compound II was identified as (3S,20R,25S)-22,26-iminocholesta-5,22 (N)-dien-3-ol (Fig. 2).

Veratramine – The molecular formula was determined as $C_{27}H_{39}NO_2$ (MW 409.61) on the basis of the high resolution ESI mass spectrum. ^{1}H NMR and ^{13}C NMR, see Table II.

From ^{1}H-NMR,^{13}C-NMR, DEPT spectra data and those of Yasuhiro Tezuka *et al.*[9] compound III was identified as (3R,23R)-14,15,16,17-tetradehydroveratraman-3,23-diol (Fig. 2).[10]

Effect of the compounds on melanization in B16F1 mouse melanoma

Verazine, epi-verazine, and veratramine showed concentration-dependent inhibition on melanin biosynthesis in B16F1 mouse melanoma cells (Fig. 3). IC_{50} of these compounds were less than 1 μg/m*l*. These are very significant decrease in melanin contents compared with other melanogenic inhibitors (Table III).

Interestingly, these compounds did not directly inhibit the enzyme tyrosinase (data not shown). The action mechanism of these compounds is under investigation.

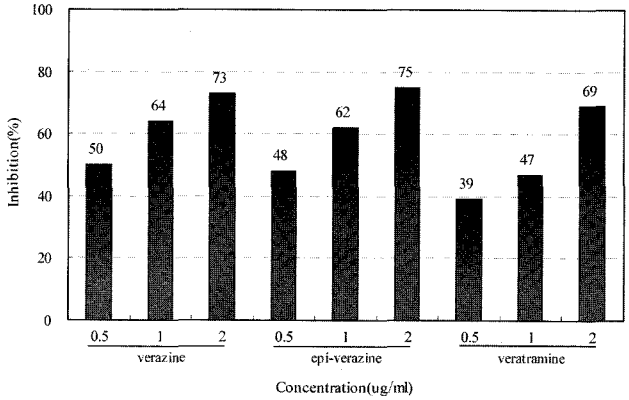

Fig. 3. Effects of verazine, epi-verazine, and veratramine on melanogenesis in B16 F1 mouse melanoma cells.

Table II. ^{1}H, and ^{13}C -NMR data for veratramine (pyridine-d_5)

Position	^{1}H	^{13}C
1	1.30td, 1.80 br d	38.5
2	1.87 dddd, 2.11 br d	32.0
3	3.84 tt	71.3
4	2.60 br dd, 2.70 br dd	43.0
5		142.5
6	5.40 br d	121.3
7	2.01 br dd, 2.56 m	30.8
8	2.94 td	41.5
9	1.80 td	57.3
10		37.2
11	2.50 dd, 2.78 dd	30.7
12		133.1
13		143.64
14		143.68
15	7.09 d	119.9
16	7.66 d	126.7
17		141.2
18	2.57 s	16.1
19	1.10 s	19.3
20	4.06 qd	35.5
21	1.64 d	21.1
22	2.84 dd	68.3
23	3.56 ddd	70.7
24	1.35 q, 2.25 br d	45.2
25	1.46 m	32.5
26	2.02 t, 2.99 br d	54.6
27	0.74 d	19.0

Toxicological studies

The toxicity potential of verazine, epi-verazine, and veratramine were studied. Cytotoxicity of these compounds was comparable to that of sodium lauryl sulfate. LLNA data showed that none of these compounds is allergenic (Table IV).

Table III. Effects of compounds I, II, and III on melanogenesis in B16 F1 mouse melanoma cells

Sample	IC_{50}
Arbutin	300 μg/ml
Kojic acid	280 μg/ml
Hydroquinone	2 μg/ml
Verazine	< 1 μg/ml
Epi-verazine	< 1 μg/ml
Veratramine	< 1 μg/ml

Table IV. Safety data for verazine, epi-verazine, and veratramine data

MTT assay (Cytotoxicity)	Verazine	IC_{50} = 0.004%
	Epi-verazine	IC_{50} = 0.004%
	Veratramine	IC_{50} = 0.003%
LLNA (Allergenicity)	Verazine	*SI = 0.97(5%), 1.14(10%)
	Epi-verazine	SI = 0.92(5%), 1.05(10%)
	Veratramine	SI = 1.17(5%), 2.09 (10%)

*SI = sensitization index.

CONCLUSION

Three compounds, showing melanogenesis inhibition in B16F1 melanoma assay, were isolated from the roots of *Veratrum nigrum*. These compounds were identified as (3S,20S,25S)-22,26-iminocholesta-5,22 (N)-dien-3-ol (verazine), (3S,20R,25S)-22,26-iminocholesta-5,22(N)-dien-3-ol (epi-Verazine) and (3R,23R)-14,15,16,17-tetradehydroveratraman-3,23-diol (veratramine) from their various spectra.

It was revealed that the compounds potently inhibited the melanogenesis in B16 F1 mouse melanoma (IC_{50} < 1 μg/ml). And the cytotoxity and allergenicity studies showed that verazine, epi-verazine, and veratramine were safe for use in cosmetics.

The action mechanism of compounds I, II, and III melanogenesis is not clear and is under investigation. These compounds were found not to directly inhibit tyrosinase. Due to both the strong inhibitory activity and safety compared to current whitening agents such as arbutin and kojic acid, these compounds can be good candidates for new skin whitening agents.

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초 록 – 국내외의 각종 피부관련 약재를 중심으로 천연물 data-base를 구축하고, 이로부터 피부 미백에 효과가 있다고 언급된 한방 약재를 추출, 용매 분획하여 얻은 분획물들을 B16 melanoma cell assay를 이용하여 미백 효과를 평가하였다. 이들 중 특히 미백효과가 우수한 여로(藜蘆, *Veratrum nigrum* L.)의 뿌리를 대상으로 성분연구하여 3개의 미백유효성분인 (3S,20S,25S)-22,26-iminocholesta-5,22(N)-dien-3-ol (verazine), (3S,20R,25S)-22,26-iminocholesta-5,22(N)-dien-3-ol (epi-verazine) and (3R,23R)-14,15,16,17-tetradehydroveratraman-3,23,diol (veratramine)을 분리, 구조분석하였다. 여로로부터 분리된 3종의 미백 유효성분은 흑화 작용의 주요 효소인 tyrosinase에 대한 작용은 확인할수 없었으나, B16 F1 mouse melanoma에 대한 멜라닌 합성 억제능은 IC_{50}<1 μg/ml로 강하게 나타났다. 또한 이와 같은 강한 멜라닌 합성 저해능을 갖는 이들 물질들의 안전성 확인을 통하여 향후 새로운 미백물질로 이용될 수 있을것으로 사료된다.