Higher Yielding Isolation of Kinsenoside in *Anoectchilus* and Its Anti-hyperliposis Effect

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Received April 12, 2000; accepted October 3, 2000

A higher concentration of kinsenoside, 3-(R)-3-\-glucopyranosylxybutanolide (1), was detected in the crude drug *Anoectchilus formosanus*, and *A. koshunensis* by HPLC analysis. A methylation reaction occurred to give methyl ester (4) when the lactone ring of 1 was cleaved by silica gel catalysis using methanol containing solvent used in the purification step resulting in difficulty to purify 1. To avoid the cleavage reaction, a reversed-phase or silica gel column without methanol was used to give a high yield of 1. In an anti-hyperliposis assay using high-fat diet rats, 1 significantly reduced the weights of body and liver, and also decreased the triglyceride level in the liver compared to those of control rats. On the other hand, the epimer of 1, 3-(S)-3-\-glucopyranoslyxybutanolide (2), trivially named goodyerose A, which was isolated from *Goodyera* species, had no effect for anti-hyperliposis. In aurothioglucone-induced obese mouse, 1 suppressed the body and liver weight increase, significantly ameliorated the triglyceride level in the liver, and also reduced the deposition of uterine fat-pads.

Key words *Anoectchilus*; kinsenoside; anti-hyperliposis effect; *Goodyera*; goodyerose A; lactone ring

The genus *Anoectchilus* (Orchidaceae) is a perennial herb, which comprise more than 35 species that are widespread in the tropical regions, from India through the Himalayas and southeast Asia to Hawaii.\(^5\) Several species have been used in Chinese folk medicines.\(^2,3\) Among them, *A. formosanus* Hayata which is only found in Taiwan and Okinawa, has been used for hypertension, lung and liver diseases, and underdeveloped children as a folk remedy.\(^4\) Since the natural sources of *A. formosanus* are becoming exhausted, other *Anoectchilus* species such as *A. koshunensis* Hayata and a different genus, *Goodyera*, are commercialized as substitutes used for the same purpose in the present market.\(^5,6\) Previously, Ito et al. isolated small amounts of kinsenoside (1) and the methyl ester of the carboxylic acid form of 1 (4) from *A. koshunensis* by Diaion and silica gel chromatography and high-performance liquid chromatography (HPLC).\(^7\) Interestingly, we isolated a great amount of 1 without 4 by silica gel column chromatography eluting with chloroform–ethanol solvent system. From these results it is easily suggested that some artificial conversion occurred during the separation procedure. This result inspired us to investigate the existence and concentrations of 1 and its analogues in *Anoectchilus*.

This paper deals with the examination of these compounds in wild and cultured plants of *A. formosanus* as well as in wild *A. koshunensis*. Quantitative analysis of 1 was also achieved using an HPLC method as an index component of *Anoectchilus*. Moreover, compound 4 was confirmed to be an artifact induced during the separation procedure, and a simple and economical method was established for purification of 1. Finally the anti-hyperliposis effects of the major component 1 in *Anoectchilus*, and 2 (goodyerose A) in *Goodyera* species,\(^8,9\) have been determined by using high-fat diet rats and the anti-hyperliposis effect of 1 also was determined by using aurothioglucone-induced obese mouse.

**MATERIALS AND METHODS**

**General Experimental Procedures** ¹H- and ¹³C-NMR spectra were recorded on a JEOL EX 270 spectrometer. FAB-MS was recorded on a JEOL AX-500 spectrometer. HPLC was carried out on a Gasukuro Kogyo Model 576, with a Shodex RI SE-61 detector and a YMC-Pack NH2 A-603 column. Silica gel 60 (70–230 and 230–400 mesh, Merck; 40–100 mm, Kanto Chemical Co., Inc.) were used for column chromatography. Kiesel-gel 60F254 (Merck) was used for analytical TLC.

**Plant Material and Extraction** *A. formosanus* was cultured in vitro in Seiwa Pharmaceuticals Ltd.\(^8,9\) The wild *A. formosanus* and *A. koshunensis* were collected in Ishigaki and Taiwan, and *G. schlechtendaliana* was collected in Ishigaki, respectively. The voucher specimens have been deposited in the Herbarium of Medicinal Plant Garden of the Graduate School of Pharmaceutical Sciences, Kyushu University. The dried powders of whole plants of cultured *A. formosanus* (20 g) were extracted with MeOH at room temperature. The MeOH extract (M-1, 6.54 g) was partitioned between CHCl3 and H2O to afford an H2O-soluble fraction (H-1, 5.88 g).

**Isolation of 1, 3 and 4** The H2O-soluble fraction (H-1, 5.88 g) was applied to an ODS (Cosmosil 75C18-OPN) column eluting with H2O to give fr. 1 (1.98 g) and fr. 2 (3.85 g). A portion of fr. 2 (1 g) was chromatographed on silica gel eluting with a gradient of CHCl3–MeOH–H2O (8 : 2 : 0.2–7 : 3 : 0.5) to give 4 (637 mg). However, when a portion of fr. 2 (1 g) was chromatographed on silica gel, but eluting with a gradient of CHCl3–EtOH (8 : 3–7 : 5) to give 1 (758 mg). Purification of fr. 1 (100 mg) by preparative TLC with CHCl3–MeOH–H2O (6 : 4 : 1) gave 3 (28 mg). The structures of 1, 3 and 4 were identified on the basis of ¹H- and ¹³C-NMR analysis compared with respective authentic samples.\(^9\)

**Extraction and Isolation of 1 and 3 from *A. koshunensis*** Wild plants of *A. koshunensis* (dried 15 g, collected in...
The MeOH extract (4.65 g) was partitioned between CHCl₃ and H₂O to afford an H₂O-soluble fraction (4.03 g), which was applied to an ODS (Cosmosil 75C₁₈-OPN) column eluting with H₂O to obtain fr. 1 (1.17 g) and fr. 2 (2.83 g). A portion of fr. 2 (1 g) was chromatographed on silica gel eluting with a gradient of CHCl₃–EtOH (8 : 3—7 : 5) to give 1 (768 mg). Purification of fr.1 (100 mg) by preparative TLC with CHCl₃–MeOH–H₂O (6 : 4 : 1) gave 3 (23 mg).

**HPLC Analysis of 1 and 4 in the Extracts of A. formosanus and A. koshunensis**  HPLC was performed by the following conditions: YMC-Pack NH₂ A-603, 250×4.6 mm i.d.; 80% CH₃CN, 1 ml/min; column temperature 25 °C; detector: Shodex RI SE-61. Under the present HPLC conditions, 4 was eluted at 6.29 min and 1 was eluted at 6.67 min. The MeOH extracts of A. formosanus (extracted from cultured plants or wild plants) and A. koshunensis were dissolved in H₂O (20 mg of each dissolved in 10 ml H₂O) and after filtering by Sep Pak C₁₈ (H₂O), 10 µl of each sample solution was injected into the HPLC system under the conditions as above, respectively.

**Quantitative Analysis of 1 by HPLC** HPLC was performed by the absolute calibration method. Stock solution was prepared by weighing accurately 20 mg of 1 and dissolving in water to make 10 ml. The solution was serially diluted to give the standard solution at the concentrations of 4, 8, 12, 16, 20 µg/10 µl and after being filtered through a 0.22-µm filter and diluted to give the standard solution at the concentrations of 4.0—20 µg/µl, and the recurrence equation and correlation coefficient were calculated by the absolute calibration method. Stock solution was calculated by the absolute calibration method. Stock solution was prepared by weighing accurately 20 mg of 1 and dissolving in water to make 10 ml. The solution was serially diluted to give the standard solution at the concentrations of 4.0—20 µg/µl, and the recurrence equation and correlation coefficient were calculated by the absolute calibration method.

**Quantitative Analysis of 1 in A. formosanus and A. koshunensis with HPLC Method** The samples of dried whole plant of Anoectochilus (1 g) were extracted with MeOH or H₂O at room temperature to give the MeOH and H₂O extracts, respectively. The extracts were dried and then dissolved in water (20 ml) after filtering by Sep Pak C₁₈ (H₂O), 10 µl of each sample solution was injected into the HPLC system under the conditions as above, and the concentration of 1 in the samples was calculated from the calibration curve.

**Conversion of 1 to 4** Compound 1 (20 mg) was chromatographed on silica gel column (10 cm×1.2 cm i.d.), eluted with CHCl₃–MeOH–H₂O (7 : 3 : 0.5), to give 4 (8 mg) and a mixture of 1 and 4, the mixture was rechromatographed on silica gel eluting with a gradient of CHCl₃–EtOH (8 : 3—7 : 5) to give 1 (5 mg) and 4 (4 mg).

**Conversion of 1 to 5** A mixture of 1 (20 mg), silica gel 60 (2 g) and CHCl₃–EtOH–H₂O (5 : 5 : 1, 5 ml) was left at room temperature for 5 weeks, then washed out by 80% EtOH. The eluate was evaporated, and the residue was chromatographed over silica gel (CHCl₃–EtOH, 7 : 3) to yield 5 (3 mg).

Ethyl 3-(R)-3-β-D-Glucopyranosyloxybutanoate (5) Amorphous, positive FAB-MS m/z: 311 [M+H]+. 1H-NMR (pyridine-d₅): δ: 5.06 (1H, d, J=7.6 Hz, G-1), 4.72 (1H, dd, J=7.2, 5.6, 5.4, 5.2 Hz, H-3), 4.52 (1H, dd, J=11.8, 2.1 Hz, G-6), 4.33 (1H, dd, J=11.5, 5.6 Hz, G-6), 4.23 (1H, m, G-3), 4.21 (1H, m, G-4), 4.18 (1H, dd, J=11.2, 5.4 Hz, H-4'), 4.13 (2H, d, J=6.9 Hz, OCH₂CH₃), 3.93 (1H, dd, J=11.2, 5.2 Hz, H-4), 3.97 (1H, m, G-2), 3.95 (1H, m, G-5), 3.01 (1H, dd, J=15.8, 5.6 Hz, H-2'), 2.95 (1H, dd, J=15.8, 7.2 Hz, H-2), 1.11 (3H, d, J=6.9 Hz, OCH₂CH₃). 13C-NMR (pyridine-d₅): δ: 14.2 (CH₃), 37.9 (C-2), 60.6 (OCH₂CH₃), 62.7 (G-6), 65.2 (C-4), 71.6 (G-4), 75.1 (G-2), 78.3 (G-3), 78.4 (G-5), 79.1 (C-3), 104.8 (G-1), 171.8 (C-1).

**Simple Purification Method of 1** The whole plant of dried A. formosanus (1 g) was extracted with H₂O (8 ml×3) at room temperature, the extract was applied to a reversed-phase column chromatography (ODS) eluting with H₂O to obtain fr. 1 and 2. Fraction 2 was applied to a silica gel column eluting with a gradient of CHCl₃–EtOH (7 : 3—7 : 5) to give 1 (158 mg).

**Isolation of Goodyerioside A (2)** The whole plant of dried G. schlechtendaliana (50 g) was treated in the same manner as the simple purification method of 1 as above, to give 2 (7.3 mg). The structure of 2 was identified by comparing its melting point, optical rotation, 1H- and 13C-NMR spectrum with that of an authentic sample.

**Anti-hyperlipidosis Effects of 1 and 2 in Rats Fed a High-Fat Diet** Six-week-old male Sprague-Dawley (SD) rats (Charles River Inc; Ibaraki, Japan, weighing approximately 170 g) were selected and randomly divided into 4 groups. All animals were maintained in an air-conditioned room. Room temperature (at 23±1°C) and humidity (approximately 55%) were controlled automatically, and with a 12 h light–dark cycle. Normal group was fed a laboratory powdered chow (Oriental Yeast Co. Ltd., Tokyo, Japan). Hyperlipidosis model animals were prepared by feeding a high-fat diet (HFD) containing 0.5% cholesterol (Wako), 0.25% cholic acid (Wako), 20% fructose (Wako) and 10% corn oil, but mineral and vitamins were balanced according to a normal commercial diet. Rats were fed the HFD for 6 days. 1 and 2 (50 mg/kg and 100 mg/kg per day, respectively) were administered orally once a day at the same time each day. The number of animals in each group was as follows: 5 in normal group, 8 in control and 1 or 2 administered groups.

**Anti-hyperlipidosis Effect of 1 in Aurothioglucose-Induced Obese Mouse** Four-week-old female ICR mice (Charles River Inc; Ibaraki, Japan) were used. Mice were given an i.p. injection of 500 mg/kg aurothioglucose (Sigma).
and after the mice weighing around 22 g were selected at random and divided into groups so that average body weight was equalized. Control group mice were fed a HFD containing 20% fructose and 10% corn oil for 6 weeks. 1 treated groups were fed a diet which mixed 1 in amount 0.1% and 0.2% of total weight of the HFD for 6 weeks, respectively. Normal group was treated without the injection of aurothioglucose and fed a laboratory powdered chow (Oriental Yeast Co. Ltd., Tokyo, Japan). The number of animals in each group was follows: 15 in normal group, 18 in control group and 20 in 1 (0.1% or 0.2%) administered groups.

Determination of Triglyceride Level  The liver triglyceride (TG) was determined by Triglyceride E-test (Wako Pure Chemical Industries. Ltd., Osaka) after extracting all lipid using the method of Folch et al. 

Histopathological Examination of the Mice Liver  A portion of the median lobe of the liver of mice were removed from the obese mice after being fed the HFD and the HFD which contained 0.1% and 0.2% of 1 for 6 weeks, respectively. For histopathological examination, 5 μm thick sections of paraffin-embedded liver slices were stained with hematoxylin-eosin (H-E) and eosin following a standard microtechnique and observed under light microscope.

Statistical Analysis  Values are expressed as means± S.E.M. and differences between the control group and other groups were examined using Duncan’s multiple range tests. The values were considered to be significantly different when the p value was less than 0.05.

RESULTS AND DISCUSSION

Ito et al. isolated 1 and 4 from A. koshunensis, and we also isolated 1, 3 and 4 from A. formosanus, respectively. However, the repeated purification of the crude extract of A. formosanus by silica gel chromatography eluting with MeOH containing solvent increased the yield of 4. In order to confirm the existence of 4 in the crude extractives of A. formosanus and A. koshunensis, the water and MeOH extracts were analyzed by HPLC and it was not found. Quantitative analysis by HPLC showed a higher concentration of 1 in A. formosanus and A. koshunensis. The contents of 1 in A. formosanus and A. koshunensis by this method were: 18% (dr. wt) in the cultured A. formosanus; 16% (dr. wt) in the wild A. formosanus; 15% (dr. wt) in the wild A. koshunensis, respectively. From these results, it is easily suggested that some artificial conversion occurred during the purification step. When compound 1 was applied to a silica gel column that was eluted with a CHCl₃–MeOH–H₂O solvent system, 4 was isolated, indicating that the purification of 1 using a long column eluting with MeOH containing solvent enhanced the yield of 4. Compound 1 completely changed into 4 by dissolving in CHCl₃–MeOH–H₂O solvent with silica gel at room temperature for one week. Furthermore, 1 was converted into a new compound 5 under the CHCl₃–EtOH–H₂O solvent system. The positive FAB-MS of 5 showed [M+H]+ ion peak at m/z 311, indicating the increase of 14 mass unit compared to that of 4. Compound 5 showed a similar ¹H- and ¹³C-NMR spectra with those of 4, except the carboxyl region. Thus, 5 was determined to be the ethyl ester of the carboxylic acid form of 1. Although the stability of 1 in H₂O or MeOH under reflux for 30 min was investigated, no chemical change was observed. According to these results, the methylation reaction occurs when the lactone ring is cleaved by the catalysis of silica gel. Based on these results, 1 was purified simply by using the reversed-phase and silica gel column eluting with no methanol containing solvent. It became evident that all Anoectochilus spp. contain 1 and 3 regardless of the differences of growth place and extraction condition. As previously discussed, the conversion of 3 to 1 was easily enhanced by mild acid treatment. On the other hand, no change of 1 into 3 occurred under various conditions (data not shown). Thus, the biogenetic conversion of 3 to 1 is suggested. From the above findings, we concluded that the yield of the major constituent, 1 depends on the purification procedure. Furthermore, it is obvious that fresh plants are recommended to be used in folk remedy. 

Since we succeeded in setting up a simple and rapid purification method for compound 1, it becomes possible to assay the pharmacological activity of 1. In an assay for anti-hyperlipemia effect using high-fat diet rats, 1 significantly ameliorated the TG level in liver. The liver and body weights were lower than those of control group. Table 1 shows the change in body weight and liver weight when 6-week-old male SD rats were fed HFD (to make hyperlipemia model rats) and the same diet plus oral administration of 1 [(50 mg/kg (being equal to 300—400 mg dried whole plant of A. formosanus) and 100 mg/kg (being equal to 600—800 mg plant)] for 6 days, respectively. The body weight increase was seen in the normal group and control group, although no difference was observed between these two groups. However, weight was suppressed in 1 administered groups. Especially, the 100 mg/kg 1 administered group exhibited significantly lower weight than the control group.

The liver weight of the control group was significantly higher than that of the normal group. However, the liver weight of both the 50 mg/kg 1 group and 100 mg/kg 1 group were significantly lower than that of normal group.

Table 1. Effect of Kinsenoside (mg/kg) on Body and Liver Weight in Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (p.o.) (mg/kg)</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>212.32±2.24</td>
<td>6.80±0.18</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>213.19±4.23</td>
<td>8.81±0.31</td>
</tr>
<tr>
<td>Kinsenoside</td>
<td>50</td>
<td>204.10±1.97</td>
<td>7.72±0.18**</td>
</tr>
<tr>
<td>Kinsenoside</td>
<td>100</td>
<td>202.18±2.39*</td>
<td>7.08±0.28**</td>
</tr>
</tbody>
</table>

***p<0.01 vs. normal group; **p<0.05, *p<0.01 vs. control group.

![Fig. 1. Effect of Kinsenoside on Triglyceride Level in Liver of Rats](Image)
was significantly lower than that of the control group. Figure 1 shows the value of TG in liver. TG, a neutral lipid, is a risk factor implicated in obesity and other diseases. The TG level in liver of the control group was significantly higher than in the normal group. The levels in the 50 mg/kg group and the 100 mg/kg group were significantly lower than the control group. When compared to TG concentration per liver protein, the same result was obtained. On the other hand, an epimer of 1, 3-(S)-3-β-D-glucopyranosylxbutanolide obtained from Goodyera spp. as the major constituent, trivially named goodyeroside A (2), was also investigated by the same assay. However, 2 had no effect for anti-hyperlipidosis. This structure-activity relationship in two epimers, 1 and 2 suggested that Goodyera species cannot be used instead of Anoectochilus species, and we believe it is a better practice to use only the original plant, and to avoid substitutes.

Table 2. Effect of Kinsenoside (1) on Liver Weight after Aurothioglucose-Induced Obese Mice for 6 Weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>n</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>18</td>
<td>1.148±0.027</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>20</td>
<td>1.448±0.031**</td>
</tr>
<tr>
<td>Kinsenoside 0.1%</td>
<td>in HFD</td>
<td>20</td>
<td>1.230±0.055**</td>
</tr>
<tr>
<td>Kinsenoside 0.2%</td>
<td>in HFD</td>
<td>20</td>
<td>1.195±0.051**</td>
</tr>
</tbody>
</table>

## p<0.01 vs. normal group; ** p<0.01 vs. control group.

The effect of 1 on anti-hyperlipidosis was also examined by using aurothioglucose-induced obese mouse. There was no significant difference in food consumption between normal and the control or 1 treated groups. This is in agreement with the literature. Diet amounts during the period were
3.93 g/d for the normal mouse, 4.18 g/d for the control mouse, 4.23 g/d and 4.09 g/d for \( \text{I} \) treated mouse. Figure 2 shows the change in body weight for normal mice, and when aurothioglucose-induced obese mice that were fed the HFD (as control) or the same diet containing 0.1% or 0.2% \( \text{I} \) for 6 weeks, respectively. Rapid weight increase was observed in the control group, while it was suppressed in the \( \text{I} \) treated groups. Especially, the 0.2% \( \text{I} \) administered group exhibited significantly lower weight than the control group. The weight increases of liver and uterine fat-pads were also observed in the control group, while in the \( \text{I} \) treated groups, they were significantly decreased (Table 2 and Fig. 3). The livers in the control group were considerably swollen, and the color and luster were those of fatty liver. These phenomena were not observed in the \( \text{I} \) treated groups. Figure 4 shows the variation in TG in liver when the obese mice were fed the HFD as the control, and the diet with \( \text{I} \) after 6 weeks. The liver TG level, which is closely associated with a fatty liver, was significantly reduced by \( \text{I} \), and in the 0.2% \( \text{I} \) treated group the level was reduced to that of the normal group, showing inhibition of lipid deposition. Figure 5 shows the typical histological views of the liver in the mice being fed the HFD and the diet with \( \text{I} \) (0.1% and 0.2%, respectively) after 6 weeks. A great deal of accumulated fatty drops was observed in the control group, while it was significantly decreased in the \( \text{I} \) administered groups. It is believed that the body weight increase was suppressed by the improvement of lipid-metabolism.

The findings of the present study indicate \( \text{I} \) may be useful for the treatment for hyperliposis. This will be further explored to clarify the mechanism of this effect.

REFERENCES