# Isolation of Pseudoprototimosaponin AIII from Rhizomes of Anemarrhena asphodeloides and Its Hypoglycemic Activity in Streptozotocin-Induced Diabetic Mice

Noboru Nakashima, Ikuko Kimura,
Masayasu Kimura, and Hiromichi Matsuura

## More About This Article

The permalink http://dx.doi.org/10.1021/np50093a006 provides access to:

- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

# ISOLATION OF PSEUDOPROTOTIMOSAPONIN AIII FROM RHIZOMES OF *ANEMARRHENA ASPHODELOIDES* AND ITS HYPOGLYCEMIC ACTIVITY IN STREPTOZOTOCIN-INDUCED DIABETIC MICE

Noboru Nakashima, Ikuko Kimura,* Masayasu Kimura,

*Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences,
Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan*

and Hiromichi Matsuura

*Institute of OTC, Wakunaga Pharmaceutical Co. Ltd., Hiroshima, Japan*

ABSTRACT.—A hot-$H_2O$ extract of rhizomes of *Anemarrhena asphodeloides*, the Japanese sino-medicine "chimo," lowered the blood glucose level in alloxan-diabetic mice. Hypoglycemic activity-guided fractionation isolated a new glycoside, pseudoprototimosaponin AIII [1], which was compared with chemically known prototimosaponin AIII [2]. These compounds exhibited hypoglycemic effects in a dose-dependent manner in streptozotocin-diabetic mice but showed no effects on glucose uptake and insulin release, suggesting that the hypoglycemic mechanism may be due to inhibition of hepatic gluconeogenesis and/or glycogenolysis.

The rhizomes of *Anemarrhena asphodeloides* Bunge (Liliaceae) are prescribed as a crude drug in traditional Chinese medicine. Known as "zhimu," it is prescribed as a remedy for diabetes in various blended preparations (Kanpō Hōzai). One of these, Byakko-ka-Ninjin-tō (Bai-Hu-Jia-Ren-Shen-Thang), consists of five crude drugs: roots of *Glycyrrhiza glabra* (kanzō), rhizomes of *A. asphodeloides* (chimo), roots of *Panax ginseng* (ninjin), gypsum fiber (sekkō), and rice (kōbei). It is effective in lowering the blood glucose level in alloxan-diabetic mice, and its hypoglycemic effect appears to be mainly dependent on the combination of "chimo" and ginseng root, which can exhibit a hypoglycemic action by itself (1). The $H_2O$ extract of "chimo" is reported to reduce the blood glucose level in normal and alloxan-diabetic rabbits (2,3) and in alloxan- and anti-insulin serum-diabetic mice (4). Although saponin (5,6) and non-saponin (7,8) compounds of "chimo" have been reported, only four glycans, anemerans A, B, C, and D, isolated from an aqueous MeOH/$H_2O$ extract of "chimo," show hypoglycemic effects in normal and alloxan-diabetic mice (9).

In an attempt to obtain new effective compounds to reduce blood glucose levels in human diabetics, the present paper deals with the isolation and purification of a hypoglycemic glycoside from the rhizomes of *A. asphodeloides*.

## EXPERIMENTAL

BLOOD GLUCOSE LEVELS.—Streptozotocin (STZ)-diabetic, alloxan-diabetic, and normal age-matched mice were used. Male ddY mice, 6 weeks (27–34 g) or 4 weeks of age (18–23 g) were injected with a 90 mg/kg bolus of alloxan monohydrate (Nacalai, Kyoto, Japan) or 150 mg/kg of streptozotocin (STZ) (Sigma, St. Louis, MO) in saline via the tail vein, and used 1 week (31–38 g) or 4 weeks (34–42 g) after injection. The blood glucose levels of these mice were uniformly more than 150 mg/dl after fasting for 13–14 h before each experiment. Non-diabetic ddY male mice were 7–8 weeks of age (33–41 g). The blood glucose levels were measured by the glucose oxidase method on a glucose analyzer (Type II, Beckman, CA). The hypoglycemic activity in alloxan- and STZ-diabetic mice was evaluated as previously described (10).

GLUCOSE UPTAKE.—Male ddY mice, 5–6 weeks of age (23–28 g), were fasted for 12 h before each experiment. A segment of isolated diaphragm was prepared as previously reported (11), and incubated for 1 h at 37° in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4), 0.5% bovine serum albumin (BSA, Fraction V, Sigma) and 8.3 mM [U-$^{14}$C]-D-glucose (14.8 kBq/ml, 9.99 GBq/mmol, Amersham, Japan). The segment was washed 4 times with ice-cold KRB buffer containing 0.5% BSA and 8.3 mM D-glucose, blotted with filter paper, and then dissolved in 1 N NaOH. Radioactivity was determined with a liquid scintillation spectrometer (300C, Packard). The protein concentration of aliquots was determined by the method described by Lowry *et al.* (12) with BSA as the standard.

INSULIN RELEASE FROM PERFUSED PANCREAS.—Male Wistar rats, 6–7 weeks of age (180–300 g), were used. Rat pancreas was prepared as previously detailed (13). Perfusion was carried out through the celiac artery with a basal medium of KRB buffer (pH 7.4) containing 0.5% BSA, 2% dextran (T-70, Pharmacia), and 2.8 mM D-glucose (14) saturated with a gas mixture of 95% $O_2$ and 5% $CO_2$.

The concentration of insulin collected from a portal vein catheter was determined using the radioimmunoassay previously reported (15).

All pharmacological data were statistically analyzed by Student's unpaired $t$-test.

ISOLATION OF PSEUDOPROTOTIMOSAPONIN AIII {1} AND PROTOTIMOSAPONIN AIII {2}.—The crude ground rhizomes (500 g, 60 mesh powder, purchased from Nihon Funmatsu, Osaka, Japan) were twice extracted with hot $H_2O$ (2 liters, 100°). After removal of the solvent by evaporation, the $H_2O$ extract (190 g, 39% yield) was defatted with $Et_2O$ (1 liter) and then $CHCl_3$ (1 liter), and dialyzed 4 times against 2 liters of $H_2O$ each time (Vinsking tube, molecular cut-off 8000, Japan). The dialyzed fraction (An-O, 26% yield) was applied to a column of highly porous polymer, MCI gel CHP20P (Mitsubishi Chemical Ind., Tokyo, Japan), to give three fractions (An-O-a, 21% yield; An-O-b, 0.34% yield; and An-O-c, 2.5% yield) by a stepwise elution of $H_2O$, 10% MeOH, and MeOH, respectively. The fraction eluted with MeOH was separated by repeated chromatography (MCI gel CHP20P, 50% MeOH and then MeOH). The MeOH-eluted fraction (An-O-c-2, 1.4% yield) was chromatographed on Si gel [CHCl₃-MeOH-$H_2O$ (7:3:0.5)] to give two glycoside fractions, timosaponin AIII {3} (0.17% yield) and a less polar fraction. The latter fraction was further separated by repeated chromatography (MCI gel CHP20P, 65% MeOH) to give a new saponin, compound 1 (0.04% yield), and a mixture of compound 2 and its methyl form. Compound 2 was proved to be identical with prototimosaponin AIII, which has been reported by Nagumo et al. (16). The mixture was heated with 30% aqueous $Me_2CO$ at 100° for 4 h, then concentrated to dryness in vacuo to obtain 2 (0.23% yield). Compound 2 (0.12 g) was dissolved in HOAc (3 ml) and heated at 90° for 20 min (17). The reaction mixture was concentrated by blowing $N_2$ gas over it at room temperature, and the residue was chromatographed on Si gel [CHCl₃-MeOH-$H_2O$ (7:3:0.5)] to give 1 (75 mg).

The nmr spectra were taken on a JEOL JNM GX-270 spectrometer in pyridine-$d_5$ using TMS as an internal standard. The fdms spectra were recorded on a JEOL JMS DX-300 mass spectrometer. Identification of the monosaccharides that resulted from acid hydrolysis was carried out as described in a previous paper (18).

A new glycoside 1 produced galactose and glucose by acid hydrolysis. The $^1H$- and $^{13}C$-nmr spectrum of 1 indicated the presence of three monosaccharide units. The fdms spectrum of 1 exhibited molecular cluster ions at $m/z$ 925 [M + Na]$^+$ and 902 [M]$^+$ and fragment peaks corresponding to a stepwise elimination of sugar units at $m/z$ 740 [M − hexosyl]$^+$ and 578 [740 − hexosyl]$^+$. The $^{13}C$-nmr spectra of 1 and 2 showed signals due to the sugar moiety at the same positions. The $^{13}C$-nmr spectrum of 1 indicated the



1                                        2

3
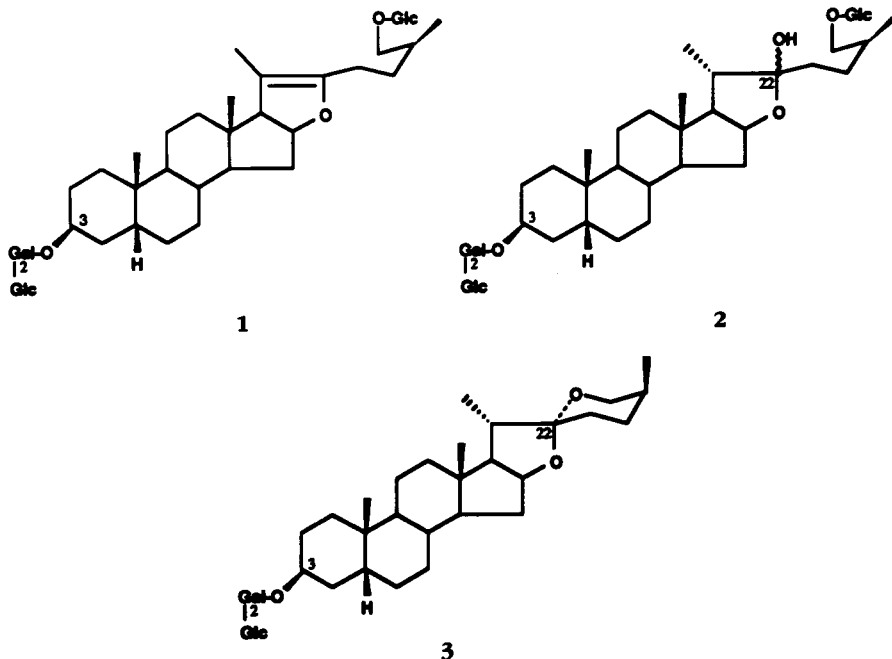
presence of C-20 ($\delta$ 103.6) and C-22 ($\delta$ 152.4) in the aglycone moiety of **1** compared with those of **2** and pseudoprotodiosgenin (19). Compound **1** was named pseudoprototimosaponin AIII.

*Pseudoprototimosaponin AIII* [**1**].—$C_{45}H_{74}O_{18} \cdot H_2O$; colorless microcrystals (from MeOH); mp 232–235°, $[\alpha]^{26}D - 15.8°$ ($c = 0.75$, pyridine); found C 58.71, H 8.24 ($C_{45}H_{74}O_{18} \cdot H_2O$ requires C 58.68, H 8.32); $^1H$ nmr $\delta$ 0.70 (3H, s), 1.00 (3H, s), 1.05 (3H, d, $J = 6.2$ Hz), 1.64 (3H, s), 4.85 (1H, d, $J = 7.7$ Hz), 4.94 (1H, d, $J = 7.7$ Hz), 5.31 (1H, d, $J = 7.7$ Hz); $^{13}C$ nmr $\delta$ 31.0, 26.9, 75.2[a], 31.0, 37.0, 27.0, 26.9, 35.2, 40.2, 35.2, 21.4, 40.2, 43.9, 54.8, 31.4, 84.6, 64.7, 17.2, 24.0, 103.6, 11.8, 152.4, 34.5, 23.7, 33.7, 75.2, 14.4 (aglycone C-1–C-27); 102.6, 81.9, 76.9[b], 69.9, 76.6[b], 62.2 (3-0-galactose C-1–C-6); 106.1, 75.6[a], 78.6[c], 71.7, 78.5[c], 62.9 (3-0-gal-glucose C-1–C-6); 105.2, 75.6[a], 78.4[c], 71.7, 78.1[c], 62.9 (26-0-glucose C-1–C-6) (values with same superscript may be reversed); fdms $m/z$ [M + Na]$^+$ 925, [M]$^+$ 902, [902 – hexosyl]$^+$ 740, [740 – hexosyl]$^+$ 578.

REFERENCE DRUGS.—Glibenclamide (Yamanouchi Pharmaceutical), $^{125}I$-insulin (Dainabot), and insulin (Sigma) were used.

## RESULTS AND DISCUSSION

Compounds with hypoglycemic activity were isolated from *A. asphodeloides* rhizomes and identified by pharmacological activity-guided fractionation (Table 1). As the crude glycoside fraction in the hot $H_2O$ extract was hypoglycemic, the fractions were separated by a combination of Si gel and reversed-phase chromatography on a highly porous polymer to give a new glycoside, pseudoprototimosaponin AIII [**1**], $C_{45}H_{74}O_{18} \cdot H_2O$, and the chemically known prototimosaponin AIII [**2**], $C_{45}H_{76}O_{19} \cdot 2H_2O$, in yields of 0.04% and 0.23%.

The hypoglycemic effects were assayed by using fasted alloxan-diabetic mice injected i.p. with 50 mg/kg of crude extracts (An-E, An-I, An-O, An-O-a, An-O-b, An-O-c, An-O-c-1, and An-O-c-2) and compounds **1–3**. The original hot $H_2O$ extract (100 mg/kg, ip) showed significant hypoglycemic activity, $84.2 \pm 4.8\%$. The control value (injected i.p. with saline) was $15.7 \pm 2.9\%$ ($N = 10$). Based on the activity-guided fractionations, the dialyzed fraction (An-O), the MeOH fraction (An-O-c), 50% MeOH fraction (An-O-c-1), and the MeOH eluate fraction (An-O-c-2) demonstrated definite hypoglycemic activity.

TABLE 1.    Hypoglycemic Effects of Crude Extracts and Compounds **1–3** from Rhizomes of *Anemarrhena asphodeloides* on Blood Glucose Level in Alloxan-Diabetic Mice.

| Fraction | Dose (mg/kg, ip) | Fall of blood glucose (%) | $n$ |
|---|---|---|---|
| An-E . . . . . . . . . . . . | 100 | $84.2 \pm 4.8$[a] | 10 |
| An-I . . . . . . . . . . . . | 50 | $60.9 \pm 4.9$ | 8 |
| An-O . . . . . . . . . . | 50 | $69.4 \pm 7.7$ | 7 |
| An-O-a . . . . . . . . . | 50 | $34.0 \pm 7.4$ | 6 |
| An-O-b . . . . . . . . | 50 | $45.3 \pm 10.5$ | 6 |
| An-O-c . . . . . . . . . | 50 | $67.1 \pm 7.3$ | 5 |
| An-O-c-1 . . . . . . . . | 50 | $66.7 \pm 10.8$ | 6 |
| An-O-c-2 . . . . . . . . | 50 | $72.1 \pm 10.3$ | 5 |
| **1** . . . . . . . . . . . | 50 | $82.8 \pm 3.4$ | 8 |
| **2** . . . . . . . . . . . | 50 | $73.4 \pm 6.6$ | 5 |
| **3** . . . . . . . . . . . | 50 | $64.5 \pm 9.4$ | 5 |
| Control (saline) . . . . . . | — | $15.7 \pm 2.9$ | 10 |

[a]Values are means $\pm$ SEM. Percent fall = (A − B)/(A − 85) × 100, where A and B are blood glucose level just before and 6 h after injection, respectively.

Compounds **1** and **2** (50 mg/kg, ip) derived from An-O-c-2 showed the highest hypoglycemic activity with 82.8 ± 3.4% and 73.4 ± 6.6% decreases in blood glucose, respectively. These components did not influence the blood glucose level in fasted normal ddY-strain mice (data not shown).

In STZ-diabetic mice, the oral hypoglycemic sulfonylurea glibenclamide lowered the blood glucose level in a dose-dependent manner. The $ID_{50}$ with 95% confidence limits was 0.33 (0.21–0.53) mg/kg. Compounds **1** and **2** also lowered the blood glucose level in a dose-dependent manner, and the $ID_{50}$'s with 95% confidence limits were 4.33 (3.05–6.13) and 6.62 (5.61–7.81) mg/kg, respectively (Figure 1).

The hypoglycemic effect of the new glycoside **1** was slightly more potent than **2** in STZ- and alloxan-diabetic mice. Compound **3** demonstrated a weak hypoglycemic effect compared with **1** and **2**.

To investigate the mechanisms of hypoglycemic action by active components, glucose uptake into isolated diaphragm muscles of mice and insulin release from isolated perfused rat pancreas were measured. Insulin-stimulated glucose uptake in the presence of 8.3 mM glucose significantly increased in a concentration-dependent manner (1.67–167 nM). The absolute amount of **1** or **2** (1 mg/ml) that stimulated glucose uptake was not significantly different from the corresponding values in controls (8.3 mM glucose alone, $n = 9$–18, data not shown). Compound **2** (1 mg/ml) slightly potentiated the glucose (16.7 mM)-induced increase of immunoreactive insulin (IRI) level in the perfused rat pancreas. Compound **2**-stimulated IRI release for 10 min (59.7 ± 7.8 ng/ml) was not significantly different from that of control (16.7 mM glucose alone, 50.7 ± 9.9 ng/ml, $n = 7$).
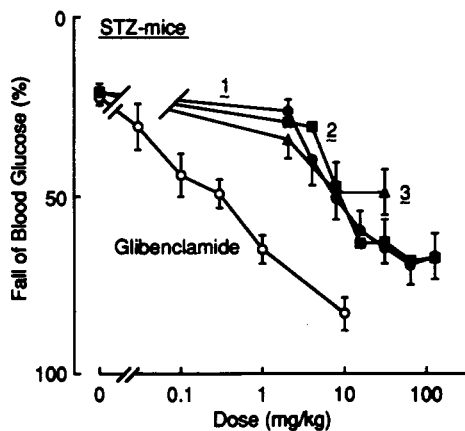


FIGURE 1.    Dose-inhibition curves of compounds **1** (●), **2** (■), **3** (▲) and glibenclamide (○) for percent lowering of blood glucose capacity in streptozotocin-diabetic mice. Each point represents the mean ± SEM of 7–12 mice. The falling of blood glucose level was calculated as the percentage of one value (after − before) against another (after − 85), where after and before represent a blood glucose level after and before test compounds in diabetic mice, and 85 represents the mean blood glucose level in normal (non-diabetic) mice.

These results suggest that the hypoglycemic action induced by the active compounds of *A. asphodeloides* may inhibit the conversion of amino acids to glucose (gluconeogenesis) in the liver or inhibit glycogenolysis.

A new glycoside, pseudoprototimosaponin AIII [1], was isolated and identified from a hot $H_2O$ extract of *A. asphodeloides* rhizomes. Compound 1 and a chemically known component, prototimosaponin AIII [2], were found to lower the blood glucose levels in a dose-dependent manner in fasted alloxan-diabetic or streptozotocin-diabetic mice. Compounds 1 and 2 did not influence blood glucose level in fasted normal ddY mice. This observation seems to reflect the characteristic of Chinese traditional medicines that the therapeutic effect is prominent only in patients with disease.

Though it has been reported that a hot $H_2O$ extract of "chimo" enhances glucose uptake in diaphragm and adipose tissues (20), the present hypoglycemic components, 1 and 2, did not enhance glucose uptake into the diaphragm muscles of normal mice. This shows that the component of "chimo" enhancing glucose uptake into diaphragm may be contained in the other crude extracts. In addition, 1 and 2 only slightly potentiated glucose-induced insulin release from perfused rat pancreas. The mechanism responsible for lowering the blood glucose level would appear to be different from that of the sulfonylureas (21–23), and evidence suggests that the mechanism may be to inhibit glycogenolysis or gluconeogenesis.

Oral hypoglycemic sulfonylureas can lower blood glucose levels in animals with or without diabetes. "Chimo" components produced a potent hypoglycemic effect in diabetic mice but not in non-diabetic mice. Therefore, the hypoglycemic effect of "chimo" components must be different from that of the sulfonylureas.

A hypoglycemic ginseng component has been found to release insulin from perfused rat pancreas (24,25). Therefore the mechanism of the hypoglycemic action of *A. asphodeloides* would appear to be different from that of ginseng.

In conclusion, the present study provides evidence that the hypoglycemic mechanism of the components derived from "chimo" may be to inhibit the conversion of amino acids to glucose (gluconeogenesis) in the liver or inhibit glucogenolysis. This finding may be useful in understanding the combined effect of Byakko-ka-Ninjinto, Kampo Hozai.

## LITERATURE CITED

1.  M. Kimura, *Proc. Symp. Wakan-Yaku*, **1**, 14 (1967).
2.  H.-K. Bin, *Nippon Yakubutsugaku Zasshi*, **11**, 22 (1930).
3.  A. Koda, H. Yoshida, H. Nagai, and M. Mizuno, *Nippon Yakurigaku Zasshi*, **67**, 223 (1971).
4.  M. Kimura, *Nippon Rinsho*, **25**, 2841 (1967).
5.  T. Kawasaki and T. Yamaguchi, *Chem. Pharm. Bull.*, **11**, 1221 (1963).
6.  A. Tanba, O. Takeda, M. Ishimaru, Y. Nakamoto, K. Yamazaki, H. Kanda, H. Nishio, T. Segawa, K. Fujimura, and J. Kuramoto, *Yakugaku Zasshi*, **108**, 555 (1988).
7.  N. Morita, M. Shimizu, and M. Fukuda, *Yakugaku Zasshi*, **85**, 374 (1965).
8.  T. Nikaido, T. Ohmoto, H. Noguchi, T. Kinoshita, H. Saitoh, and U. Sankawa, *Planta Med.*, **43**, 18 (1981).
9.  M. Takahashi, C. Konno, and H. Hikino, *Planta Med.*, **51**, 100 (1985).
10. M. Kimura, I. Waki, O. Tanaka, Y. Nagai, and S. Shibata, *J. Pharmacobio-Dyn.*, **4**, 402 (1981).
11. M. Kimura, T. Naitoh, S. Kobayashi, and I. Kimura, *J. Pharmacobio-Dyn.*, **15**, 17 (1992).
12. O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
13. M. Kimura, N. Nakashima, and I. Kimura, *Jpn. J. Pharmacol.*, **52**, 579 (1990).
14. A. Niki, H. Niki, and I. Miwa, *Endocrinology*, **105**, 1051 (1979).
15. M. Kimura, J. Suzuki, and K. Amemiya, *Endocrinol. Jpn.*, **26**, 185 (1978).
16. S. Nagumo, S. Kishi, T. Inoue, and M. Nagai, *Yakugaku Zasshi*, **111**, 306 (1991).
17. R. Tschesche and G. Wulff, in: "Fortschritte der Chemie Organischer Naturstoffe." Ed. by W. Hers, H. Grisebach, and G.W. Kirby, Springer-Verlag, Wien, 1973, Vol. 30, p. 486.
18. H. Matsuura, T. Ushiroguchi, Y. Itakura, N. Hayashi, and T. Fuwa, *Chem. Pharm. Bull.*, **36**, 3659 (1988).

19.  Y. Hirai, S. Sanada, Y. Ida, and J. Shoji, *Chem. Pharm. Bull.*, **34**, 82 (1986).
20.  E. Nagata and M. Kimura, *Japana Centra Revuo Medicina*, **276**, 346 (1971).
21.  B. Hellman and I.B. Taljedal, in: "Handbook of Experimental Pharmacology." Ed. by A.V. Has-selblatt and F.V. Bruchhausen, Springer Verlag, Berlin, 1975, Vol. 32, p. 175.
22.  O.G. Kolterman, *Diabetes Metab. Rev.*, **3**, 399 (1987).
23.  A.E. Boid III, *Diabetes*, **37**, 847 (1988).
24.  M. Kimura, I. Waki, T. Chujo, T. Kikuchi, C. Hiyama, K. Yamazaki, and O. Tanaka, *J. Pharmacobio-Dyn.*, **4**, 410 (1981).
25.  T. Zhang, M. Hoshino, K. Iguchi, J. Ishikawa, T. Mochizuki, N. Takatsuka, C. Yanaihara, M. Yokota, G.H. Greeley, and N. Yanaihara, *Biomed. Res.*, **11**, 49 (1991).