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A Novel Insulin-releasing Substance, Phanoside, from the Plant *Gynostemma pentaphyllum**

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Extracts from *Gynostemma pentaphyllum* Makino (Cucurbitaceae), a Southeast Asian herb, has been reported to affect numerous activities resulting in antitumor, cholesterol-lowering, immunopotentiating, antioxidant, and hypoglycemic effects. We have isolated one active compound by ethanol extraction, distribution in *n*-butyl alcohol/water, solid phase extraction/separation, and several rounds of reverse phase high pressure liquid chromatography. We have shown by NMR and mass spectrometry that this active compound is a novel saponin, a gypenoside, which we have named phanoside (21-,23-epoxy-,3β-,20-,21-trihydroxydammar-24-ene-3-*O*-([α-D-rhamnopyranosyl(1→2)]-[β-D-glycopyranosyl(1→3)]-β-D-lyxopyranoside)), with a molecular mass of 914.5 Da. Phanoside is a dammarane-type saponin, and four stereoisomers differing in configurations at positions 21 and 23 were identified, each of which were found to stimulate insulin release from isolated rat pancreatic islets. We have also found that the stereoisomers are interconvertible. Dose-dependent insulin-releasing activities at 3.3 and 16.7 mM glucose levels were determined for the racemic mixture containing all four stereoisomers. Phanoside at 500 μM stimulates insulin release *in vitro* 10-fold at 3.3 mM glucose and potentiates the release almost 4-fold at 16.7 mM glucose. At these glucose levels, 2 μM glibenclamide stimulates insulin release only 2-fold. Interestingly, β-cell sensitivity to phanoside is higher at 16.7 mM than at 3.3 mM glucose, although insulin responses were significantly increased by phanoside below 125 μM only at high glucose levels. Also when given orally to rats, phanoside (40 and 80 mg/ml) improved glucose tolerance and enhanced plasma insulin levels at hyperglycemia.

Plant extracts have been used in traditional medicine since ancient times for treatment of various diseases including a disease similar to type 2 diabetes. Several hundred plants are known to have antidiabetic properties and a large number of compounds from plant extracts have been reported to have beneficial effects for treatment of diabetes (1, 2). The phytochemicals with hypoglycemic activity can be classified as alkaloids, flavonoids, glycosides/steroids/terpenoids, polysaccharides, proteins, saponins, and miscellaneous other compounds (3). A large number of crude plant extracts and purified substances from plants have been tested in clinical trials for treatment of diabetes (4, 5). However, effects with crude extracts remain obscure if the active principles in the extracts are not characterized.

Gynostemma pentaphyllum Makino (Cucurbitaceae) is a herb that is widely used in Southeast Asian folk medicine and is gaining popularity also in other countries as an herbal medicine. *G. pentaphyllum* extracts reportedly have many effects, such as lowered cholesterol, immunopotentiation, as well as antitumor, antioxidant, and hypoglycemic effects (6–9). Although there are substances in *G. pentaphyllum* extract that belong to different compound classes, a large group of substances in these extracts is saponins. Saponins isolated from *G. pentaphyllum* are also known as gypenosides. Currently, there are about 90 different gypenosides isolated and characterized. Several representatives of gypenosides are also found in extracts of other plants, such as species of ginseng (10, 11). Ginseng extracts have been widely studied and, similar to *G. pentaphyllum* extracts, exhibit antidiabetic effects via hypoglycemic properties (12).

Although several types of compounds have hypoglycemic properties, the mechanisms for achieving this effect are different. There is a growing body of evidence that saponins, although active in several systems, are responsible for modulation of glucose disposal and insulin release (13). For example, crude saponin fractions of *G. pentaphyllum* extracts have been shown to have hypoglycemic and hypolipidemic effects in rats (8). However, the active principles responsible for hypoglycemia are not known.

In the present study, we demonstrated that ethanol extracts of *G. pentaphyllum* strongly stimulate insulin secretion from isolated rat pancreatic islets. We have isolated a novel insulin-releasing substance from this extract, determined the structure of four active isomers by NMR and mass spectrometry, and characterized its effects on insulin release both *in vitro* and *in vivo* in the rat.

MATERIALS AND METHODS

Extraction—An ethanol extract of *G. pentaphyllum* was supplied by the Institute of Material Medica, Hanoi, Vietnam. The parts of *G. pentaphyllum* were dried in sunlight before pounding into powder. The powder was extracted with 70% ethanol in a Soxhlet extractor for 4 cycles. The ethanol was evaporated under reduced pressure to give a crude extract. 15 g of the crude extract was dissolved in 200 ml of water and centrifuged for 20 min at 3000 × *g*. The supernatant was collected, diluted with 200 ml of water, and extracted with 400 ml of *n*-butyl alcohol with a magnetic stirrer for 10 min. Water and *n*-butyl alcohol (Merck) were allowed to separate for 30 min in a separating funnel. The

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n-butyl alcohol phase was collected, and the solvent was evaporated until most of the *n*-butyl alcohol had been removed. The residue from the *n*-butyl alcohol extraction was diluted with 1000 ml of water. Portions of 500 ml were mixed with 200 g of Amberlite XAD-16 HP (Supelco, Bellefonte, PA) and stirred for 30 min. The unabsorbed material was removed by filtration, and the Amberlite was collected and washed with water. The adsorbed material was eluted from the Amberlite with aqueous acetonitrile (Riedel-de Haën) at different concentrations (25, 60, and 80% acetonitrile).

Chromatography—The subsequent two high pressure liquid chromatography (HPLC)[1] steps were carried out on an ÄKTAexplorer and the last on an ÄKTApurifier HPLC system (Amersham Biosciences AB, Uppsala, Sweden). The bioactivity was located in the 60% acetonitrile fraction (total volume 500 ml). Portions (150 ml) of this fraction were diluted with 400 ml of water, applied to a Vydac C18 column (22 × 250 mm; 218TP152022), and eluted with a linear gradient of 20–57.5% B (eluent A, water; eluent B, 95% acetonitrile) in 4.5 column volumes (CV) at a flow rate of 25 ml/min. The fractions were analyzed for insulin-releasing activity. In the second chromatographic step, the active fraction from the first reverse phase HPLC separation was rechromatographed on a SOURCE 15RPC 16/90 column in a linear gradient of 32–40% B in 9.33 CV at a flow rate of 8 ml/min.

In the last chromatographic step, the active fraction was purified on an ÄKTApurifier HPLC system using a C18 column (ODS-AP, 10 × 100 mm, 5 μm, YMC, Germany) and the same solvent system described above employing a gradient of 40–41% B in 4 CV at 6 ml/min. The structure of the pure component was elucidated using ^{1}H-^{1}H and ^{1}H-^{13}C two-dimensional NMR spectroscopy and mass spectrometry.

NMR—NMR spectra were recorded with a Bruker DMX-600 spectrometer equipped with a cryoprobe in Me_2SO-d_6 solution at 25 °C. Chemical shifts are reported in parts/million relative to residual solvent signal ($\delta(^1H)$, 2.50 parts/million; $\delta(^{13}C)$, 39.5 parts/million). Two-dimensional spectra recorded included DQF-COSY, ROESY, TOCSY, sensitivity-enhanced ^{13}C HSQC, and ^{13}C-^{1}H HMBC. Pulsed-field gradients were used for all ^{13}C correlation spectra. ^{13}C HMBC spectra were recorded with a coupling evolution delay for the generation of multiple-bond correlations set to 62.5 ms. All two-dimensional spectra were run with a data matrix of 4096 × 1024 points, giving τ_{2max} = 250 ms for the ^{1}H nucleus in the acquisition dimension and τ_{1max} = 200 ms for ^{1}H or τ_{1max} = 50 ms for ^{13}C for the indirect dimension. Prior to Fourier transform, the data matrix was zero-filled twice, and a multiplication by shifted sine-bell window function was applied. For ^{13}C-HMBC, the magnitude spectra were calculated.

Mass Spectrometry—Positive ion mass spectra were recorded on an Ettan ESI-ToF electrospray ionization time-of-flight mass spectrometer (Amersham Biosciences AB, Uppsala, Sweden) using a capillary exit voltage of 150 V. Samples of chromatographic fractions were infused at 10–50 μl/min via the electrospray ionization source using a syringe pump. For alkali metal adduct measurements, a 50-μl aliquot of the chromatographic fraction was mixed with 2 μl of 5 μM LiCl, NaCl, or KCl solution in water and sprayed into the mass spectrometer.

Insulin Release Assay—Male Wistar rats, weighing 250–280 g, were from a commercial breeder (B & K Universal, Sollentuna, Sweden). The animals were kept at 22 °C with free access to food and water before being sacrificed for isolation of their pancreatic islets. The islets were isolated by collagenase (Roche Applied Science) digestion of the pancreas, followed by hand picking (14). The islets were then cultured for 24 h in RPMI 1640 medium (Flow Lab Ltd., Irvine, UK) containing 11 mM glucose, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Batch incubations of the islets were performed in Krebs-Ringer bicarbonate (KRB) buffer containing 118.4 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ (equilibrated with 5% CO_2/95% O_2, pH 7.4), 0.2% bovine serum albumin, 10 mM HEPES, and 3.3 or 16.7 mM glucose. Following pre-incubation for 30 min in KRB at 3.3 mM glucose, batches of 3 islets were incubated for 60 min in KRB with 3.3 or 16.7 mM glucose and different concentrations of the crude ethanol extracts or the purified compounds. After the batch incubations, aliquots of the medium were analyzed for insulin content by radioimmunoassay (15).

Bioactivity of Phanoside in Vivo—Normal male Wistar rats (120 ± 32 g) and an intraperitoneal glucose tolerance test were used to study the *in vivo* effect of 10, 40, and 80 mg/kg phanoside. In rats fasted overnight, phanoside in Me_2SO (2.5%, 1 ml/100 g body weight) was given to the animals orally 90 min before the intraperitoneal injection of glucose (3 g/kg body weight). The control group was given only Me_2SO orally. Blood glucose concentrations were measured in blood taken after a small tail vein incision at 90 min and immediately before (0 min) and 15, 30, 60, and 120 min after the injection of glucose. To study the effect of phanoside (40 and 80 mg/kg body weight) on insulin secretion *in vivo*, vein plasma samples were drawn before administration of the compound (-90 min), before injection of glucose (0 min), and 30 min thereafter for measurement of immunoreactive insulin.

RESULTS

Purification—Purification of the novel compound was guided by monitoring the insulin release from the isolated rat pancreatic islets as described under "Materials and Methods." The crude ethanol extract of *G. pentaphyllum* was dissolved in water, and the solution was extracted with *n*-butyl alcohol. After separation of the water/*n*-butyl alcohol phases and lyophilization of an aliquot, the major part of the activity was detected in the *n*-butyl alcohol phase. In the following purification procedure, most of the *n*-butyl alcohol was removed under reduced pressure and the extract adsorbed to Amberlite XAD-16. The material trapped on the resin was eluted first with water and then sequentially with three different acetonitrile/water mixtures containing 25, 60, and 80% acetonitrile. The activity was found in the eluate containing 60% acetonitrile. This fraction was further purified by reverse phase HPLC. After addition of 3 volumes of water, the fraction was chromatographed on a preparative C18 column (Fig. 1*A*). The active fraction was further purified on a polyaromatic column (Fig. 1*B*). The material from the active fraction was used for characterization of the insulin-releasing activity described below. However, upon further purification using a high resolution C18 column, the material was resolved into four components, reflected as four *peaks* in Fig. 2*A*. All of these four peak fractions were found to be active in the insulin release assay at both 3.3 mM and 16.7 mM glucose (Fig. 2, *B* and *C*).

NMR Spectra—Structure elucidations of all four peak fractions (Fig. 2) were accomplished on the basis of two-dimensional NMR ^{1}H-^{1}H and ^{1}H-^{13}C spectra taken in Me_2SO-d_6 solution. According to the NMR spectra, all fractions consisted of two products, one of which was not stable and slowly transformed to the other component. A careful analysis of spectra taken at different times allowed determination of the structure of all components. Comparison of ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H DQF-COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC spectra led to the conclusion that the structure contains three different sugar moieties: (*a*) lyxose, to which both of the other two sugars are attached at position 2; (*b*) rhamnose; and (*c*) glucose, at position 3. In turn, lyxose is connected to the steroid-type triterpene skeleton through oxygen at position 3 of triterpene. The tetrahydrofurane ring is linked to the triterpene skeleton at position 17. The tetrahydrofurane moiety is unusual; it has an isopropylidene group and two hydroxyl groups attached (Fig. 3*A*). Chemical shifts, the multiplicity of signals, and the ^{13}C NMR data all indicated β-configuration at the anomeric positions for rings A and C ($^3J(1–2)$ = 3 and 6.8 Hz, respectively) but α-configuration for the rhamnosyl unit B ($^3J(1–2) \sim 1$ Hz). All three sugars are in pyranose form. The linkage sites and the sequence of saccharides and the aglycone were deduced from HMBC experiment. The HMBC experiment made it possible to confirm the stereochemistry of the steroid-type triterpene skeleton and the tetrahydrofurane moiety at position 17. In addition, the strong NOEs in ^{1}H-^{1}H ROESY spectra between 30-CH_3 and H-17α confirmed the β-orientation of the tetrahydrofurane ring.

[1] The abbreviations used are: HPLC, high pressure liquid chromatography; CV, column volumes; DQF, double quantum-filtered; ROESY, rotating Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond correlation; KRB, Krebs-Ringer bicarbonate; NOE, nuclear Overhauser effect; iNOS, inducible isoform of nitric-oxide synthase.

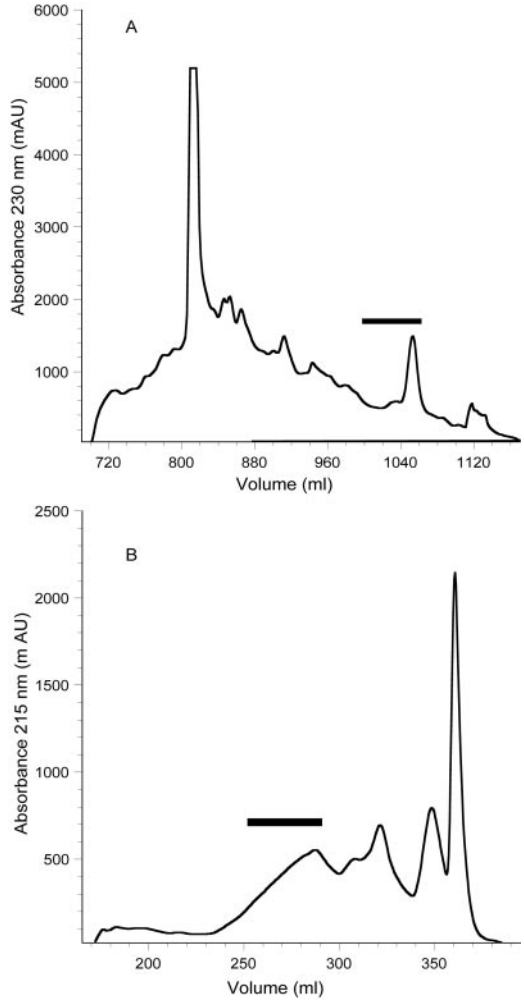

FIG. 1. **Purification of the insulin-releasing compound from *G. pentaphyllum*.** *A*, the fraction eluted with 60% acetonitrile after Amberlite XAD-16 extraction (150 ml) was diluted with 400 ml water and chromatographed on a Vydac C18 22 × 250 mm column with a linear gradient of 20–57.5% B in 4.5 CV using a solvent system A, water; B, 95% acetonitrile/water. The active fraction collected for further purification is indicated by the *bar*. *B*, the bioactive fraction was collected and rechromatographed on a SOURCE 16/90 column with a linear gradient of 32–40% B in 9.33 CV followed by 40–95% in 3 CV at 8 ml/min. The active fraction is indicated by the *bar*. This material was found to be pure and was used for characterization of its bioactivity. *AU*, absorbance units

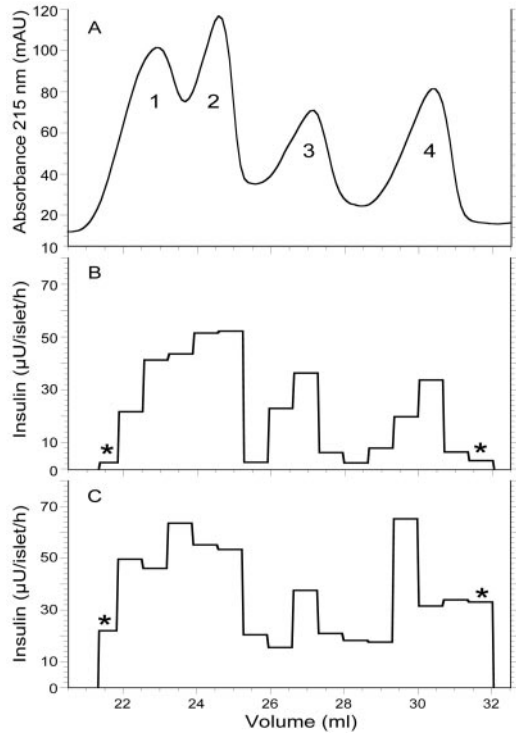

FIG. 2. **Separation of optical isomers of the active compound using a high resolution reverse phase high pressure liquid chromatography column.** *A*, the active fraction from the chromatography shown in Fig. 1*D* was rechromatographed on a YMC ODS-AP column (100 × 10 mm inner diameter, 5 μm, 300 Å). Gradient 40–41% B in 4 CV at 6 ml/min. The material resolved into four distinct peak fractions denoted as *1–4*. *B*, insulin-releasing activity of separate fractions from the chromatography above at 3.3 mM glucose. Control values obtained without the addition of phanoside are indicated with *asterisks*. An aliquot of each fraction was lyophilized and assayed for bioactivity. *C*, insulin-releasing activity of separate fractions from the chromatography above at 16.7 mM glucose. Control values obtained without the addition of phanoside are indicated with *asterisks*. An aliquot of each fraction was lyophilized and assayed for bioactivity. *U*, unit; *AU*, absorbance units

Inspection of spectra taken at different times after solubilization in Me_2SO revealed a slow interchange in time between the isomers in peak fractions 1 and 4, whereas isomers of peak fractions 2 and 3 constituted the second pair of the slowly interconverting mixture (Fig. 2*A*). Between fractions 1/4 and fractions 2/3, the interconversion rate was too slow to be noted under the experimental NMR conditions that were applicable. From the inspection of one-dimensional spectra, we immediately saw that for all isomers, there are no differences between the triterpene and sugar parts of the molecule and that the main differences had to be related to the orientation of the substituents in the tetrahydrofurane ring. The stereo-orientation of these substituents were established from ^1H-^1H ROESY spectra. In Fig. 3, *arrows* indicate the long-range NOE connectivities observed for all four components. Fractions 1/4 and 2/3, as shown in Fig. 2, differ by α/β orientation of the isopropylidene substituent relative to the steroid ring. For fractions 2/3, the isopropylidene groups have β-orientation, but for fractions 1/4, that group is located α relative to the steroid ring. The long range NOEs made it possible to deduce that OH groups at positions 20 and 21 for the stable, more populated, isomers are located at opposite sides of the 5-membered ring, but at the same side for the unstable, less populated, isomers at equilibrium conditions. Consequently, the *cis*-orientation of OH substituents in the ring markedly destabilized the structure. The unchanged β-stereo-orientation of OH-20 relative to the steroid ring in all isomers was evidenced by strong NOEs between H-12β and H-21; OH-21 and from OH-20 to H-13. This conclusion was supported by negative evidence. No NOEs from OH-20 to H-22α could be observed in any case.

Mass Spectrometry—In the mass spectrometric analysis of phanoside, a very weak signal was detected for the protonated singly charged compound $[M + H]^+$, (*m/z* = 915.501, calculated value 915.532) (Fig. 4, *A* and *B*). Instead, several intense characteristic peaks for phanoside adducts and its fragments were observed at *m/z* 937.519 (corresponding to $[M + Na]^+$); 897.499 ($[M - H_2O + H]^+$); 879.488 ($[M - 2H_2O + H]^+$); 457.356 ($[aglycone]^+$, calculated value 457.368); 439.356 ($[aglycone - H_2O]^+$, calculated value 439.358), and 421.343 ($[aglycone - 2H_2O]^+$, calculated value 421.347) in addition to several unidentified fragment peaks. The fragmentation occurs at the capillary exit area of the mass spectrometer.

It is known that saponins, being neutral molecules, do not readily ionize by proton attachment. In contrast, they tend to yield relatively stable complexes with alkali metals, which can then be detected by mass spectrometry. Therefore this property was utilized to determine the molecular mass of phanoside. The

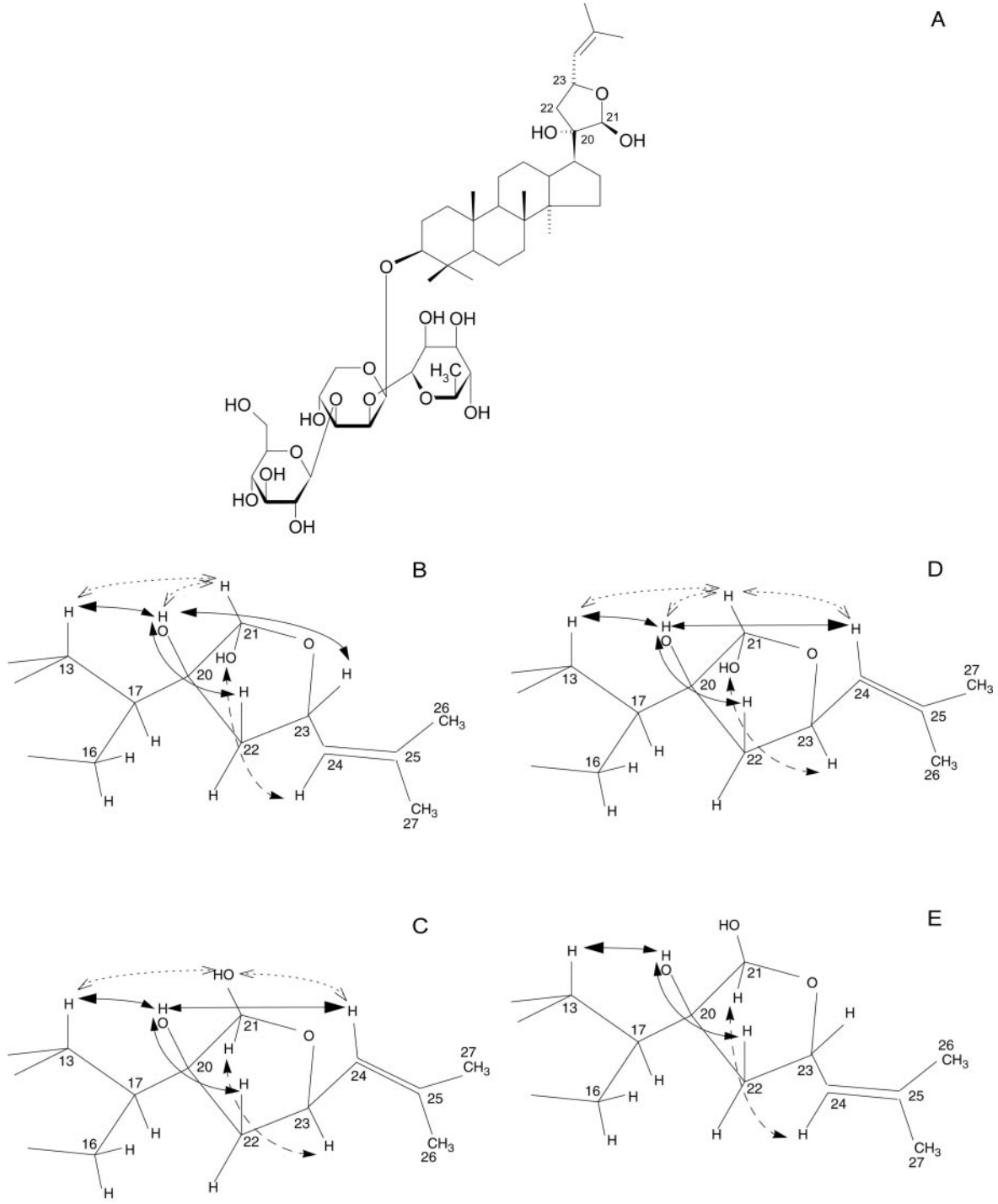

FIG. 3. **Structural analysis of the active compound.** *A*, the structure of the isolated compound. The *numbering* used is indicated. *B–E*, long range NOEs identified within the tetrahydrofurane moiety defining the stereo-orientation of the substituents relative to the five-membered ring. The NOE-connected protons are indicated by *arrows*. Fraction 1, stable isomer (*B*); fraction 3, unstable isomer (*C*); fraction 2, stable isomer (*D*); fraction 4, unstable isomer (*E*). Fraction numbers refer to those in Fig. 2*A*.

compound solution from chromatographic fractions containing water and acetonitrile was mixed with different alkali metal chloride solutions (Na^+, K^+, and Li^+) and the mass spectra recorded (Fig. 4, *C* and *D*). In the spectrum obtained with Na^+, the signal amplitude increased about 10 times, and the fragmentation peaks observed without metal ion addition disappeared (Fig. 4*C*). A similar mass spectrum was obtained with K^+, and the metal adduct peak shifted according to the mass difference between Na^+ and K^+ (Fig. 4*D*). A similar experiment was made with Li^+ (data not shown). In these experiments, the following mass values were obtained: $[M + Na]^+ = 937.545$ (calculated value 937.514); $[M + K]^+ = 953.516$ (calculated value 953.488); $[M + Li]^+ = 921.558$ (calculated value 921.540, mass spectrum not shown). The experimental mass values of the metal adducts of intact phanoside and its fragment ions are in excellent agreement with the calculated values and thus confirm the structure of phanoside determined by NMR.

Insulin Release from Isolated Pancreatic Islets—An ethanol extract of *G. pentaphyllum* increased insulin secretion from isolated rat islets at both basal (3.3 mM) and stimulating (16.7 mM) glucose concentrations. The activity depended on the concentration of the extract. Thus, 2 and 4 mg/ml (but not 1 mg/ml)

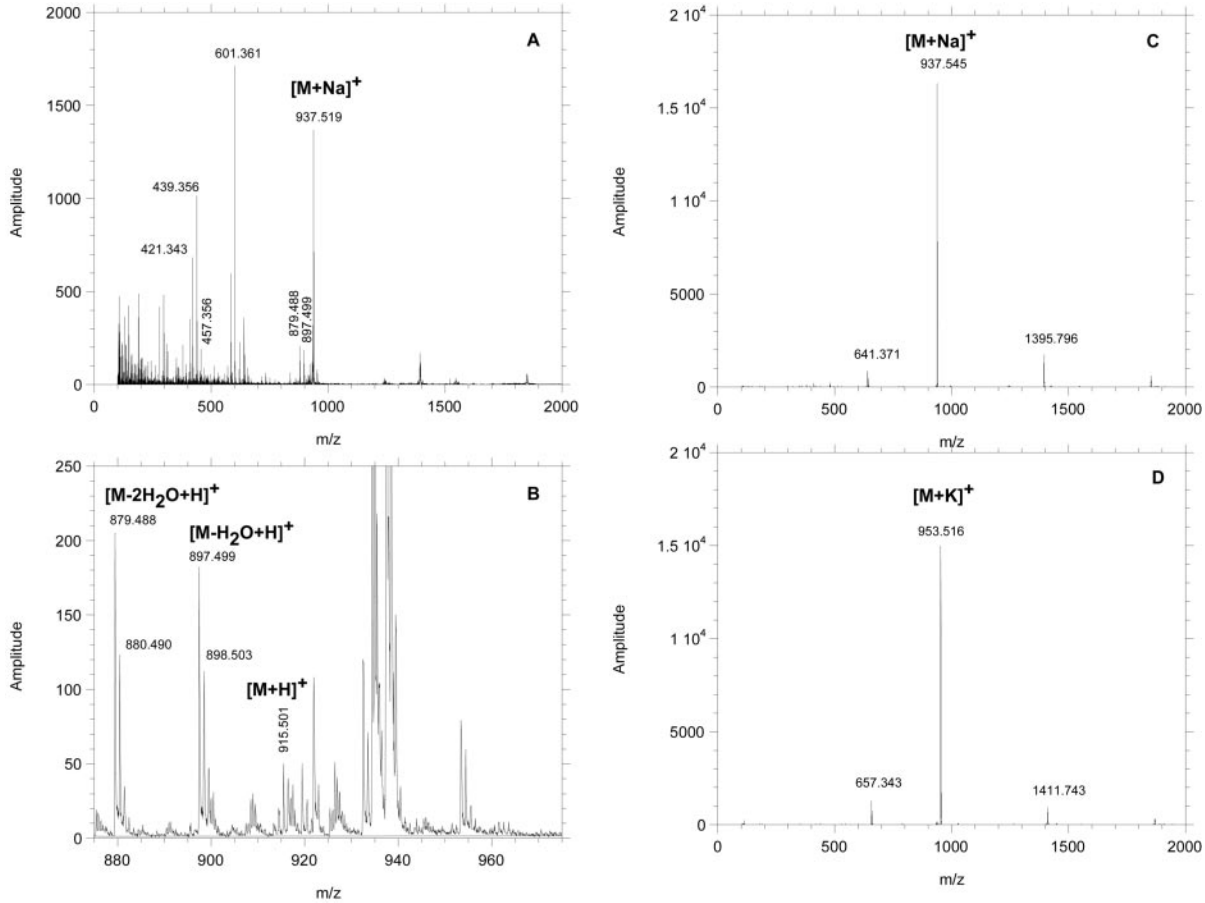

FIG. 4. **Analysis of the active compound by electrospray ionization time-of-flight mass spectrometry.** *A*, a chromatographic fraction from *peak 2* in Fig. 2*A* was infused at 50 μl/min without any additions. Several characteristic fragment ions are detected. *B*, zoomed portion of *A*. Protonized ions of phanoside and its two water-loss ions are indicated. *C*, 50 μl of the same fraction was mixed with 2 μl of 5 mM NaCl and infused into the mass spectrometer. Na^+ adduct peak of phanoside is indicated. *D*, 50 μl of the same fraction was mixed with 2 μl of 5 mM KCl and infused into the mass spectrometer. K^+ adduct peak of phanoside is indicated.

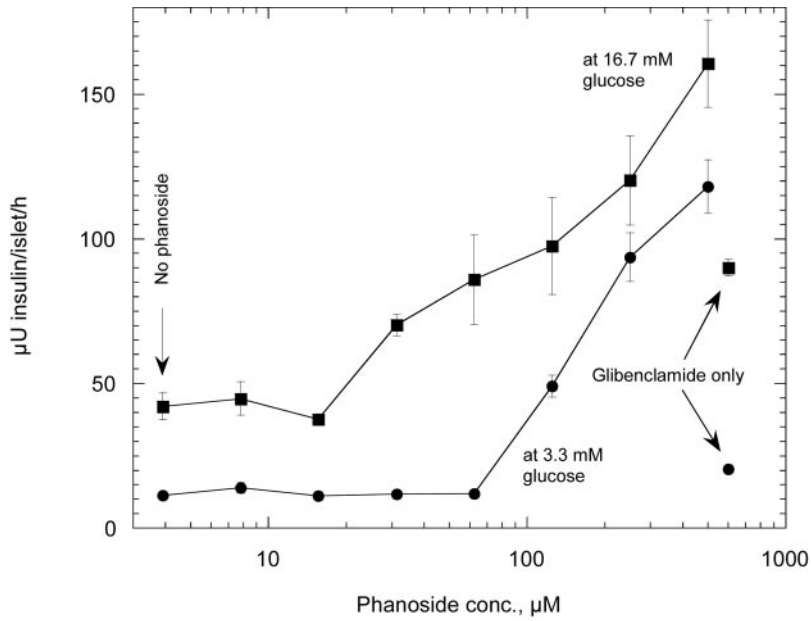

FIG. 5. **Effect of phanoside on insulin secretion from isolated rat pancreatic islets.** Phanoside was added at indicated concentrations (7.8, 15.6, 31.3, 62.5, 125, 250, 500 μM) in the presence of 16.7 mM glucose (*filled squares*) or 3.3 mM glucose (*filled circles*). For comparison, glibenclamide was assayed at 2 μM concentration without the addition of phanoside. The *leftmost points* indicate control values at each glucose concentration without the addition of phanoside. Results (mean ± S.E.) of 5–6 incubations in each condition. All values over 50 microunits (μU) insulin/islet/h are significant *versus* no addition at $p < 0.001$. Effect of glibenclamide at 3.3 mM glucose is significant *versus* no addition at $p < 0.01$.

of extract increased insulin release at 3.3 mM glucose from 12.1 ± 1.8 to 63.4 ± 5.0 and 103.0 ± 15.4 microunits/islet/h, respectively ($n = 4$, $p < 0.001$ for both), and at 16.7 mM glucose from 29.2 ± 6.5 to 42.4 ± 4.0 and 94.8 ± 9.8 microunits/islet/h, respectively ($n = 4$, $p < 0.05$).

The fractions of the purified compound from the fraction indicated in Fig. 1*B* by the *horizontal bar* were finally used to study the effect on insulin release (Fig. 5). At 3.3 mM glucose, 125 μM and higher concentrations of the gypenoside were stimulatory, whereas at 16.7 mM glucose, significant stimulation of

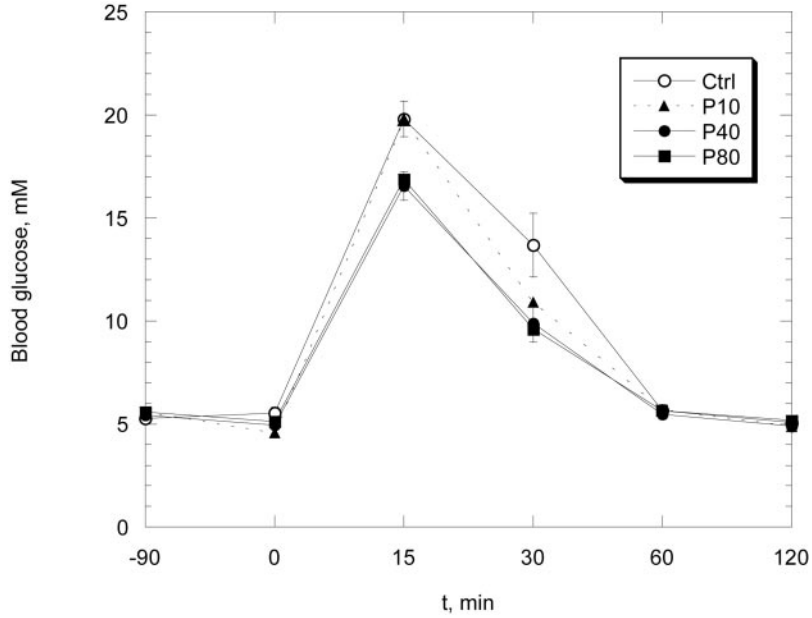

FIG. 6. **The hypoglycemic effect of phanoside on normal Wistar rats.** Normal Wistar rats were given phanoside orally at 10, 40, and 80 mg/kg (indicated as *P10*, *P40*, and *P80*, respectively) in Me_2SO (2.5%). The control (*Ctrl*) group was given only Me_2SO. Ninety min after drug administration, glucose (3 g/kg) was injected intraperitoneally, and blood glucose levels were measured at −90, 0, 15, 30, 60, and 120 min after glucose loading. Data represent mean ± S.E. (n = 10); S.E. is indicated for control and phanoside 40 mg/kg groups only.

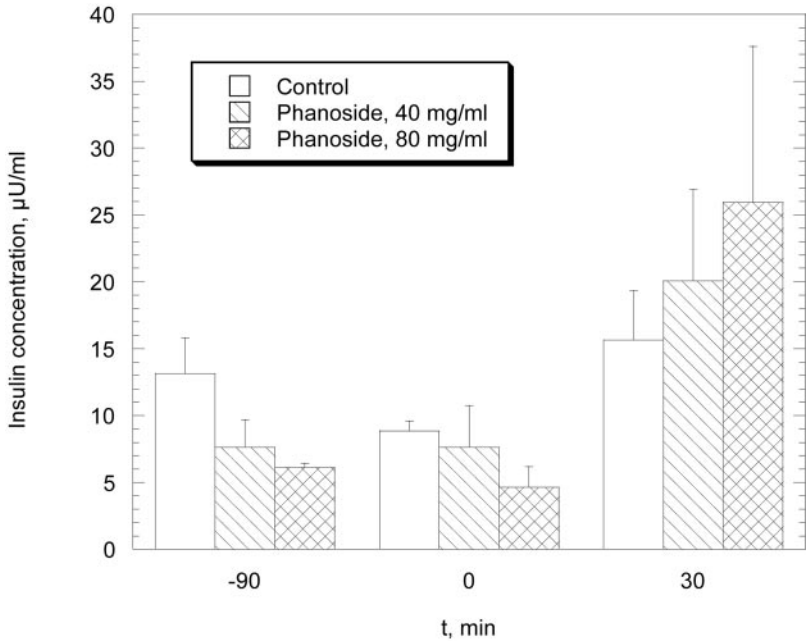

FIG. 7. **The effect of phanoside on insulin secretion of normal Wistar rats after loading of glucose.** Normal Wistar rats were given phanoside orally in Me_2SO (2.5%), and the control group was given only Me_2SO. Ninety min after drug administration, glucose (3 g/kg) was injected intraperitoneally. Plasma insulin concentrations were measured at −90, 0, and 30 min after glucose loading. Data represent mean ± S.E. (n = 4).

insulin release was noted already at the compound concentration of 31 µM ($p < 0.001$). At the highest concentration tested, 0.5 mM, the compound stimulated insulin release 10-fold at 3.3 mM glucose and almost 4-fold at 16.7 mM glucose. These effects were severalfold stronger than the insulin release induced by a sulfonylurea, glibenclamide, added at a nearly maximally effective concentration.

In Vivo Effects of Phanoside—Before the administration of phanoside and 90 min thereafter, when glucose was injected (0 min), blood glucose levels were similar in all groups of rats injected with 10, 40, or 80 mg/kg phanoside and in control rats (Fig. 6). Fifteen min after the intraperitoneal injection of glucose, blood glucose levels were increased to 15–20 mM in all rats. However, phanoside treatment (40 and 80, but not 10 mg/kg) decreased significantly the blood glucose concentrations compared with controls ($p < 0.001$ and $p < 0.05$, respectively; n = 10 in each group). This difference remained also at 30 min ($p < 0.01$ and $p < 0.05$, respectively). Thus, at the higher doses, 40 and 80 mg/kg, phanoside improved glucose tolerance in healthy rats. In a smaller number of rats (n = 4), plasma insulin levels were similar in phanoside-treated rats (40 and 80 mg/kg) and control rats at −90 min and 0 min (Fig. 7). Thirty min after glucose loading, the increment in plasma insulin levels was greater in phanoside-treated than in control rats.

DISCUSSION

A novel compound with insulin-releasing activity was isolated from an ethanol extract of the Southeast Asian herb *G. pentaphyllum* and demonstrated to be a gypenoside, which we have named phanoside, having a molecular mass of 914.5 Da. Because in preliminary studies the herbal extract administered orally to mice decreased blood glucose levels, we assumed that the hypoglycemic agent would be a nonpeptide compound that could be absorbed via the gut. This conclusion therefore agrees with our isolated novel gypenoside, a saponin-like substance that we have isolated from the extract. This assumption is furthermore supported by the fact that the crude extract exerted a dose-dependent insulin-stimulating effect on

isolated pancreatic islets and that we were able to track this biological activity throughout the whole purification procedure.

A large number of saponins, called gypenosides, has been isolated previously from *G. pentaphyllum*. The gypenoside isolated in this study has a relatively unusual structure compared with the majority of *G. pentaphyllum* saponins known. However, a quite similar compound has been isolated previously from this plant grown in China (16), but no biological activity has been reported. The compound had the same core structure but had an aldehyde group at position 19 and different sugar moieties.

Our studies with purified phanoside demonstrated a potent insulin-releasing activity that is both initiatory (*i.e.* eliciting insulin release at a nonstimulatory glucose concentration of 3.3 mM) and potentiatory (*i.e.* enhancing the stimulatory effect of 16.7 mM glucose). We compared these effects with a sulfonylurea (glibenclamide) that is known to initiate insulin secretion also at low glucose levels (17). At 2 μM concentration, glibenclamide has been shown to stimulate insulin release *in vitro* to a nearly maximal extent (18). It seems that phanoside at maximal effective concentration is a severalfold more potent initiator than the sulfonylurea.

In support of our findings in isolated islets, purified phanoside, given orally, significantly improved glucose tolerance in normal rats. Interestingly, this effect was only obvious in the presence of hyperglycemia. The decrease in blood glucose concentrations after administration of phanoside was paralleled by augmented incremental insulin responses. Thus, it appears that the hypoglycemic effect *in vivo* is mainly attributed to the stimulation of insulin secretion.

The mechanism by which phanoside stimulates insulin secretion remains to be clarified. However, it has been shown recently that extract of *G. pentaphyllum* containing gypenosides suppresses nitric oxide synthesis by inhibiting iNOS enzymatic activity and attenuating NFκB-mediated iNOS protein expression (19). Because inhibitors of nitric-oxide synthase activity, such as Nω-nitro-L-arginine methyl ester (L-NAME), was demonstrated to stimulate insulin secretion in isolated rat islets (20), it is possible that phanoside increases insulin release by the inhibition of iNOS in the β-cells. However, this effect is controversial because it has also been shown that gypenosides from *G. pentaphyllum* release nitric oxide from endothelial cells, which is antagonized by the iNOS inhibitor L-NAME (21); although this experiment was conducted with a pool of all gypenosides from *G. pentaphyllum*. On the other hand, it has been demonstrated that nitric oxide increases insulin release from pancreatic β-cells (22). Therefore the effect of phanoside may be mediated by direct release of nitric oxide in the β-cells.

Extracts of *G. pentaphyllum* and/or isolated gypenosides have been reported to exert a variety of effects, such as the inhibition of inflammation (7), anti-oxidant (6) and lipid-lowering effects (23), as well as anti-carcinogenic (9) and pro-apoptotic properties (24). In previous investigations of *G. pentaphyllum*, Hu *et al.* (25) have isolated dammarane-type glycosides structurally related to the ginseng saponins. *Panax ginseng* berry extract has been shown to reduce blood glucose and body weight in mice (12). In fact, saponin fractions of *P. ginseng* and *G. pentaphyllum* were demonstrated to have similar effects on hyperglycemia and hyperlipidemia (8). In the *P. ginseng* berry extract, an antihyperglycemic component, ginsenoside R_e, was identified (13).

In conclusion, from the plant *G. pentaphyllum* we have isolated a novel, biologically active substance with the molecular mass 914.5 Da, a gypenoside, which we have named phanoside. Phanoside is a potent initiator and potentiator of insulin secretion both *in vivo* and *in vitro* in the rat.

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A Novel Insulin-releasing Substance, Phanoside, from the Plant *Gynostemma pentaphyllum*

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