Extracts from *Gynostemma pentaphyllum* Makino (Curcurbitaceae), 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-β-L-20,21-trihydroxydammar-24-ene-3-O-[(α-L-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 (Curcurbitaceae) 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 Medicina, 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
A Novel Insulin-releasing Compound

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 Haen) at different concentrations (25, 60, and 80% acetonitrile).

Chromatography—The subsequent two high pressure liquid chromatography (HPLC) steps were carried out on an AKTAexplorer and the last on an AKTApurifier 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; 218TP152922), and eluted with a linear gradient of 20–57.5% B (elucent 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 recrystallized on a SOURCE 15RPC 16/60 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 AKTAexplorer 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 1H- and 13C NMR spectroscopy and mass spectrometry.

NMR—NMR spectra were recorded with a Bruker DMX-600 spectrometer equipped with a cryoprobe in Me2SO-d6 solution at 25 °C. Chemical shifts are reported in parts/million relative to residual solvent signal (δH = 4.6 ppm, δC = 41.0 ppm). Two-dimensional NMR spectra recorded included DQF-COSY, ROESY, TOCSY, sensitivity-enhanced 13C HSQC, and 1H-13C HMBC. Pulsed-field gradients were used for all 13C correlation spectra. 13C NMR spectra were recorded with a coupling evolution delay for the generation of multiple-bond correlations with 62.5 ms. All two-dimensional spectra were run with a recycle delay of 200 ms for 1H or 100 ms for 13C 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 13C-HMBC, the magnitude spectra were calculated.

Mass Spectrometry—Positive ion mass spectra were recorded on an Etten 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 a flow rate of 8 ml/min.

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. 1A). The active fraction was further purified on a polyaromatic column (Fig. 1B). 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. 2A. 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 1H-1H and 1H-13C spectra taken in Me2SO-d6 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 1H-1H TOCSY, 1H-1H DQF-COSY, 1H-13C HSQC, and 1H-13C 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 tetrahydrofuran ring is linked to the triterpene skeleton at position 17. The tetrahydrofuroine moiety is unusual; it has an isopropyliened group and two hydroxyl groups attached (Fig. 3A).

Chemical shifts, the multiplicity of signals, and the 13C NMR data all indicated β-configuration at the anomeric positions for rings A and C (δJ(1–2) = 3 and 6.8 Hz, respectively) but α-configuration for the rhamnose unit B (δJ(1–2) = 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 tetrahydrofuroine moiety at position 17. In addition, the strong NOEs in 1H-1H ROESY spectra between 30-CH3 and H-17α confirmed the β-orientation of the tetrahydrofuroine ring.

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; DQF-COSY, double quantum coherence; HMBC, heteronuclear multiple bond correlation; KR, Krebs-Ringer bicarbonate; NOE, nuclear Overhauser effect; iNOS, inducible isofom of nitric oxide synthase.

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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-stereo-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 OH-20 and H-22, 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 \([M + H]^+\), \((m/z = 915.501, \text{calculated value } 915.532)\) (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 \([\text{aglycone}^+\), calculated value 457.368]; 439.356 \([\text{aglycone} – H_2O]^+\), calculated value 439.358], and 421.343 \([\text{aglycone} – 2H_2O]^+\), 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
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. 4C). A similar mass spectrum was obtained with K\(^+\), and the metal adduct peak shifted according to the mass difference between Na\(^+\) and K\(^+\) (Fig. 4D). 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)
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. 1B 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
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 vitro 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 Rb1, 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 vitro and in vivo in the rat.

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

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