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Identification of Cytotoxic Constituents of *Narthecium ossifragum* Using Bioassay-Guided Fractionation

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Narthecium ossifragum, a member of the Liliaceae family, contains phytochemicals that have hepatotoxic and nephrotoxic activity in several ruminant species. 3-Methoxyfuran-2(5*H*)-one has previously been isolated as the principal nephrotoxin, and its toxicity has been confirmed in vivo. However, previous investigations into the nephrotoxicity of *N. ossifragum* both in vivo and in vitro indicate that other phytochemical factors might contribute to the nephrotoxicity of the plant. In this study, the cytotoxicity in renal tubular cells (LLC-PK1) was measured using an aqueous extract from the plant and fractions from chromatographic separation to identify the cytotoxic constituents of the extract. In an iterative process two different groups of compounds were identified as the major cytotoxic principles in LLC-PK1 cells: steroidal saponins (primarily di- and trisaccharides of sarsasapogenin) and 5-hydroxy-4-methoxyfuran-2(5*H*)-one. Up to a concentration of 880 μg/mL (7.7 M) 3-methoxyfuran-2(5*H*)-one was not cytotoxic. The cytotoxicity of the saponins was abolished upon hydrolysis, indicating that the carbohydrate moiety of the molecule is a prerequisite for toxicity on the cellular level. The results of the present study have two important implications: first, the results question the direct involvement of 3-methoxyfuran-2(5*H*)-one in the nephrotoxicity of *N. ossifragum*; second, the findings should induce future investigations into the possible role of saponins in *N. ossifragum*-related nephrotoxicosis observed in ruminants that graze on this plant.

KEYWORDS: Alveld; cytotoxicity; furanones; hepatotoxin; Liliaceae; *Narthecium* spp.; nephrotoxin; neutral red; saponins

INTRODUCTION

Narthecium ossifragum (L.) Huds. is the only species of the seven to nine validly described species of the genus that occurs in Scandinavia, and it is also found in the northern parts of Spain and Portugal, western France, northwestern Germany, the United Kingdom, Ireland, and the Faeroe Islands (*1*). The plant has for several hundred years been linked to a disease in lambs called alveld (literally "the elf's fire"), which is a hepatogenous photosensitivity disease (*2*). Hepatogenous photosensitization occurs when a toxin, normally produced by a higher plant, a fungus or an alga, causes liver damage or liver dysfunction that results in retention of the photosensitizing agent phylloerythrin. Phylloerythrin is a metabolic product of chlorophyll produced by rumen microorganisms, and an acute inflammatory response of the skin is induced when phylloerythrin reacts with sunlight (*3*). It has been suggested that the steroidal saponins (**Figure 1**) found in *N. ossifragum* are the cause of the disease, even though there exists experimental evidence that opposes this theory (*2*, *4*−*6*). Several other plants containing steroidal saponins have been suggested to cause hepatogenous photosensitization of sheep (*3*). These plant species include *Agave lecheguilla*, *Brachiaria decumbens*, *Panicum* spp., and *Tribulus terrestris* (*3*).

In addition to containing liver-damaging toxins, *N. ossifragum* is known to cause kidney damage in several ruminant species, and 3-methoxyfuran-2(5*H*)-one has been isolated as its principal nephrotoxin (*7*). Aqueous extracts from 30 g of *N. ossifragum* per kilogram of live weight given to goats, calves, lambs, moose and red deer resulted in acute tubular degeneration and necrosis in the renal proximal tubules (*8*). The effects of 3-methoxyfuran-2(5*H*)-one-containing extracts and fractions have recently been studied in vitro in renal tubular cells (*9*). Results from the latter study cast doubts on the hypothesis that 3-methoxyfuran-2(5*H*)-one is the major toxic principle of the plant extract in vitro. Furthermore, through the past decades a number of compounds have been isolated from *Narthecium* spp. that may possess biological activity on the cellular level, among them several other furanone compounds, flavonoids, carotenoids, lignans, sterols, and saponins **(Figure 1)** (*1*, *10*−*12*).

The objective of this study was to identify the water-soluble phytochemicals of *N. ossifragum* that induce toxicity in renal tubular cells. To achieve this, LLC-PK1 cells were repeatedly exposed to plant extracts, fractions, and purified compounds in order to trace the cytotoxicity of specific plant constituents.

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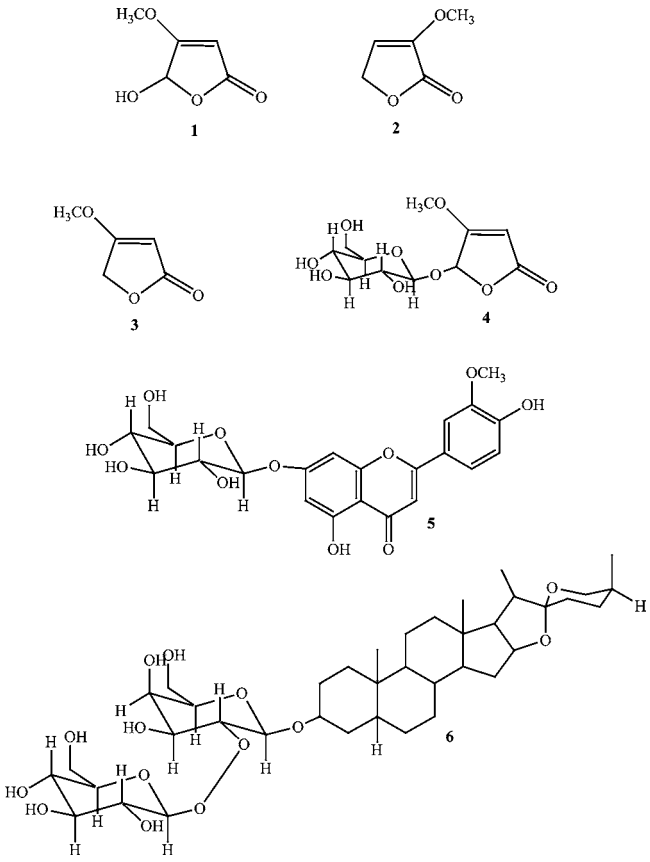

Figure 1. Compounds that have been isolated and identified from *Narthecium* spp.: (**1**) 5-hydroxy-4-methoxyfuran-2(5*H*)-one; (**2**) 3-methoxyfuran-2(5*H*)-one; (**3**) 4-methoxyfuran-2(5*H*)-one; (**4**) 4-methoxyfuran-2(5*H*)-one 5-(*β*-D-glucoside); (**5**) chrysoeriol 7-*O*-*β*-D-glucopyranoside, a flavanoid glucoside; (**6**) disaccharide of sarsasapogenin.

MATERIALS AND METHODS

Plant Material and Extraction. *N. ossifragum* flower stems were collected during the flowering period in different geographic locations in Norway (*13*). The plant material was frozen at −20 °C on the day it was collected and kept frozen until it was extracted. An aqueous extract was produced as follows: liquid nitrogen was added to the flower stems with blossoms, and the brittle material was chopped finely in an R 301 Ultra food processor (Robot Coupe, S.A., Montceau en Bourgogne, France). The chopped material was suspended in purified water (1 L per 500 g of wet plant material) and shaken in bottles on a universal flask-shaking machine for 2 h. The mixture was centrifuged in 250 mL cups at 4 °C for 15 min at 10000 rpm on a centrifuge. The supernatant was vacuum-filtered through a no. 1 filter (Whatman, Brentford, Middlesex, U.K.). The extraction was repeated with half the amount of water, the supernatant was filtered as above, and the extracts were combined, resulting in a final volume of approximately 1.2 L per 500 g of wet plant material. The aqueous extract was frozen at −26 °C until further processing.

HPLC-Electrospray Tandem Quadrupole Mass Spectrometry of 3-Methoxyfuran-2(5*H*)-one. The crude *N. ossifragum* extract was diluted 10000 times and injected (20 μL aliquots) into a 2695 Separation Module (Waters, Milford, MA) interfaced with a Quattro Ultima PT mass spectrometer (Micromass, Manchester, U.K.), equipped with an electrospray interface. Separation was achieved with a 50 × 2.1 mm i.d., 3 μm, Atlantis dC_{18} column (Waters) using a mobile phase consisting of 0.1% HCOOH in methanol (A) and 0.1% HCOOH (B) and a flow rate of 0.3 mL/min. A linear gradient was applied to the column starting with 100% B to 1:9 A/B over 6 min. The column was then flushed with A for 3 min, before a return to the starting conditions. Under these conditions, 3-methoxyfuran-2(5*H*)-one eluted from the column after 3.5 min.

The mass spectrometer was tuned by continuous infusion of an aqueous solution containing about 1 μg/mL of 3-methoxyfuran-2(5*H*)-one into a mobile phase consisting of 5:95 A/B. For pneumatically assisted electrospray (+) ionization, the spray capillary voltage was set to 3.5 kV. Other important source parameters included a cone voltage of 80 V, a source temperature of 100 °C, a desolvation temperature of 250 °C, a cone gas flow of approximately 100 L/h, and a desolvation gas flow of approximately 600 L/h. The mass spectrometer was run in the selected ion recording mode by continuous monitoring of the signal corresponding to the protonated molecular ion $[M + H]^+$ of 3-methoxyfuran-2(5*H*)-one (*m*/*z* 115). Linear regression was used for calibration of the instrument in the concentration range of 1.96−245 ng/mL, the low point of the calibration being at the approximate limit of detection (3 × signal/noise). Pure standard material of 3-methoxyfuran-2(5*H*)-one for tuning and calibration was available from earlier experiments and had been isolated and purified using the method of Langseth et al. (*7*).

General Fractionation Procedure. Aliquots of the aqueous extract of *N. ossifragum* were fractionated by semipreparative HPLC using a Gilson system (Gilson, Middleton, WI) consisting of a model 321 pump, a 232XL sampling injector, a 402 syringe pump, and a 206 fraction collector. A HP 1100 series UV−vis diode array detector (Hewlett-Packard, Waldbronn, Germany) was used for the continuous monitoring of the absorption at 200, 226, and 350 nm. Chromatography was performed on a 250 × 10 mm ACE 5 C_{18} column (Advanced Chromatography Technologies, Aberdeen, Scotland). For all fractionations and purifications, separation was achieved using combinations of linear gradient elution (5 mL/min) or isocratic elution (4 mL/min) with a binary mobile phase consisting of methanol (A) and water (B), both containing 0.005% HCOOH. For initial fractionation of the aqueous extract into three main fractions (F I, F II, F III), a linear gradient was used starting with 3:97 A/B to 25:75 A/B over 10 min; isocratic elution with 100% A was then maintained for 3 min before the eluent was switched to the starting conditions. The mobile phase composition was subsequently varied depending on which of the three fractions was targeted. Aliquots of 450 μL of the crude aqueous extracts were injected, and the fractions were collected according to **Figures 2**−**4**. The fractions were evaporated to dryness using either rotary evaporation or lyophilization and reconstituted in water such that the final solution was 20-fold more concentrated than the injected sample. Typically, the residue of 10 450-μL injections was dissolved in 225 μL of water. Likewise, an aliquot of the crude extract was also concentrated 20-fold and served as the positive control for cell culture testing.

Electrospray Ion Trap Mass Spectrometry. All extracts and fractions were analyzed using HPLC coupled to a Finnigan LCQ Classic ion trap mass spectrometer or a Finnigan LTQ linear ion trap mass spectrometer, with an electrospray interface operated in the positive mode (Thermo Electron, San Jose, CA). The instruments were run in the full-scan mode in various mass ranges, depending on the fraction (50−1500 u maximum range). The electrospray interface of both instruments was operated with a spray voltage of 4.5 kV, and a sheath gas and auxiliary gas rate of 60/40 (LCQ/LTQ) and 5 units, respectively (approximately 60/40 and 5 mL/min, respectively). The heated capillary was set to a temperature of 200/250 °C (LCQ/LTQ), whereas the capillary voltage and the tube lens offset were 30/45 V (LCQ/LTQ) and 15/90 V (LCQ/LTQ), respectively. The LTQ mass spectrometer was used for multiple fragmentation experiments either by using data-dependent scanning or direct fragmentation of selected precursor ions. Ions destined for fragmentation were isolated with an isolation width of 2 *m*/*z* units, the activation Q was set to 0.15 or 0.25, and the activation time was set to 30 ms. For all runs on the LTQ, a Finnigan Surveyer PDA Plus (Thermo Electron) photodiode array detector was coupled in-line and operated in the wavelength range 200−500 nm to gather UV data.

Chromatography was performed using an Atlantis dC_{18} column (Waters) for the analysis of extracts and fractions for furanones, whereas 50 × 2.1 mm i.d., 3.5 μm, SunFire C_{18} or Symmetry C_{18} columns (Waters) were used for general screening or analysis of fraction F III and its subfractions.

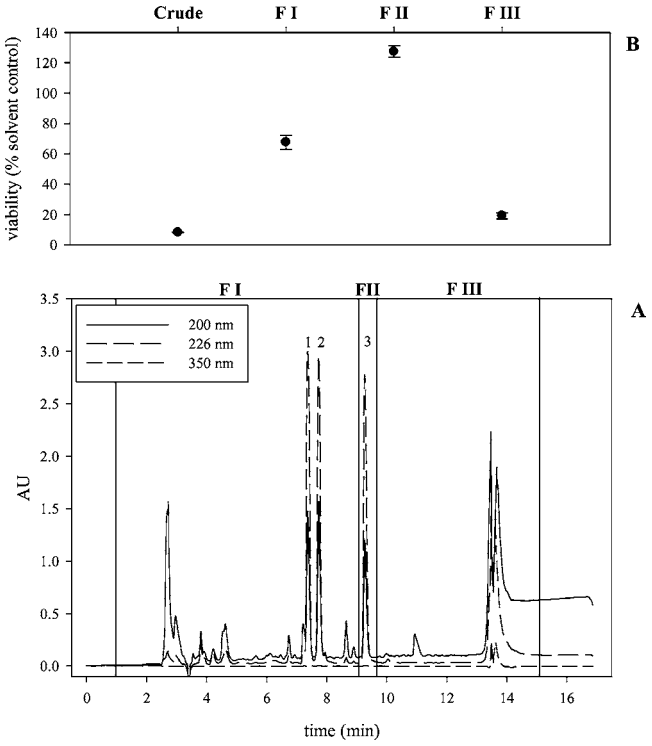

Figure 2. (**A**) Initial fractionation of the crude aqueous *N. ossifragum* extract into three fractions: F I containing 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (**1**) and 5-hydroxy-4-methoxy-2(5*H*)-furanone (**2**); F II containing 3-methoxy-2(5*H*)-furanone (**3**); and F III containing pigments and steroidal saponins. (**B**) Cytotoxicity of the fractions in LLC-PK1 cells using the Neutral Red cytotoxicity assay. Error bars represent the standard error of triplicate measurements.

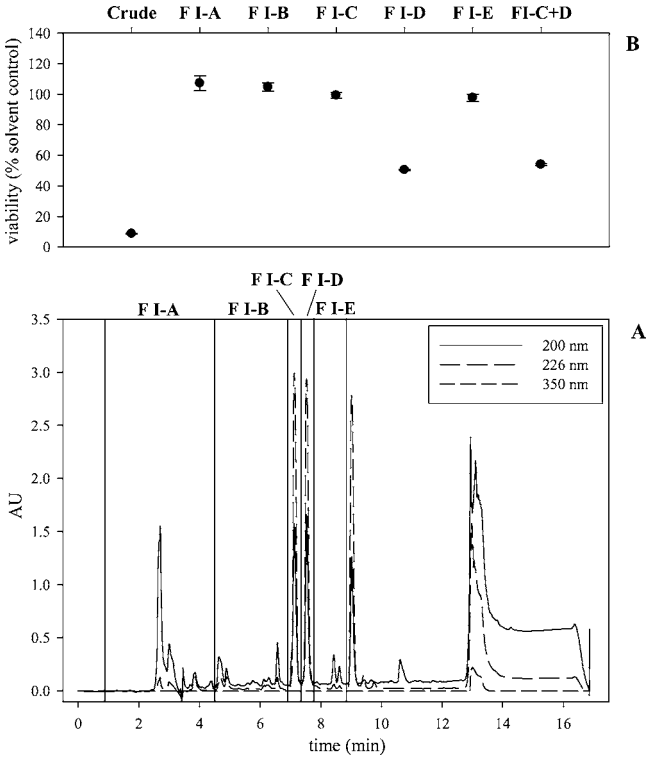

Figure 3. (**A**) Fractionation of the crude aqueous *N. ossifragum* extract into five fractions: F I-A and F I-B containing unknown compounds, F I-C containing mostly 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside), F I-D containing mostly 5-hydroxy-4-methoxyfuran-2(5*H*)-one, and F I-E containing unknown compounds. (**B**) Cytotoxicity of the fractions in LLC-PK1 cells using the Neutral Red cytotoxicity assay. Error bars represent the standard error of triplicate measurements.

Isolation and Purification of 4-Methoxyfuran-2(5*H*)-one 5-(β-D-Glucoside), 5-Hydroxy-4-methoxyfuran-2(5*H*)-one, and 3-Methoxyfuran-2(5*H*)-one. The three furanones were purified in a two-step procedure using semipreparative reversed-phase HPLC with the ACE 5 column and the same Gilson system as described above. The crude *N. ossifragum* extract (750 μL) was injected into a mobile phase consisting of 8:92 A/B. The column was eluted isocratically for 7 min, and the three furanones were collected manually in three separate fractions. The column was then flushed with pure A for 3 min before the procedure was repeated. The fractions were then, after evaporation to dryness (Rotavapor) and dissolving in water, rerun on the same column by injecting 450 μL aliquots into a mobile phase consisting of 1:9 A/B. Each furanone was again collected manually and after evaporation of the solvent obtained as white crystalline material. Approximately 1 mg of 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) and 3-methoxyfuran-2(5*H*)-one was purified for structural characterization using NMR spectroscopy, whereas 27.8 mg of 5-hydroxy-4-methoxyfuran-2(5*H*)-one was purified for structural characterization and dose−response measurements.

Isolation of Steroidal Saponins from F III. Steroidal saponins were purified in a two-step procedure by semipreparative reversed-phase HPLC using the same Gilson system as above. The crude *N. ossifragum* extract (450 μL) was injected into a mobile phase consisting of 35:65 A/B and applied to the ACE 5 column. The column was eluted using a linear gradient to 100% A over 16 min; isocratic elution with 100% A was then maintained for 2 min before the eluent was switched to the starting conditions. The fraction between 16.8 and 17.8 min was collected. Combined fractions were evaporated to dryness (Rotavapor), and the residue was dissolved in 1:1 methanol/water. This solution (450 μL aliquots) was applied to a 250 × 10 mm i.d., 5 μm, SunFire C_8 column (Waters), and the column was eluted with a linear gradient starting with 7:3 A/B to 100% A over 10 min at a flow rate of 4 mL/min. Isocratic elution with 100% A was maintained for 5 min before the eluent was switched to the starting conditions. The fraction from 8.5 to 10 min was collected and comprised di- and trisaccharides of smilagenin and sarsasapogenin (ca. 1:10 of smilagenin/sarsasapogenin aglycone and 1:4 of disaccharide/trisaccharide per HPLC-MS). Collected fractions amounted to approximately 1 mg of saponin mixture.

Acid Hydrolysis of Saponins and HPLC-MS of Sarsasapogenin. To compare the cytotoxicity of saponins and sapogenins, the complete combined saponin-containing fractions F III-I and F III-J, obtained from 9 mL of the aqueous *N. ossifragum* extract, were evaporated and dissolved in 2 mL of methanol. The solution was divided between two Reacti-vials and evaporated. The residue in one vial was dissolved in 225 μL of methanol and directly used for the exposure of LLC-PK1 cells, and the residue in the second vial was hydrolyzed using the method of Wilkins et al. (*14*), with modifications. The residue was dissolved in 1 mL of 1 M HCl, and the vial was placed in an oven that was heated to 90 °C for 90 min. After cooling, the sapogenins were extracted with 3 × 2 mL of diethyl ether. The combined ether phases were transferred to a conical tube and evaporated under a gentle stream of nitrogen, and the residue, composed of the sapogenins, was dissolved in 225 μL of methanol. This solution was then used for the exposure of LLC-PK1 cells. Aliquots of all solutions were retained for HPLC-MS analysis.

The successful deglycosylation of the saponins was controlled both by analysis of the aqueous phase for saponins using HPLC-MS and by analysis of the ether extract for sarsasapogenin. Sarsasapogenin reference material was obtained from Sigma (St. Louis, MO). The Finnigan LTQ mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) interface, operated in the positive mode, was used for sarsasapogenin analysis. The APCI interface was operated with a vaporizer temperature of 300 °C, a discharge current of 4.5 μA, and sheath gas and auxiliary gas rates of 33 and 10 units, respectively (approximately 33 and 10 mL/min, respectively). The heated capillary was set to a temperature of 250 °C, whereas the capillary voltage and the tube lens offset were 47 and 125 V, respectively. The mass analyzer was operated in the full-scan mode in the mass range of 380−425 u. Chromatography was performed using

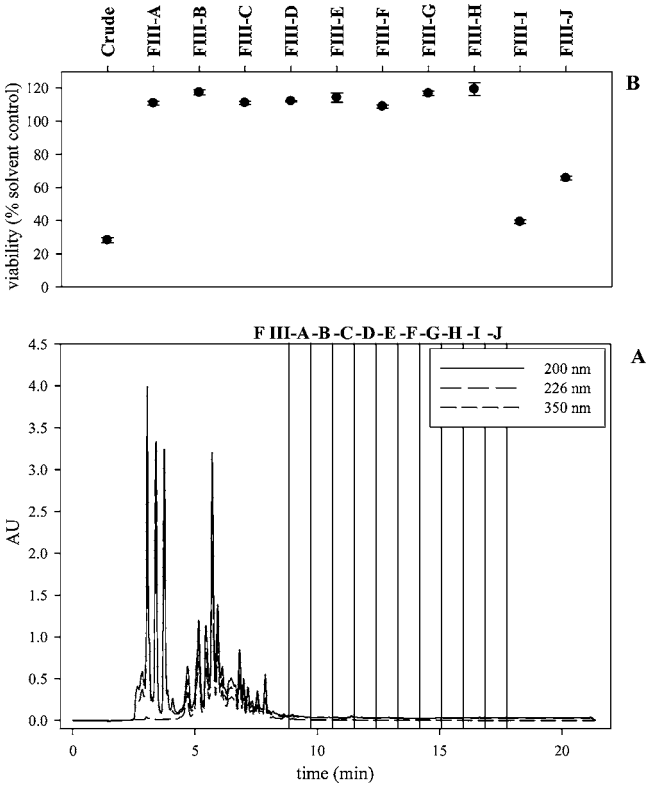

Figure 4. (**A**) Fractionation of the crude aqueous *N. ossifragum* extract into 10 fractions; F III-I and F III-J contained cytotoxic compounds that were eventually identified as di- and trisaccharides of sarsasapogenin and smilagenin. (**B**) Cytotoxicity of the fractions in LLC-PK1 cells using the Neutral Red cytotoxicity assay. Error bars represent the standard error of triplicate measurements.

a 50 × 2.1 mm i.d., 3.5 μm Symmetry C_{18} column (Waters), which was eluted using a linear gradient starting with 1:1 A/B to 100% A over 8 min; isocratic elution with 100% A was then maintained for 3 min before the eluent was switched to the starting conditions.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra of 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (approximatgely 1 mg) and 5-hydroxy-4-methoxyfuran-2(5*H*)-one (approximately 1 mg) were obtained from solutions in 0.5 mL of deuterated water (Chiron AS, Trondheim, Norway), whereas NMR spectra of 3-methoxyfuran-2(5*H*)-one (>1 mg) were obtained from a solution in 0.5 mL of chloroform-d_1 (Sigma). All spectra were acquired on an Avance DRX 500 MHz NMR spectrometer (Bruker BioSpin, Germany) applying a 5 mm TXI (^{1}H/^{13}C,^{15}N-^{2}H) triple-resonance inverse probe, equipped with a Z-gradient coil.

NMR assignments of 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) and 5-hydroxy-4-methoxyfuran-2(5*H*)-one were obtained from examination of ^{1}H, ^{13}C, DEPT135, g-COSY, TOCSY, g-HSQC, g-HMBC, and NOESY data (**Table 1**). The data were processed using Bruker XWIN NMR software (version 3.5).

NMR spectra of steroidal saponins (ca. 1 mg, sarsasapogenin trisaccharide/sarsasapogenin disaccharide/smilagenin trisaccharide/smilagenin disaccharide; about 1:0.25:0.1:0.025) were obtained from solutions in 50 μL of methanol-d_4 (Cambridge Isotope Laboratories, Inc., Andover, MA) and 50 μL of pyridine-d_5 (Cambridge Isotope Laboratories) in 1.7 mm tubes, 1.3 mm i.d., (Hilgenberg, Maisfeld, Germany) using a Bruker MATCH clamp holder. NMR assignments of the saponins were obtained from examination of ^{1}H, ^{13}C, APT, COSY45, TOCSY, g-HSQC, g-HMBC, g-H2BC, and NOESY spectral data. NMR spectra of sarsasapogenin (Sigma) were obtained from a solution of approximately 3 mg in 0.5 mL of pyridine-d_5 and assigned from ^{1}H, ^{13}C, and DEPT135 spectra by comparison with literature data (*15*). NMR spectra of 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (approximately 1 mg), dissolved in 0.5 mL of pyridine-d_5 (Cambridge Isotope Laboratories) were inferred from ^{1}H, ^{13}C, DEPT135, g-COSY, TOCSY, g-HSQC, g-HMBC, and NOESY (**Table 2**). The spectra were acquired on an Avance AV 600 MHz NMR spectrometer or an Avance AV II 600 MHz NMR spectrometer (Bruker BioSpin, Silberstreifen, Germany) with a 5 mm CP-TCI (^{1}H/^{13}C, ^{15}N-^{2}H) triple-resonance inverse cryo probe, equipped with a Z-gradient coil. The data on the latter two instruments were processed using Bruker TOPSPIN (version 1.3) software.

Spectra were acquired in WG5-Economy 5 mm tubes (Wilmad) if not indicated otherwise. Chemical shifts, determined at 298 K, are reported relative to internal C*H*D$_2$OD (3.31 ppm), C*D*$_3$OD (49.0 ppm), C*H*Cl$_3$ (7.24 ppm), C*D*Cl$_3$ (77.0 ppm), CD(CD)$_2$(C*H*)$_2$N (8.71 ppm), and CD(CD)$_2$(C*D*)$_2$N (149.8 ppm), respectively. The spectra acquired in D$_2$O are reported relative to external 3-trimethylsilylpropionic acid sodium salt-d_4 (0.00 ppm in both ^{1}H and ^{13}C NMR spectra).

Cell Culture. LLC-PK1 cells were originally obtained from the European Collection of Cell Cultures (ECACC 86121112) at passage 196. The cells were obtained from proximal tubules in porcine kidneys. The cells used in the present experiments were grown from passages numbers 200−215 in Eagle's Dulbecco Modified Medium (DMEM) (Cambrex, Verviers, Belgium) with 10% newborn calf serum, 2% glutamine, penicillin (100 IU/mL), and streptomycin (100 mg/mL). The cells were maintained as monolayers in 75 cm^2 cell culture flasks with filter screw caps (Techno Plastic Products, Trasadingen, Switzerland) at 37 °C in an atmosphere of 5% CO_2/95% air.

Cytotoxicity Assay. Cell viability and lysosomal activity were assessed using the Neutral Red cytotoxicity assay (Xenometrix, Allschwil, Switzerland). LLC-PK1 cells were diluted to 2 × 10^5 cells/mL with DMEM, and 100 μL (2 × 10^4 cells) of the suspension was transferred to the 60 inner wells of a 96-well plate (Greiner bio-one, Frickenhausen, Germany). The outer wells of the plates were filled with 200 μL of medium. The cells were grown overnight to allow the cells to attach. On the next day, 100 μL of fresh medium containing 10% of the prepared dilutions of the different fractions was added to the cell cultures in triplicates. After an incubation period of 24 h, the medium was removed and replaced with 200 μL of medium containing 1.7% of the Neutral Red dye, which in accordance with the manufacturer's recommendation had been preincubated overnight at 37 °C and centrifuged prior to use. The cells were further incubated for 3 h to allow viable cells to take up the dye into the lysosomes. The Neutral Red-containing medium was then removed, and the cells were treated with wash and fixing solutions from the assay kit. Finally, the cells were lysed using 200 μL of the assay kit solubilization solution. The Neutral Red concentration in the lysates was determined photometrically on a Victor^2 Multilabel Counter (Perkin-Elmer Inc., Boston, MA) at 540 nm. Cell growth control (medium with cells) and solvent control (10% purified water in medium) were run for each experiment. To establish the dose−response relationship for 5-hydroxy-4-methoxyfuran-2(5*H*)-one in LLC-PK1 cells, 27.8 mg of the compound was weighed and dissolved in 220 μL of water. The cell culture assay was performed in triplicate as described above, and the cells were exposed to concentrations in the range from 0.02 mg/mL (0.15 mM) to 10.4 mg/mL (79.9 mM).

To compare the cytotoxicity of saponins and sapogenins, both were dissolved in methanol, and the cells were exposed to 5% of the solutions. The solvent control for this experiment was then 5% methanol in DMEM.

RESULTS AND DISCUSSION

Quantification of 3-Methoxyfuran-2(5*H*)-one. 3-Methoxyfuran-2(5*H*)-one was quantified in the diluted aqueous *N. ossifragum* extract with HPLC−tandem quadrupole mass spectrometry. Four-point calibration plots were linear over 2 orders of magnitude ($R^2 > 0.997$), and residuals (19% maximum) were evenly distributed around the line of regression. When the protonated molecular ions of 3-methoxyfuran-2(5*H*)-one (*m*/*z* 115) were fragmented in Q2, using argon as the collision gas, the major fragment of the molecule appeared with a *m*/*z* ratio of 55. This fragment is probably the result of loss of CO after opening of the lactone ring and cleavage of the methoxy group.

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for the Furanones Found in the Aqueous *N. ossifragum* Extract

	4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (D_2O)		5-hydroxy-4-methoxy-furan-2(5*H*)-one (D_2O)		3-methoxyfuran-2(5*H*)-one ($CDCl_3$)	
position	δ H[a]	δ C[a]	δ H[a]	δ C[a]	δ H[b]	δ C[b]
2		176.2		177.1		168.0
3	5.44 (s)	92.7	5.38 (s)	92.1		146.9
4		181.0		182.4	6.12 (t, J = 2.1 Hz)	112.6
5	6.17 (s)	101.1	6.05 (s)	97.3	4.76 (d, J = 2.1 Hz)	67.5
OCH_3	4.00 (s)	63.2	3.99 (s)	63.1	3.79 (s)	58.1
1′	4.78 (d, J = 8.0 Hz)	103.7				
2′	3.38 (dd, J = 9.4, 8.0 Hz)	75.5				
3′	3.53 (dd, J = 9.4, 9.4 Hz)	78.3				
4′	3.42 (dd, J = 9.4, 9.4 Hz)	72.1				
5′	3.50 (ddd, J = 9.4, 5.7, 2.2 Hz)	79.4				
6′	3.91 (dd, J = 12.5, 2.2 Hz) 3.74 (dd, J = 12.5, 5.7 Hz)	63.4				

[a] ^{1}H and ^{13}C NMR shifts relative to 3-trimethylsilylpropionic acid sodium salt-d_4 (*H*DO = 4.82 ppm). [b] ^{1}H and ^{13}C NMR shifts relative to C*H*Cl_3 = 7.24 and *C*DCl_3 = 77.00 ppm.

Table 2. ^{1}H and ^{13}C NMR Assignments for the 5*S* Isomer of 4-Methoxyfuran-2(5*H*)-one 5-(β-D-Glucoside)

position	δ H[a] (pyridine-d_5)	δ C[b] (pyridine-d_5)
2	5.41 (1H, s)	170.6
3		90.3
4	6.47 (1H, s)	178.3
5	3.67 (3H, s)	98.3
OCH_3	5.29 (1H, d, J = 7.9 Hz)	59.5
1′	4.09 (1H, dd, J = 8.8, 7.9 Hz)	103.5
2′	4.21 (1H, dd, J = 8.8, 8.8 Hz)	74.8
3′	4.27 (1H, dd, J = 8.8, 8.8 Hz)	78.2
4′	3.91 (1H, ddd, J = 8.8, 5.0, 2.3 Hz)	70.8
5′	4.48 (1H dd, J = 12.0, 2.3 Hz)	79.2
6′a	4.34 (1H dd, J = 12.0, 5.0 Hz)	62.0
6′b		62.0

[a] ^{1}H NMR shifts relative to $CD(CD)_2(CH)_2N$ = 8.71 ppm. [b] ^{13}C NMR shifts relative to $CD(CD)_2(CH)_2N$ = 149.8 ppm.

However, using the *m*/*z* 115>55 transition for the detection of the compound in the multiple reaction monitoring mode increased the limit of detection significantly (ca. 100 times), and therefore the mass analyzer was operated in the selected ion recording mode, which allowed for the strong dilution of the extract (1/10000). Possible matrix signal enhancement/suppression effects were not investigated because such strong dilution minimizes or abolishes matrix effects. The plant extract was found to contain 440 μg/mL of 3-methoxyfuran-2(5*H*)-one, or 1.3 mg/g of plant material.

Bioassay-Guided Fractionation. 3-Methoxyfuran-2(5*H*)-one has previously been isolated as the principal nephrotoxin from *N. ossifragum* (*7*). Hence, it has been hypothesized that this compound also is the principal toxin in the in vitro bioassays, which had been used by Wisløff et al. (*9*) during toxicological characterizations of extracts from the plant. The results in the latter study were, however, variable, and it was doubted if the observed effects were solely due to 3-methoxyfuran-2(5*H*)-one. It was therefore assumed that other factors may contribute to the in vitro toxicity of the *N. ossifragum* extracts. Previously, Langseth et al. (*7*) expressed uncertainty about the toxicity of 3-methoxyfuran-2(5*H*)-one, and it was found that highly purified 3-methoxyfuran-2(5*H*)-one was not as toxic as less purified material. This supports the hypothesis that there might be other factors that contribute to the nephrotoxicity of the plant. On the basis of these considerations, the crude aqueous *N. ossifragum* extract was separated into three main fractions using semipreparative reversed-phase HPLC: a fraction containing compounds with less retention than 3-methoxy-2(5*H*)-furanone; a fraction containing 3-methoxyfuran-2(5*H*)-one among minor impurities; and a fraction containing compounds with higher retention than 3-methoxyfuran-2(5*H*)-one (**Figure 2**). Fraction F I contained, among unknown compounds, two other furanones, which were easily recognized as such because of their UV spectra. These were isolated and purified, and their chemical structures were determined as 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) ("narthesid") and 5-hydroxy-4-methoxyfuran-2(5*H*)-one on the basis of ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (**Table 1**) (*12*).

LLC-PK1 cells were exposed to the concentrated crude extract and concentrated fractions F I, F II, and F III, and the cytotoxicity was assessed measuring Neutral Red uptake. Cells that were exposed to fraction F II were exposed to 880 μg/mL (or 7.7 M) of 3-methoxyfuran-2(5*H*)-one. The effect of this concentration was more proliferative than cytotoxic (**Figure 2**). Langseth et al. (*7*) isolated 3-methoxyfuran-2(5*H*)-one as the principal nephrotoxin from *N. ossifragum* and confirmed its toxicity in goats. The activity of the compound in vivo, but not in vitro, may be due to several reasons. Most likely seems the possibility that this furanone is activated metabolically in vivo. It has in many cases been shown that the parent compound is not toxic in a living organism but rather its hepatic metabolite(s) (*16*, *17*). Future studies on the metabolism of 3-methoxyfuran-2(5*H*)-one should test this hypothesis.

Both fractions F I and F III were cytotoxic. Of the two fractions, F III was the most cytotoxic, whereas the cytotoxicity of F I was about 30% that of F III. Fractions F I and F III were subsequently subjected to fractionations in an iterative process by narrowing and retesting of fractions and subfractions in the bioassay to locate and isolate the cytotoxic principles in the extract.

Fraction F I. *N. ossifragum* is known to contain up to four furanone compounds: 3-methoxyfuran-2(5*H*)-one, 4-methoxyfuran-2(5*H*)-one, and 5-hydroxy-4-methoxyfuran-2(5*H*)-one and its glucoside [4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside)] (*1*, *7*, *12*). Whereas 4-methoxyfuran-2(5*H*)-one could not be detected in the aqueous plant extract, the other three compounds were present in approximately equal amounts as far as can be assessed from their relative peak areas from HPLC-PDA-MS runs (**Figure 5**). The integrity of the compounds was verified by one- and two-dimensional NMR spectroscopy, and the ^{1}H and ^{13}C NMR data were compared with the literature data for the identical compounds (*12*). Even though not all of the NMR spectroscopic data matched with those reported in the literature,

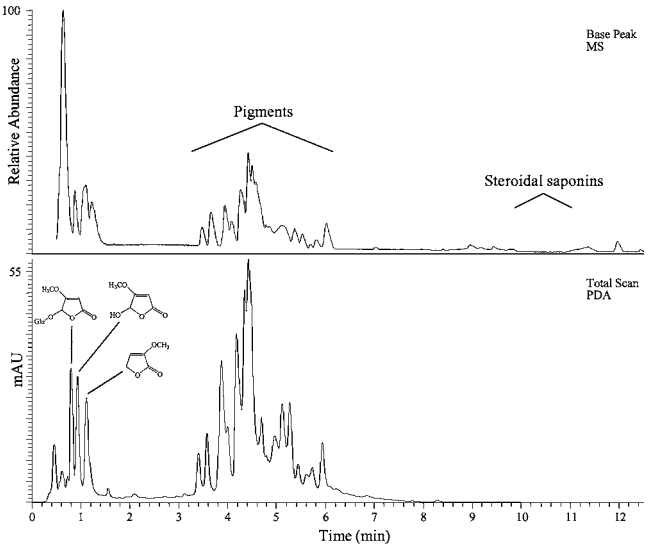

Figure 5. Chromatogram from HPLC-PDA-MS (200–500 nm; 80–1000 u) of the crude aqueous *N. ossifragum* extract.

the three isolated furanones were the same as those previously isolated. Inoue et al. (*10*) presented ^{1}H and ^{13}C NMR data for the 5*S* isomer of 4-methoxyfuran-2(5*H*)-one 5-(*β*-D-glucoside) from *N. asiaticum*. Comparison of their assignments with the NMR spectroscopic data of the identical compound from *N. ossifragum* shows that both plant species contain the same optical isomer. However, several of the NMR assignments presented by Inoue et al. (*10*) are interchanged or misinterpreted (**Table 2**). These are the H-3/H-5 and the H-3′/H-4′ proton shifts as well as the C-3′/C-5′ carbon shifts. The correct assignment for the H-3/H-5 protons was confirmed by HMBC and NOESY. The HMBC spectrum, obtained in pyridine-d_5, showed correlations between H-1′ (5.29 ppm) and C-5 (98.3 ppm). The corresponding HMBC correlation between H-5 (6.47 ppm) and C-1′ (103.5 ppm) was also obvious, whereas no HMBC correlation could be detected between H-3 (5.41 ppm) and C-1 or between H-1 and C-3 (90.3 ppm). Further evidence for our assignment was obtained from NOESY in pyridine-d_5: there was a clear NOE correlation between H-5 (6.47 ppm) and H-1′ (5.29 ppm), but no correlation between H-3 and H-1′. The HMBC and NOESY data acquired in D_2O did show the same relationships. However, whereas Inoue et al. (*10*) reported an incorrect shift assignment for H-3 and H-5, the corresponding carbon shifts are reported correctly. The ^{1}H NMR shift assignments for H-3′ and H-4′, as presented by Inoue et al. (*10*), were likewise interchanged. This was supported by NOESY, acquired in pyridine-d_5. There was a NOE correlation between H-1′ and the signal at 4.21 ppm (in addition to a NOE to H-5′ at 3.91 ppm), whereas there was a NOE correlation between H-2′ and the signal at 4.72 ppm. For *β*-D-glucose, this is expected for the signal at 4.21 ppm being H-3′ and the signal at 4.27 ppm being H-4′. This assignment was further verified from the HMBC correlations, but not as clearly or unambiguously. Furthermore, Inoue et al. (*10*) interchanged the C-3′/C-5′ carbon shifts, as identified by HSQC, and presented two different ^{13}C shift values for C-5. One of these belongs to C-4′, and not C-5 (**Table 2**). Tschesche and Hoppe (*12*) reported confusing ^{1}H NMR data (pyridine-d_5) for the 5*S* isomer of 4-methoxyfuran-2(5*H*)-one 5-(*β*-D-glucoside), which they named narthesid A. In that paper, the ^{1}H resonance for OCH_3 was interchanged with H-5 (H-4 in the original paper). Also, the ^{1}H NMR shift for H-3 (H-2 in the original paper) was reported at 4.71 ppm, which

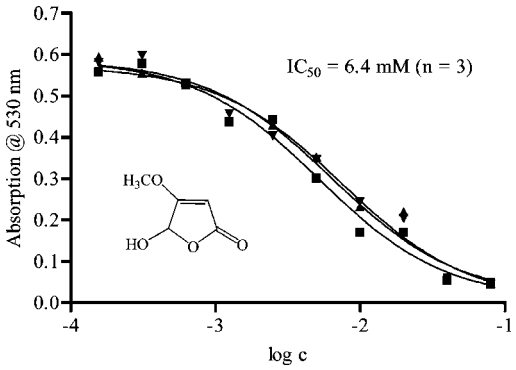

Figure 6. Sigmoidal dose–response plots from the 24 h exposure of LLC-PK1 cells with 5-hydroxy-4-methoxyfuran-2(5*H*)-one.

is far from the shift value of 5.41 ppm, which was found in the present study in the same solvent.

To trace the cytotoxic effect of fraction F I to specific compounds, a new fractionation was set up, which isolated 5-hydroxy-4-methoxyfuran-2(5*H*)-one and its glucoside into separated fractions, whereas the rest of F I was collected in three other fractions (**Figure 3**). The cytotoxic effect was traced to F I-D containing 5-hydroxy-4-methoxyfuran-2(5*H*)-one among minor impurities using the Neutral Red bioassay (**Figure 3**). Of the three furanones, both 5-hydroxy-4-methoxyfuran-2(5*H*)-one and 4-methoxyfuran-2(5*H*)-one 5-(*β*-D-glucoside) have previously shown to be biologically active in bioassays. Tschesche and Hoppe (*12*) found that 5-hydroxy-4-methoxyfuran-2(5*H*)-one had antibiotic activity against *Bacillus subtilus*. More recently, it was shown that 4-methoxyfuran-2(5*H*)-one 5-(*β*-D-glucoside) exhibited good antifungal activity against *Microsporum canis* and *Trichophyton mentagrophytes* and limited antifungal and antiyeast activity against *Penicillium roqueforti* and *Candida glabrata* (*18*). 5-Hydroxy-4-methoxyfuran-2(5*H*)-one was subsequently purified by isocratic semipreparative HPLC of fraction F I-D, and its dose−response relationship in LLC-PK1 cells was established using the Neutral Red bioassay (**Figure 6**). The inhibition concentration (IC_{50}) of 5-hydroxy-4-methoxyfuran-2(5*H*)-one was calculated to be 0.8 mg/mL (6.4 mM). The implications of this finding are difficult to assess because no data about the bioactivity of the compound in vivo are available. However, because the plant might contain milligrams per gram amounts of 5-hydroxy-4-methoxyfuran-2(5*H*)-one, its contribution to the observed nephrotoxicity in *N. ossifragum* grazing animals must be considered.

Fraction F III. The chromatograms from HPLC-PDA-MS runs of fraction F III were dominated by signals caused by structurally related compounds in the *m*/*z* 563−623 range (**Figure 5**). Their similar UV spectra with an absorption maximum around 340 nm and the presence of signals due to multiple water losses in their fragmentation spectra suggested that these might be polyphenolic pigments.

The fractionation procedure was modified such that the related unknown compounds of fraction F III were partitioned into five subfractions, which were tested for cytotoxicity in LLC-PK1. Additionally, the isocratic flush of the column with methanol at the end of the run was collected in a separate fraction and also tested for cytotoxicity. Whereas the five subfractions were not cytotoxic in LLC-PK1 using the Neutral Red assay, the fraction from the column flush resulted in about 90% cell death. A final fractionation experiment focused therefore on the constituents of the *N. ossifragum* extract with the highest retention on the C_{18} column, and 10 fractions were collected. Cytotoxic activity was spread over two fractions (F III-I + F

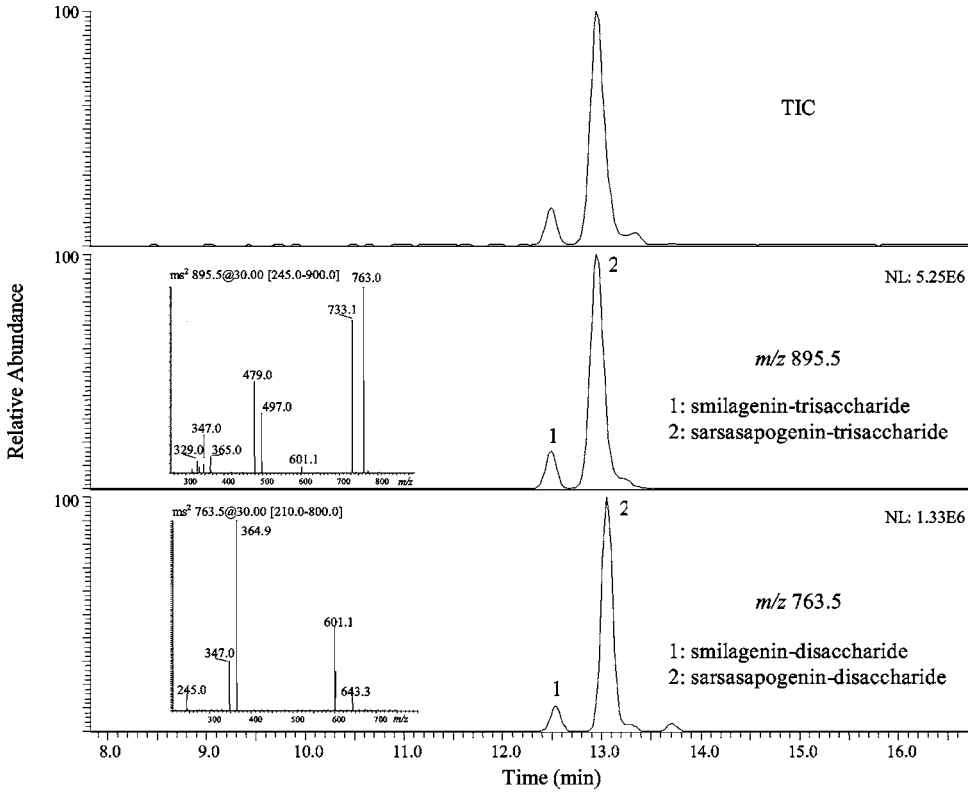

Figure 7. Chromatogram from HPLC-MS of the cytotoxic saponin fraction composed of di- and trisaccharides of smilagenin and sarsasapogenin. Insets represent MS^2 spectra from fragmentation of $[M + Na]^+$. Fragment principal peak assignments-MS^2 of *m/z* 895.5: 763, $[M -$ pentose $+ Na]^+$; 733, $[M -$ hexose $+ Na]^+$; 497, $[2 \times$ hexose + pentose $+ H_2O + Na]^+$; 479, $[2 \times$ hexose + pentose $+ Na]^+$; 365, $[2 \times$ hexose $+ H_2O + Na]^+$, 347, $[2 \times$ hexose $+ Na]^+$, 329, $[2 \times$ hexose $- H_2O + Na]^+$; fragment principal peak assignments-MS^2 of *m/z* 763.5: 643, $[M - C_8H_{15}O_2 + Na]^+$, possibly from cleavage over the E-ring; 601, $[M -$ pentose $+ Na]^+$; 365, $[2 \times$ hexose $+ H_2O + Na]^+$; 347, $[2 \times$ hexose $+ Na]^+$; 245, [hexose $-$ O—CH═CH—OH $+ Na]^+$.

III-J in **Figure 4**). Although the PDA detector did not detect any constituents in these fractions, the HPLC-MS chromatograms showed a pair of signals (peak area about 1:10) with *m/z* 895.5 as the major constituent of both fractions. The relative concentration in the two fractions was in accordance with their measured relative cytotoxicity. The mass spectra of the same peaks showed additional weaker signals at *m/z* 890.5 and 873.5, and therefore it was concluded that the base peak of the compound (*m/z* 895.5) was its sodium adduct $[M + Na]^+$. The same fractions contained a lesser amount of a pair of compounds with a base peak at *m/z* 763.5. MS^2 and MS^3 experiments revealed that the two compounds possess an identical basic structure (**Figure 7**). For the identification of the compounds, the combined fractions F III-I and F III-J were further purified, and the final isolate was analyzed by one- and two-dimensional ^1H and ^{13}C NMR experiments. Initial NMR experiments were carried out in methanol-d_4. A cluster of proton resonances in the range of 3.0−4.1 ppm suggested the presence of sugar moieties. Also, the NMR spectra indicated a saturated molecule, which is coincident with the absence of UV absorbance. Because *Narthecium* spp. are known to contain steroidal saponins, an obvious assumption was that the cytotoxic fractions F III-I and F III-J contained these biologically active compounds that are widespread in the plant kingdom (*1*, *10*, *19*, *20*). Steroidal saponins were not a primary target of the bioassay-guided fractionation because the water solubility of these compounds is relatively low. The assumption that the cytotoxic constituents of fractions F III-I and F III-J were saponins was supported by the MS^n fragmentation spectra (**Figure 7**). These were dominated by signals caused by carbohydrate fragments with the typical mass differences of 132 u for a pentose unit and 162 u of a hexose unit (*21*−*23*). The mass difference between the *m/z* 895.5 and 763.5 species was 132 u and hence indicative for a pentose unit. Detailed NMR data of *Narthecium asiaticum* saponins and *N. ossifragum* sapogenins are available in the literature (*10*, *24*). To compare the NMR data from the literature with the NMR data of the purified cytotoxic fractions (F III-I and F III-J), ^1H and ^{13}C NMR experiments were rerun with pyridine-d_5 as the solvent. This identified the main component of the fraction as a trisaccharide of sarsasapogenin. Sarsasapogenin was also available as reference material, which allowed further verification. The compound possesses a 25*R* configuration, whereas the 25*S* isomer of the same compound is named smilagenin. The ^{13}C shifts of C-20 to C-27 of the potential major saponin in the extract were in excellent agreement with the data published for the 25*R* configuration of the steroidal skeleton (*10*, *15*). However, the NMR spectra of the saponin fraction also displayed, although with much less intensity, signals that belonged to the same steroidal skeleton with the 25*S* configuration (smilagenin) (*10*, *15*).

Stabursvik (*1*) and Čeh and Hauge (*25*) found that the saponins of *N. ossifragum* may be composed of four different sugars, namely, the hexoses D-glucose and D-galactose and the pentoses D-arabinose and D-xylose. The ^{13}C NMR data for the hexoses of the *N. ossifragum* saponins in fractions F III-I and F III-J were in good agreement with those presented by Inoue et al. (*10*) for a D-glucose/D-galactose side chain. However, the NMR data for the pentose unit could not be assigned completely, and it is not clear whether the sapogenin trisaccharides of the cytotoxic fractions solely were composed of arabinose or xylose, or a mixture of both. Saponins are known to act by disintegrating cell membranes, and their permeability and lytic effects have been reported in the literature (*26*). The structure−activity relationship for saponins regarding their effects on cell mem-

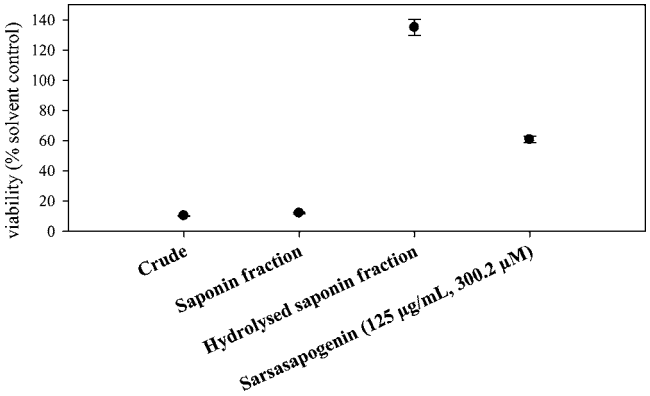

Figure 8. Direct comparison of the cytotoxicity of saponins and sapogenins in LLC-PK1 cells using the Neutral Red cytotoxicity assay. Cells that were exposed to the saponin hydrolysate were exposed to approximately 2.3 μg/mL (5.5 μM) of sarsasapogenin. Error bars represent the standard error of triplicate measurements.

branes is complex and yet not fully understood. For example, the hemolytic action of saponins is believed to be caused by the affinity of the aglycone moiety for membrane sterols (*27*). In addition, the side chains present on the aglycone, for example, sugar chains or acyl residues, are supposed to contribute to their effects on membranes (*26*). The carbohydrate chains of *N. ossifragum* saponins obviously are of major importance for the observed cytotoxicity in LLC-PK1 cells because the toxicity of the saponin fraction was totally abolished after acid hydrolysis (**Figure 8**). This is an important finding for the possible effects of saponins in vivo as previous metabolism studies in sheep have shown that saponins from *N. ossifragum* are predominantly hydrolyzed in the rumen to free sapogenins. A further oxidation and reduction at C-3 to the epi-analogues of the parent sapogenins take place in the liver before conjugation and excretion of epi-sapogenin conjugates (*28*). The role of the saponins or sapogenins in relation to alveld and related hepatogenous photosensitization diseases is still not clarified. Whether the saponins or their metabolites are hepatotoxic per se, thus causing alveld in lambs, or precipitate as insoluble calcium salts of epi-sapogenin glucuronides in the liver and bile of lambs with alveld as a secondary effect of liver damage induced by another factor is disputed (*2*, *4*, *5*, *29*−*31*). Also, sapogenins were not found to be excreted via the kidney in lambs or sheep (*15*, *24*, *28*).

There are two important conclusions to this study. The first is the finding that it is 5-hydroxy-4-methoxyfuran-2(5*H*)-one that has cytotoxic potential rather than 3-methoxyfuran-2(5*H*)-one. Because the former furanone is of higher polarity than the latter it could be postulated that conversion of 3-methoxyfuran-2(5*H*)-one to a more polar metabolite during biotransformation can result in a more toxic product. This would explain the toxicity of 3-methoxyfuran-2(5*H*)-one in vivo and its apparently low toxicity in vitro. The second important conclusion to the study is that the cytotoxicity of the saponins questions if there is an additional link between saponins and disease etiology, namely, the involvement of saponins in the kidney damage that has been observed in goats, lambs, and calves after dosage with *N. ossifragum* plant material. It should therefore be the subject of future research to find out whether ruminants are able to hydrolyze saponins completely and if saponins may be absorbed in the intestinal tract and excreted via the kidney.

ABBREVIATIONS USED

APT, attached proton test; COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; g-HMBC, pulsed field gradient heteronuclear multiple bond correlation; g-HSQC, pulsed field gradient heteronuclear single quantum coherence; g-H2BC, pulsed field gradient heteronuclear two-bond correlations; NOE, nuclear Overhauser effect; TOCSY, total correlation spectroscopy.

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LITERATURE CITED

(1) Stabursvik, A. A phytochemical study of *Narthecium ossifragum* (L.) Huds. Doctoral Thesis, Norges Tekniske Vitenskapsakademi, Trondheim, Noway, 1959.

(2) Ender, F. Undersøkelser over alveldsykens etiologi (Etiological studies on "alveld"—a disease involving photosensitization and icterus in lambs). *Nordisk Vet.-Med.* **1955**, *7*, 329−377.

(3) Flåøyen, A. Plant-associated hepatogenous photosensitization diseases. In *Natural and Selected Synthetic Toxins: Biological Implications*; Gaffield, W., Tu, A. T., Eds.; ACS Symposium Series 745; American Chemical Society: Washington, DC, 2000; pp 204−219.

(4) Abdelkader, S. V.; Ceh, L.; Dishington, I. W.; Hauge, J. G. Alveld-producing saponins. II. Toxicological studies. *Acta Vet. Scand.* **1984**, *25*, 76−85.

(5) Flåøyen, A.; Hjorth, T. H.; Grønstøl, H.; Karlsen, J. Failure to induce toxicity in lambs by administering saponins from *Narthecium ossifragum*. *Vet. Res. Commun.* **1991**, *15*, 483−487.

(6) Wisløff, H.; Wilkins, A. L.; Scheie, E.; Flåøyen, A. Accumulation of sapogenin conjugates and histological changes in the liver and kidneys of lambs suffering from alveld, a hepatogenous photosensitization disease of sheep grazing *Narthecium ossifragum*. *Vet. Res. Commun.* **2002**, *26*, 381−396.

(7) Langseth, W.; Torgersen, T.; Kolsaker, P.; Romming, C.; Jantsch, T. G.; Mantle, P. G.; Pearce, J.; Gibson, S. E.; Goicochea, M. G.; Flåøyen, A. Isolation and characterization of 3-methoxy-2(5*H*)-furanone as the principal nephrotoxin from *Narthecium ossifragum* (L.) Huds. *Nat. Tox.* **1999**, *7*, 111−118.

(8) Wisløff, H.; Flåøyen, A.; Ottesen, N.; Hovig, T. *Narthecium ossifragum* (L.) Huds. causes kidney damage in goats: morphologic and functional effects. *Vet. Pathol.* **2003**, *40*, 317−327.

(9) Wisløff, H.; Gharehnia, B.; Flåøyen, A.; Andersen, K. J. Effects of 3-methoxy-2(5*H*)-furanone-containing extracts from *Narthecium ossifragum* (L.) Huds. on renal tubular cells in vitro. *Toxicon* **2007**, *49*, 368−377.

(10) Inoue, T.; Mimaki, Y.; Sashida, Y.; Kobayashi, M. Structures of toxic steroidal saponins from *Narthecium asiaticum* Maxim. *Chem. Pharm. Bull.* **1995**, *43*, 1162−1166.

(11) Stabursvik, A.; Holen, B. Saxosterol, a new dihydroxy cholesterol from *Narthecium ossifragum*. *Phytochemistry* **1988**, *27*, 1893−1895.

(12) Tschesche, R.; Hoppe, H.-J. Über Narthecid A und B, zwei diastereomere Lacton-Glucoside und ihr antibiotisch wirksames Aglykon aus Beinbrech (*Narthecium ossifragum* Huds.). *Chem. Ber.* **1971**, *104*, 3573−3581.

(13) Lid, J. *Narthecium* Huds. In *Norsk og Svensk Flora*; Det Norske Samlaget: Oslo, Norway, 1974.

(14) Wilkins, A. L.; Miles, C. O.; Smith, B. L.; Meagher, L. P.; Ede, R. M. GC-MS method for analysis of steroidal sapogenins in plant and animal samples associated with ovine photosensitisation. In *Plant-Associated Toxins. Agricultural, Phytochemical and Ecological Aspects*; Colegate, S. M., Dorling, P. R., Eds.; CAB International: Wallingford, U.K., 1994.

(15) Flåøyen, A.; Wilkins, A. L.; Sandvik, M. Ruminal metabolism in sheep of saponins from *Yucca schidigera*. *Vet. Res. Commun.* **2002**, *26*, 159−169.

(16) Burgat-Sacaze, V.; Rico, A. Bioactivation and bound residues. *Food Addit. Contam.* **1984**, *1*, 121−129.
(17) Williams, D. P. Toxicophores: investigations in drug safety. *Toxicology* **2006**, *226*, 3−13.
(18) Loader, J. I. Chemical aspects of saponin bioactivity and ovine photosensitization diseases. Doctoral Thesis, The University of Waikato, New Zealand, 2004.
(19) Cheeke, P. R. Anti-inflammatory and anti-arthritic effects of *Yucca schidigera*: a review. *J. Inflamm.* **2006**, *3*, 1−17.
(20) Yeskaliyeva, B.; Mesaik, M. A.; Abbaskhan, A.; Kulsoom, A.; Burasheva, G. S.; Abilov, Z. A.; Choudhary, M. Q.; Atta, U. R. Bioactive flavonoids and saponins from *Climacoptera obtusifolia*. *Phytochemistry* **2006**, *67*, 2392−2397.
(21) Ahn, M. J.; Kim, J. Identification and quantification of steroidal saponins in *Polygonatum* species by HPLC/ESI/MS. *Arch. Pharm. Res.* **2005**, *28*, 592−597.
(22) Li, B.; Abliz, Z.; Fu, G. M.; Tang, M. J.; Yu, S. S. Characteristic fragmentation behavior of some glucuronide-type triterpenoid saponins using electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 381−390.
(23) Li, R.; Zhou, Y.; Wu, Z. J.; Ding, L. S. Esi-Qqtof-Ms/Ms and Apci-It-Ms/Ms analysis of steroid saponins from the rhizomes of *Dioscorea panthaica*. *J. Mass Spectrom.* **2006**, *41*, 1−22.
(24) Flåøyen, A.; Wilkins, A. L.; Deng, D.; Brekke, T. Ovine metabolism of saponins: evaluation of a method for estimating the ovine uptake of steroidal saponins from *Narthecium ossifragum*. *Vet. Res. Commun.* **2001**, *25*.
(25) Ĉeh, L.; Hauge, J. G. Alveld-producing saponins I. Chemical studies. *Acta Vet. Scand.* **1981**, *22*, 391−402.
(26) Francis, G.; Kerem, Z.; Makkar, H. P. S.; Becker, K. The biological action of saponins in animal systems: a review. *Br. J. Nutr.* **2002**, *88*, 587−605.
(27) Glauert, A. M.; Dingle, J. T.; Lucy, J. A. Action of saponin on biological membranes. *Nature* **1967**, *196*, 953−955.
(28) Flåøyen, A.; Wilkins, A. L. Metabolism of saponins from *Narthecium ossifragum*−a plant implicated in the aetiology of alveld, a hepatogenous photosensitization of sheep. *Vet. Res. Commun.* **1997**, *21*, 335−345.
(29) Kellerman, T. S.; Erasmus, G. L.; Coetzer, J. A.; Brown, J. M.; Maartens, B. P. Photosensitivity in South Africa. VI. The experimental induction of geeldikkop in sheep with crude steroidal saponins from *Tribulus terrestris*. *Onderstepoort J. Vet. Res.* **1991**, *58*, 47−53.
(30) Mathews, F. P. Lecheguilla (*Agave lecheguilla*) poisoning in sheep, goats, and laboratory animals. *Tex. Agric. Exp. Stn. Bull.* **1937**, *No. 554*, 5−36.
(31) Miles, C. O.; Wilkins, A. L.; Munday, S. C.; Flåoyen, A.; Holland, P. T.; Smith, B. L. Identification of insoluble salts of the β-D-glucuronides of episarsasapogenin and epismilagenin in the bile of lambs with alveld, and examination of *Narthecium ossifragum*, *Tribulus terrestris*, and *Panicum miliaceum* for sapogenins. *J. Agric. Food Chem.* **1993**, *41*, 914−917.

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