New Nitric Oxide Inhibitory Spirostane Glycosides from Polygonatum kingianum
Collett & Hemsl

Tran Thi Thu Ha\textsuperscript{1,2}, and Phan Van Kiem\textsuperscript{3,4}

Abstract
Two new spirostane glycosides, (25R)-12β-hydroxyspirost-5-en-3β-yl O-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (I) and (25S)-spirost-5-en-7-one-3β-yl O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (2), and a known spirostane glycoside, funkioside C (3), were isolated from the roots of Polygonatum kingianum Collett & Hemsl. (Asparagaceae). Their structures were determined by extensive analysis of mass spectrometry high resolution electron spray ionization mass spectrum and nuclear magnetic resonance spectral data, as well as by comparison of the spectral data with those reported in the literature. Compound 2 showed inhibitory effects on nitric oxide production in the lipopolysaccharide stimulated RAW 264.7 cells with an IC\textsubscript{50} value of 8.78 ± 0.05 \textmu M compared to a value of 7.12 ± 0.08 \textmu M for the positive control compound, N\textsuperscript{G}-monomethyl-L-arginine.

Keywords
Polygonatum kingianum, polygokingiaside C, polygokingiaside D, nitric oxide inhibitor, spirostane glycoside

Introduction
The phytochemistry of plants belonging to the genus Polygonatum is mainly characterized by steroidal sapogenins (furostan and spirostan aglycone skeleton), such as kингianosides A-D, funkioside C, furostanol sapogenins, 22-hydroxywattinoside C, (25R)-kingianoside G, (25R,5)-pratioside D1, (25R,5)-kingianoside A, kingianoside J, and kingianoside K, and some of which show many interesting activities such as antiviral, antitumor activity, anti-inflammatory, and anti-diabetes, variable effects on the immune system and anticoagulant activity.\textsuperscript{1-10} In our research program to screen medicinal plants with good activity for seedling through in vitro propagation, P kingianum plant has been chosen for the study. Continuing our studies on bioactive compounds from P kingianum plant,\textsuperscript{11} we report herein the isolation, the structure elucidation of 3 steroidal saponins (1-3), and their 3 anti-inflammatory activity evaluated by their inhibition of NO production in LPS stimulated RAW 264.7 cells. Compounds 1 and 2 have not been phytochemically investigated before.

Results and Discussion
Compound 1 was obtained as a colorless amorphous powder and positive in the Liebermann-Burchard reaction. It showed a quasimolecular ion peak at \textit{m}/\textit{z} 777.4034 [M + Na]\textsuperscript{+} (calcd. for [C\textsubscript{39}H\textsubscript{62}O\textsubscript{14}Na]\textsuperscript{+}, 777.4032, \textDelta = +0.2 ppm) in the high-resolution electron spray ionization mass spectrum (HR-ESI-MS), indicating its molecular formula of C\textsubscript{39}H\textsubscript{62}O\textsubscript{14} and 9\textdegree of unsaturation (see Supplemental Figure S1). The IR spectrum of 1 exhibited the presence of hydroxy (3371 cm\textsuperscript{-1}) and C–O–C (1056 cm\textsuperscript{-1}) groups. The nuclear magnetic resonance (NMR) spectra of 1 were similar to the corresponding spectra of 3 except for the additional signal of a methine carbonyl group (\textdelta\textsubscript{H} 3.30/ \textdelta\textsubscript{C} 80.3) in the NMR spectra of 1.

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suggesting that 1 was a spirostan glycoside having 2 sugar moieties (see Supplemental Figures S2 and S3).4,7,9,11,12 The methine carbon signal at $\delta_C$ 82.1/441.41 (m) and the quaternary carbon signal at $\delta_C$ 110.8 were typical for C-16 and C-22, respectively, of the spirostan skeleton.4,7,9,11,12 In the heteronuclear multiple bond correlation (HMBC) spectrum, the H3 to 18 protons correlated to the oxygenated methine carbon at $\delta_C$ 80.3 confirming that the hydroxy group attached to C-12. Detailed analysis of the $^1$H NMR and $^{13}$C NMR spectra, combined with the heteronuclear single quantum coherence (HSQC) and HMBC spectra of 1 (see Supplemental Figures S4 and S5) indicated that the aglycone of this compound was similar to that of pratioside E1, isolated from Polygonatum prattii4,7,9,11,12. The carbon chemical constants of H-6 and H-17 of this compound were further indicated from the large proton coupling rotations.14 Thus, compound 1 was determined to be (25R)-12$\beta$-hydroxyspirost-5-en-3$\beta$-yl O-$\beta$-D-glucopyranosyl-

| Pos. | $\delta_C$ | $\delta_{1H}$ (mult., $J$ in Hz) | Pos. | $\delta_C$ | $\delta_{1H}$ (mult., $J$ in Hz) |
|------|------------|---------------------------------|------|------------|---------------------------------|
| 1    | 38.5       | 1.11 (m), 1.89 (m)              | 2    | 37.5       | 1.24 (m, H$_{\alpha}$), 2.00 (m, H$_{\beta}$) |
| 2    | 30.7       | 1.62 (m), 1.92 (m)              | 3    | 30.4       | 1.76 (m), 2.05 (m)              |
| 3    | 80.0       | 3.56 (m)                        | 4    | 78.7       | 3.73 (m)                        |
| 4    | 39.7       | 2.28 (dd, 12.5, 11.5)           | 5    | 39.7       | 2.49 (dd, 12.5, 11.5)           |
| 5    | 142.0      | -                               | 6    | 169.0      | -                               |
| 6    | 122.6      | 5.38 (brd, 3.0)                 | 7    | 126.4      | 5.72 (brs 1.5)                  |
| 7    | 32.9       | 1.55 (m), 2.04 (m)              | 8    | 204.1      | -                               |
| 8    | 31.8       | 1.63 (m)                        | 9    | 46.1       | 2.51 (m)                        |
| 9    | 51.3       | 1.00 (m)                        | 10   | 51.3       | 1.55 (m)                        |
| 10   | 38.1       | -                               | 11   | 39.9       | -                               |
| 11   | 31.4       | 1.50 (m), 1.70 (m)              | 12   | 22.1       | 1.64 (m), 1.66 (m)              |
| 12   | 80.3       | 3.30 (*)                        | 13   | 39.9       | 1.20 (m), 1.79 (m)              |
| 13   | 46.9       | -                               | 14   | 42.2       | -                               |
| 14   | 56.4       | 1.12 (m)                        | 15   | 50.9       | 1.43 (m)                        |
| 15   | 32.5       | 1.42 (m), 2.03 (m)              | 16   | 34.8       | 2.81 (dd, 7.5, 7.0), 1.42 (*)   |
| 16   | 82.1       | 4.41 (m)                        | 17   | 82.0       | 4.47 (m)                        |
| 17   | 63.2       | 1.91 (m)                        | 18   | 62.5       | 1.73 (*)                        |
| 18   | 11.0       | 0.80 (s)                        | 19   | 16.9       | 0.84 (s)                        |
| 19   | 19.8       | 1.08 (s)                        | 20   | 17.7       | 1.28 (s)                        |
| 20   | 43.7       | 1.90 (m)                        | 21   | 43.4       | 1.89 (m)                        |
| 21   | 14.0       | 1.05 (d, 7.0)                   | 22   | 14.8       | 1.03 (d, 7.0)                   |
| 22   | 110.8      | -                               | 23   | 111.1      | -                               |
| 23   | 31.8       | 1.40 (m), 1.72 (m)              | 24   | 29.9       | 1.65 (m), 1.45 (m)              |
| 24   | 29.9       | 1.43 (m), 1.64 (m)              | 25   | 26.7       | 1.44 (m), 2.05 (m)              |
| 25   | 31.5       | 1.62 (m)                        | 26   | 28.5       | 1.70 (m)                        |
| 26   | 67.8       | 3.34 (dd, 10.0, 9.0), ax        | 27   | 66.1       | 3.30 (dd, 10.0, 1.2)            |
| 27   | 17.5       | 0.81 (d, 7.0)                   | 28   | 16.4       | 3.97 (dd, 10.0, 3.0)            |

Asterisk indicates overlapped signals.
Table 2. NMR Spectroscopic Data for the Sugar Moieties of 1 and 2 in Deuterated Methanol.

| Pos. | δC (multiplicity, J in Hz) | δH (multiplicity, J in Hz) |
|------|----------------------------|----------------------------|
| 1    |                            |                            |
| 3-O-gal | 1’ | 103.0 | 4.34 (dd, 7.5) |
|       | 2’ | 73.2 | 3.49 (ddd, 9.0, 7.5) |
|       | 3’ | 75.2 | 3.58 (dd, 9.0, 9.0) |
|       | 4’ | 80.3 | 4.08 (dd, 3.0) |
|       | 5’ | 75.7 | 3.28 (m) |
|       | 6’ | 61.3 | 3.63 (dd, 12.0, 5.0) |
|       |    |     | 3.88 (dd, 12.0, 2.0) |
| 4’-O-glc | 1” | 106.1 | 4.51 (dd, 7.5) |
|       | 2” | 75.7 | 3.54 (s) |
|       | 3” | 78.3 | 3.38 (dd, 9.0, 9.0) |
|       | 4” | 72.0 | 3.23 (dd, 9.0, 9.0) |
|       | 5” | 78.0 | 3.32 (dd, 9.0, 9.0) |
|       | 6” | 63.2 | 3.60 (dd, 12.0, 5.0) |
|       |    |     | 3.91 (dd, 12.0, 2.0) |
| 2’-O-glc | 1’’’ | 106.2 | 4.69 (dd, 8.0) |
|       | 2’’’ | 76.3 | 3.29 (s) |
|       | 3’’’ | 77.6 | 3.41 (s) |
|       | 4’’’ | 70.8 | 3.24 (s) |
|       | 5’’’ | 78.7 | 3.36 (m) |
|       | 6’’’ | 62.0 | 3.82 (dd, 12.0, 5.0) |
|       |    |     | 3.98 (dd, 12.0, 2.0) |

Asterisk indicates overlapped signals.

(1→4)-β-D-galactopyranoside and named polygokningiaside C (Tables 1 and 2, Figure 1).

Compound 2 was obtained as a colorless amorphous powder, positive in the Liebermann-Burchard reaction, and its molecular formula was deduced to be C_{45}H_{74}O_{19} based on the HR-ESI-MS results (see Supplemental Figure S10). The IR spectrum of 2 exhibited the presence of hydroxyl (3381 cm\(^{-1}\)), carbonyl (1667 cm\(^{-1}\)), and C-O-C (1070 cm\(^{-1}\)) groups. The NMR spectra of 2 (see Supplemental Figures S11 and S12) were similar to those of 1 except for the disappearance of the 12-OH group signals and additional typical signals of one hexose unit [δC/δH: 106.2/4.69, 76.3/3.29, 73.7/3.14, 70.8/3.41, 78.7/3.36, 62.0/(3.98 and 3.82)] and 1 carbonyl group (δC 204.1). The signals at δC 169.0, 126.4, and 204.1 were assigned for C-5, C-6, and C-7, respectively, by comparing with the corresponding values of 7-oxodioscin\(^{10}\) and further confirmed by the HMBC correlations from H\(_1\) to 19 (δH 1.28) to C-5 (δC 169.0), from H-6 (δH 5.72) to C-4 (δC 39.7)/C-8 (δC 46.1), and from H-8 (δH 2.51) to C-7 (δC 204.1). The methine carbonyl signals at δC 82.0/δH 4.47 (m) and the quaternary carbon at δC 111.1 were typical for C-16 and C-22 of the spirostan skeleton.\(^{9,11,12}\) The (25S)-configuration was suggested by comparing the carbon chemical shifts of C-24, C-25, C-26 of 2 with those of OJV-IV (25S) and OJV-III (25R),\(^{13}\) and further confirmed by the small J\(_{1H,13C}\) values (J = 3.0 Hz, 1.2 Hz). Moreover, the aglycone of 2 was determined to be spirostan-3β-hydroxy-5-en-7-one, similar to the aglycone of kinganioside K isolated from P kingianum,\(^{4}\) confirmed by analyzing the 1H-1H COSY and HMBC spectra as shown in Supplemental Figure S17 (see Supplemental Figures S14 and S15). Furthermore, H\(_3\) to 19 (δH 1.28) had NOESY cross peak to H\(_{5r-1}\) (δH 2.00) indicating that H\(_{5r-1}\) was at δH 1.24, which had NOESY cross-peaks to H-3 (δH 3.73). This confirmed Hα-3 (see Supplemental Figure S14). The additional sugar was suggested to link to C-2' indicated from HSQC cross-peaks of H-1 (δH 4.57)/C-1 (δC 104.9) and H-2 (δH 3.55)/C-2 (δC 85.0) (see Supplemental Figure S11), the 1H-1H COSY cross peak of H-1'' (δH 4.57) and H-2'' (δH 3.55), and from the HMBC correlation from H-1' (δH 4.69) to C-2' (δC 85.0). In addition, HMBC correlations from H-1' to C-4' and from H-1' to C-3 determined the second sugar attached to C-4' of the galactose, and the galactose linked to C-3 of the aglycon. The large J values of the anomeric proton (J = 7.5-8.0 Hz) indicated β-glycoside linkages for the sugar moieties. Finally, acid hydrolysis of 2 obtained D-glucose and D-galactose, which were identified by TLC comparison with the authentic samples and from the positive sign of the optical rotations.\(^{14}\) Thus, compound 2 was determined to be (25S)-spirost-5-en-7-one-3β-yl O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside and named polygokningiaside D (Tables 1 and 2, Figure 1).

Compound 3 was identified as fukinioside C.\(^{7}\) The NMR spectral data of 3 were consistent with those previously reported in the literature (see Supplemental Figures S15 and S16).

The anti-inflammatory activity of compounds 1 to 3 was evaluated by their ability to inhibit NO production in LPS stimulated RAW 264.7 cells. Each compound was assessed at a concentration of 20 µM. No cytotoxic effect on the RAW
264.7 cells was found after treating the cells with compounds 1 to 3 (20 µM). L-NMMA (N\textsuperscript{G}-monomethyl-L-arginine) was used as a positive control. Compound 2 exhibited the highest inhibitory, 68.3%, comparing to the positive control, L-NMMA: 83.1% (Supplemental Table S1). Therefore, compound 2 was further evaluated for its IC\textsubscript{50} value. Compound 2 showed the inhibitory effects on NO production with an IC\textsubscript{50} value of 8.78 ± 0.05 µM compared to a value of 7.12 ± 0.08 µM for the positive control compound, L-NMMA.

**Material and Methods**

**General Experimental Procedures**

The general experimental procedures are the same as described in our previous work.\textsuperscript{11} Refer to Supplemental Material.

**Plant Material**

The plant materials are the same as described in our previous work.\textsuperscript{11}

**Extraction and Isolation**

Continue studying the water layer as described in our previous work,\textsuperscript{11} the fraction IPK2C was chromatographed on a silica gel column eluting with CH\textsubscript{2}Cl\textsubscript{2}/MeOH/H\textsubscript{2}O (3/1/0.1,v/v/v) to give 5 subfractions (IPK2C1-IPK2C5). The IPK2C3 was further chromatographed by HPLC using a J\textsuperscript{’}sphere ODS H-80, 250 mm × 20 mm column, MeCN in H\textsubscript{2}O (30%, v/v), and a flow rate of 3 mL/min to yield compounds 1 (17 mg) and 3 (14.5 mg). The IPK2C4 was further chromatographed by HPLC using a J\textsuperscript{’}sphere ODS H-80, 250 mm × 20 mm column, MeCN in H\textsubscript{2}O (35%, v/v), and a flow rate of 3 mL/min to yield compound 2 (14.0 mg).

**Polygokingiaside C (1).** Colorless amorphous powder, [\(\alpha\)]\textsubscript{D}\textsuperscript{25} −43.5° (c 0.05, MeOH); IR (KBr) \(v_{\text{max}}\): 3371 (broad), 2928, 2873, 1455, 1056 cm\textsuperscript{−1}. HR-ESI-MS \(m/z\) 777.4032 [M + Na]+ (calcd. for [C\textsubscript{39}H\textsubscript{62}O\textsubscript{14}Na]+, 777.4032, \(\Delta = +0.2\) ppm); \(\text{\textsuperscript{1}H NMR (CD}_{3}\text{OD, 500 MHz)}\) and \(\text{\textsuperscript{13}C NMR (CD}_{3}\text{OD, 125 MHz)}\) data are given in Tables 1 and 2.

**Polygokingiaside D (2).** Colorless amorphous powder, [\(\alpha\)]\textsubscript{D}\textsuperscript{25} −52.0° (c 0.05, MeOH); IR (KBr) \(v_{\text{max}}\): 3381 (broad), 2930, 1667, 1452, 1070 cm\textsuperscript{−1}. HR-ESI-MS \(m/z\) 949.4192 [M + 35\text{Cl}]+ (calcd. for [C\textsubscript{45}H\textsubscript{70}O\textsubscript{19}35\text{Cl}]+, 949.4200, \(\Delta = −0.8\) ppm, \(m/z\) 951.4194 [M + 37\text{Cl}]+ [calcd. for [C\textsubscript{45}H\textsubscript{70}O\textsubscript{19}37\text{Cl}]+, 951.4170, \(\Delta = +2.5\) ppm]. \(\text{\textsuperscript{1}H NMR (CD}_{3}\text{OD, 500 MHz)}\) and \(\text{\textsuperscript{13}C NMR (CD}_{3}\text{OD, 125 MHz)}\) data are given in Tables 1 and 2.

![Figure 1. Chemical structure of compounds 1 to 3.](image-url)
Acid Hydrolysis of Compounds 1 and 2. Compounds 1 and 2 (each, 9.0 mg) were dissolved in 0.5 mL of 6 M HCl and heated at 60°C for 1.5 h. After cooling, the mixtures were extracted with EtOAc. The acid aqueous layer was neutralized with 1 M NaOH and freeze-dried. Two sugars were identified as glucose and galactose by comparison with authentic samples by TLC using MeCOEt–isoPrOH–Me2CO–H2O (20:10:7:6). After preparative TLC of the sugar mixture (for each 1 and 2, 3.0 mg) using this solvent, each isolated sugar was filtered to eliminate SiOH residue. The optical rotation of each purified sugar was measured to afford glucose and galactose as +19.5 (c 0.15, H2O) and +45.7 (c 0.15, H2O), respectively. By comparing the optical rotations with D-glucose [α]D+18.0 (c 0.1, H2O) and D-galactose [α]D+45.0 (c 0.08, H2O), the glucose and galactose in compounds 1 and 2 were determined to have D-configurations.14

Nitric Oxide Assay

Refer to Supplemental Material.

Conclusions

Phytochemical study on the methanol extracts of the roots of *P. kingianum* Collett & Hemsl. (Asparagaceae) led to the isolations of 2 new spirostane glycosides, (25R)-12β-hydroxyspirost-5-en-3β-yl O-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (1) and (25S)-spirost-5-en-7-one-3β-yl O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-β-D-galactopyranoside (2), and a known spirostane glycoside, funkioside C (3). Their structures were determined by extensive analysis of HR-ESI-MS and NMR spectral data, as well as by comparison of the spectral data with those reported in the literature. The anti-inflammatory activity of the isolated compounds was evaluated by their inhibition of NO production in LPS stimulated RAW 264.7 cells. Interestingly, compound 2 showed inhibitory effects on NO production with an ICα0 value of 8.78 ± 0.05 µM compared to a value of 7.12 ± 0.08 µM for the positive control compound, L-NMMA. These results suggested the selection of the medicinal plant varieties for further breeding in the future.

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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**Abbreviations**

- NMR: nuclear magnetic resonance
- COSY: correlation spectroscopy
- HRESIMS: high-resolution electrospray ionization mass spectrometry
- HMBC: heteronuclear multiple bond correlation
- HSQC: heteronuclear single quantum coherence
- NOESY: nuclear Overhauser effect spectroscopy
- NO: nitric oxide
- LPS: lipopolysaccharide.