New Cardenolides from the Seeds of *Adonis aestivalis*

Satoshi Kubo,^a Minpei Kuroda,^{*,a} Yukiko Matsuo,^a Daichi Masatani,^a Hiroshi Sakagami,^b and Yoshihiro Mimaki^a

^aDepartment of Medicinal Pharmacognosy, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan; and ^bDivision of Pharmacology, Department of Diagnostic and Therapeutic Sciences, School of Dentistry, Meikai University; 1-1 Keyaki-dai, Sakado, Saitama 350-0283, Japan. Received May 29, 2012; accepted June 30, 2012

Chemical investigation of the seeds of *Adonis aestivalis* has led to the isolation of a new cardenolide (3 β ,5 α ,14 β ,17 β -tetrahydroxycard-20,22-enolide) (1), two new glycosides (2, 3) of 1, and a new strophanthidin hexaglycoside (4), together with a known compound, strophanthidin 3-*O*- β -D-glucopyranoside (5). The structures of 1–4 were determined by 1D- and 2D-NMR spectroscopic analysis and the results of hydrolytic cleavage. The isolated compounds (1–5) were examined for their cytotoxic activity against neoplastic HSC-2, HSC-3, HSC-4, and HL-60 cells, as well as HGF, HPLF, and HPC normal cell lines. Compounds 2, 4, and 5 were found to display selective cytotoxicity toward malignant tumor cell lines. Although the morphological observations of HL-60 and HSC-2 cell deaths by 2, 4, and 5 revealed changes characteristic of apoptosis, neither DNA degradation nor activation of caspase-3 was observed. Our findings demonstrated that 2, 4, and 5 may trigger caspase-3-independent apoptotic cell death in HL-60 and HSC-2 cells.

Key words *Adonis aestivalis*; Ranunculaceae; cardenolide; cytotoxic activity; malignant tumor cell line; apoptosis

Our previous phytochemical studies have revealed that plants belonging to family Ranunculaceae such as *Eranthis cilicica*,¹⁾ *Cimicifuga racemosa*,^{2,3)} *Helleborus orientalis*,⁴⁾ *Clematis chinensis*,⁵⁾ and *Pulsatilla chinensis*^{6,7)} contain a variety of steroid and triterpene glycosides. Examples include oleanane octa- and nonaglycosides, named eranthisaponins A

and B, from *Eranthis cilicica*,¹⁾ and cycloartane glycosides, named neocimicigenosides A and B, from *Cimicifuga racemosa*,²⁾ which promote corticotropin releasing factor (CRF)-stimulated adrenocorticotrophic hormone (ACTH) secretion from AtT-20 cells. A novel spirostane glycoside from *Helleborus orientalis* has a unique structure of oxygen atoms at C-1,

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: kurodam@toyaku.ac.jp

© 2012 The Pharmaceutical Society of Japan

C-3, C-21, C-23, and C-24 of the spirostanol skeleton with a new acetylated tetraglycoside, a diglycoside, and a monosaccharide at C-1, C-21, and C-24 of the aglycone, respectively.⁴⁾ Recently, several new chromone glycosides were isolated from *Eranthis cilicica* and characterized.⁸⁾ Over some years, other research groups have isolated several triterpene glycosides from *Thalictrum fortunei*,^{9,10)} *Nigella damascene*,¹¹⁾ and *Cimicifuga Rhizome*,¹²⁾ and furostanol and flavonoid glycosides from *Helleborus viridis*.¹³⁾ As part of our continuing search for bioactive compounds from plants belonging to family Ranunculaceae, we investigated the MeOH extract of the seeds of *Adonis aestivalis* L., which is native to Europe, resulting in the isolation of a new cardenolide (**1**), two new glycosides (**2**, **3**) of **1**, and a new strophanthidin hexaglycoside (**4**), together with a known compound, strophanthidin 3-*O*- β -D-glucopyranoside (**5**). The cytotoxic activity of the isolated compounds was evaluated against human malignant cell lines (HSC-2, HSC-3, HSC-4, and HL-60) and normal human cells (HGF, HPC, and HPLF). The expected modes of action of **2**, **4**, and **5** are also reported.

The seeds of *A. aestivalis* were extracted with hot MeOH. The MeOH extract was passed through a porous-polymer polystyrene resin (Diaion HP-20) column, and the eluted fraction was subjected to column chromatography using silica gel and octadecylsilanized (ODS) silica gel, giving compounds **1**–**5**. Compound **5** was identified as strophanthidin 3-*O*- β -D-glucopyranoside.¹⁴⁾

Compound **1** was obtained as an amorphous solid. Its molecular formula was determined as $C_{23}H_{34}O_6$ on the basis of the high resolution (HR)-electrospray ionization (ESI)-time-of flight (TOF)-MS data (m/z : 429.2253 [$M+Na$]⁺). The IR spectrum suggested the presence of hydroxy (3369 cm^{-1}) and carbonyl (1739 cm^{-1}) groups. A cardenolide skeleton was evident from the characteristic ^1H - and ^{13}C -NMR spectra of **1** (Table 1), which displayed signals for an α,β -unsaturated- γ -lactone group [δ_{H} 6.32 (brs), 5.25 (1H, dd, $J=18.3, 1.8\text{ Hz}$), and 5.11 (1H, dd, $J=18.3, 1.8\text{ Hz}$); δ_{C} 174.0 (C), 173.2 (C), 116.7 (CH), and 73.4 (CH_2)], two tertiary methyls (δ_{H} 1.28, 1.17; δ_{C} 16.9, 13.1), eight methylenes, three methines including an oxygenated methine (δ_{C} 67.5), and five quaternary carbons, three of which were oxygenated (δ_{C} 87.8, 86.7, 74.1). The spectroscopic data of **1** are suggestive of a cardenolide derivative with four hydroxy groups. The unequivocal structure of **1** was determined by the following spectroscopic analysis. The connectivity of the protonated carbons (C-1 through C-4; C-6 through C-9; C-9, C-11, and C-12; C-15 and C-16) was determined from the ^1H - ^1H shift correlation spectroscopy (COSY), total correlated spectroscopy (TOCSY), and ^1H -detected heteronuclear multiple-quantum coherence (HMQC) spectra. The carbon–carbon connections through the nonprotonated carbon atoms were established by the following correlations of the ^1H -detected heteronuclear multiple-bond connectivity (HMBC): C-5 (δ_{C} 74.1) with H-4eq (δ_{H} 2.18), H-6eq (δ_{H} 1.74), and Me-19 (δ_{H} 1.17); C-10 (δ_{C} 40.3) with H-4eq, H-6ax (δ_{H} 2.03), and Me-19; C-13 (δ_{C} 51.9) with H-12eq (δ_{H} 1.20), H-12ax (δ_{H} 1.11), H-15 (δ_{H} 2.04), H-16 (δ_{H} 2.37), and Me-18 (δ_{H} 1.28); C-14 (δ_{C} 87.8) with H-8 (δ_{H} 2.00), H-15, H-16 and Me-18; and C-17 (δ_{C} 86.7) with H-15, H-16, and Me-18 (Fig. 1). These data identified hydroxy groups at C-3, C-5, C-14, and C-17. The structure of the γ -lactone moiety and its connection of C-20 to C-17 of the D ring part were confirmed by HMBC correlations

Fig. 1. The Plane Structure of **1**

Fig. 2. NOE Correlations of **1**

between the C-23 carbonyl carbon (δ_{C} 174.0) and the H-21 oxymethylene protons (δ_{H} 5.25, 5.11)/H-22 olefinic proton (δ_{H} 6.32), C-17 and H-22, and between the C-20 olefinic carbon (δ_{C} 173.2) and H-21/H-22. The phase-sensitive nuclear Overhauser effect spectroscopy (NOESY) correlations between Me-19 and H-1eq (δ_{H} 1.71)/H-2ax (δ_{H} 1.89)/H-6ax/H-8/H-11ax (δ_{H} 1.55), and between H-1ax (δ_{H} 2.10) and H-9 (δ_{H} 1.91), allowed determination of the A/B *trans* and B/C *trans* ring connections (Fig. 2). Furthermore, NOE correlations from Me-18 to two hydroxy groups at C-14 (δ_{H} 6.51) and C-17 (δ_{H} 6.69) and to H-21b (δ_{H} 5.11), and from H-22 to H-12ax, H-12eq, and H-16, confirmed the C/D *cis* ring junction and 17 β -hydroxy configuration. The orientation of the oxygen atom at C-3 was determined to be in the β form on the basis of the H-3 coupling constants ($^3J_{\text{H-2ax,H-3}}=14.1\text{ Hz}$, $^3J_{\text{H-3,H-4ax}}=12.4\text{ Hz}$) and NOE correlations between H-3 and H-2ax/H-4ax. From the analysis of these spectral data, the structure of **1** was designated as 3 β ,5 α ,14 β ,17 β -tetrahydroxycard-20(22)-enolide.

Compound **2** was shown to have a molecular formula $C_{29}H_{44}O_{11}$ on the basis of the HR-ESI-TOF-MS data (m/z : 591.2780 [$M+Na$]⁺). The ^1H - and ^{13}C -NMR signals arising from the aglycone moiety of **2** were almost superimposable with those of **1**. However, the molecular formula of **2** was higher than that of **1** by $C_6H_{10}O_5$. In addition, a signal assignable to an anomeric proton of a hexopyranosyl group was observed at δ_{H} 5.18 (d, $J=7.9\text{ Hz}$). Enzymatic hydrolysis of **2** with naringinase gave **1** and D-glucose. Identification of the D-glucose, including its absolute configuration, was carried out by direct HPLC analysis of the hydrolysate. When

Table 1. ¹H-NMR Data for **1–3** in C₅D₅N

1			2		3	
Positions	δ_{H}	J (Hz)	δ_{H}	J (Hz)	δ_{H}	J (Hz)
1 ax	2.10 ddd	14.1, 14.1, 3.3	1.96 m		1.96 m	
1 eq	1.71 brd	14.1	1.63 brd	14.4	1.64 brd	14.1
2 ax	1.89 dddd	14.3, 14.1, 14.1, 3.7	1.76 m		1.76 m	
2 eq	2.12 brd	14.3	2.23 brd	14.1	2.21	
3	4.74 m	$W_{1/2}$ =26.6	4.86 m	$W_{1/2}$ =23.8	4.81 m	$W_{1/2}$ =22.4
4 ax	2.50 dd	12.8, 12.4	2.47 dd	12.4, 12.2	2.46 dd	12.5, 12.1
4 eq	2.18 dd	12.8, 4.5	2.27 dd	12.4, 3.8	2.21	
5	—		—		—	
6 ax	2.03 m		1.98 m		1.99 m	
eq	1.74 brd	13.2	1.66 brd	13.4	1.64	
7 ax	1.49 dddd	13.6, 13.2, 13.2, 4.4	1.36 m		1.37 dddd	13.8, 13.0, 13.0, 3.6
7 eq	2.27 brdd	13.2, 2.4	2.20 brdd	13.2, 2.2	2.21	
8	2.00 ddd	11.8, 11.8, 3.5	1.94 m		1.94 ddd	12.0, 12.0, 2.8
9	1.91 ddd	11.8, 11.8, 3.5	1.78 ddd	11.8, 11.8, 3.3	1.77 ddd	12.0, 12.0, 2.8
10	—		—		—	
11 ax	1.27 dddd	13.4, 12.6, 12.6, 3.3	1.21 dddd	13.3, 12.5, 12.5, 2.7	1.20 m	
11 eq	1.55 brdd	13.4, 3.3	1.42 brdd	13.3, 2.7	1.43 brdd	13.3, 3.2
12 ax	1.11 ddd	13.4, 13.4, 3.3	1.01 ddd	13.2, 13.2, 3.3	1.03 ddd	13.0, 13.0, 3.0
12 eq	1.20 brd	13.4	1.13 brd	13.2	1.15 brd	13.5
13	—		—		—	
14	—		—		—	
15 (2H)	2.04 m		1.97 m		1.98 m	
16 (2H)	2.37 brdd	8.0, 8.0	2.37 brdd	9.4, 6.4	2.39 brdd	8.0, 8.0
17	—		—		—	
18	1.28 s		1.24 s		1.25 s	
19	1.17 s		1.11 s		1.11 s	
20	—		—		—	
21 a	5.25 dd	18.3, 1.8	5.25 dd	18.3, 1.4	5.26 dd	18.3, 1.8
21 b	5.11 dd	18.3, 1.8	5.10 dd	18.3, 1.4	5.11 dd	18.3, 1.8
22	6.32 brs		6.35 brs		6.37 brs	
23	—		—		—	
14-OH	6.51 s		6.48 s			
17-OH	6.69 s		6.70 s			
1'			5.08 d	7.9	5.04 d	7.8
2'			4.06 dd	8.8, 7.9	4.06 dd	8.7, 7.8
3'			4.27 dd	8.8, 8.8	4.30 dd	8.8, 8.7
4'			4.30 dd	8.8, 8.8	4.40 dd	9.3, 8.8
5'			3.93 m		3.89 m	
6' a			4.47 dd	11.7, 2.0	4.56 dd	11.9, 3.5
6' b			4.39 dd	11.7, 4.8	4.36 dd	11.9, 2.6
1''					5.23 d	7.9
2''					4.12 dd	8.6, 7.9
3''					4.23 dd	8.7, 8.6
4''					4.21 dd	9.0, 8.7
5''					4.01 m	
6'' a					4.54 dd	11.5, 2.4
6'' b					4.31 dd	11.5, 5.6

the ¹³C-NMR spectrum of **2** was compared with that of **1**, a downfield shift of C-3 (+8.0 ppm) and upfield shifts of C-2 (−4.0 ppm) and C-4 (−2.5 ppm) were detected. These results indicated that **2** was a β-D-glucopyranoside (Glc) of **1**. Furthermore, a long-range correlation was observed between the anomeric proton of Glc and C-3 of the aglycone at δ_{C} 75.5 in the HMBC spectrum of **2**. The structure of **2** was

thus confirmed as 3β-[(O-β-D-glucopyranosyl)oxy]-5α,14β,17β-trihydroxycard-20(22)-enolide.

Compound **3** exhibited a molecular formula of C₃₅H₅₄O₁₆ on the basis of the HR-ESI-TOF-MS data (m/z : 753.3320 [M+Na]⁺). The ¹H- and ¹³C-NMR spectral data showed that the structure of the aglycone moiety of **3** was identical to that of **2**. The molecular formula of **3** differed from that of

Fig. 3. HMBC Correlations of the Sugar Moiety of **4**

2 by $C_6H_{10}O_5$, and the 1H -NMR spectrum showed signals for two anomeric protons at δ_H 5.23 (d, $J=7.9$ Hz) and 5.04 (d, $J=7.8$ Hz). Enzymatic hydrolysis of **3** with β -glucosidase produced a partial hydrolysate, identified as **2**, along with D-glucose. Analysis of the 1H - 1H COSY spectrum, starting from each anomeric proton, and the HMQC spectrum of **3** led to the assignment of all proton and carbon signals due to the sugar moieties. The 1H - and ^{13}C -NMR shifts thus assigned were indicative of a terminal β -D-glucopyranosyl unit (Glc'') and an inner β -D-glucopyranosyl residue glycosylated at C-4 (Glc'). In the HMBC spectrum, the anomeric proton signal (H-1) of Glc'' at δ_H 5.23 showed a long-range correlation with C-4 of Glc' at δ_C 81.3, whereas the H-1 signal of Glc' at δ_H 5.04 was correlated to C-3 of the aglycone at δ_C 75.5. Thus, the diglycoside of glucosyl-(1 $\rightarrow$ 4)-glucosyl unit was shown to be attached to C-3 of the aglycone. The structure of **3** was determined as 3 β -[(*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-glucopyranosyl)oxy]-5 α ,14 β ,17 β -trihydroxycard-20(22)-enolide.

Compound **4** had a molecular formula of $C_{61}H_{96}O_{28}$ on the basis of the HR-ESI-TOF-MS data (m/z : 1299.6085 [$M+Na$] $^+$). The IR spectrum of **4** showed the absorption bands for hydroxy (3393 cm^{-1}) and carbonyl (1737 cm^{-1}) groups. The 1H -NMR spectrum displayed signals for a methyl group at δ_H 1.00 (s, Me-18), an aldehyde group at δ_H 10.40 (s, H-19), an oxymethylene group at δ_H 5.29 and 5.03 (each dd, $J=18.0$, 1.3 Hz, H₂-21), and an olefinic group at δ_H 6.13 (brs, H-22), which are characteristic of the cardenolide skeleton, as well as signals for six anomeric protons at δ_H 5.39 (dd, $J=9.3$, 1.2 Hz), 5.37 (dd, $J=9.4$, 1.3 Hz), 5.19 (d, $J=7.8$ Hz), 5.10 (d, $J=7.8$ Hz), 4.84 (brd, $J=10.2$ Hz), and 4.74 (dd, $J=9.7$, 1.2 Hz). The methyl carbon signals at δ 18.7, 18.5 $\times$ 2 and 18.1, and proton signals at δ_H 1.67 (d, $J=6.4$ Hz), 1.39 (d, $J=6.2$ Hz), 1.37 (d, $J=6.2$ Hz), and 1.30 (d, $J=6.2$ Hz) suggested that **4** possessed four deoxy sugars. Acid hydrolysis of **4** with 0.025 M H_2SO_4 gave 3 β ,5 β ,14 β -trihydroxy-19-oxocard-20(22)-enolide (strophanthidin),¹⁵ and D-oleandrose, D-diginose, D-digitoxose, and D-glucose as the carbohydrate components. Severe overlapping of the proton signals for the sugar moieties excluded the possibility of a complete assignment in a straightforward way using conventional 2D-NMR methods, such as 1H - 1H COSY, 2D TOCSY, and HMQC spectra. Analysis of the 1D TOCSY spectra followed by interpretation of the 1H - 1H COSY, heteronuclear single quantum coherence (HSQC)-TOCSY, and HMQC spectra allowed the exact sugar sequences of the monosaccharides to be determined. The 1H -NMR subspectra of the individual monosaccharide units were obtained by using selective irradiation of easily identifiable anomeric proton

signals at δ_H 5.39, 5.37, 5.19, 5.10, 4.84, and 4.74, as well as irradiation of other non-overlapping proton signals at δ_H 1.67, 1.39, 1.37, and 1.30 in a series of 1D TOCSY experiments.^{16,17} Subsequent analysis of the 1H - 1H COSY spectrum resulted in the sequential assignment of all proton resonances due to the six monosaccharide units, including identification of their signal multiplet patterns and coupling constants. The proton resonances were correlated with those of the corresponding one-bond coupled carbons using the HMQC and HSQC-TOCSY spectra, leading to unambiguous assignment of the carbon shifts. A comparison of the carbon chemical shifts thus assigned with those reported in the literature,^{18–20} taking into account the known effects of the *O*-glycosylation shift, indicated that **4** contained two 4-substituted β -D-digitoxopyranosyl (4C_1) units (Dgt' and Dgt''), a 4-substituted β -D-oleandropyranosyl (4C_1) unit (Ole'''), a 4-substituted β -D-diginopyranosyl (4C_1) unit (Dgn'''), a 6-substituted β -D-glucopyranosyl (4C_1) unit (Glc'''), and a terminal β -D-glucopyranosyl (4C_1) unit (Glc'''). The β -orientations of the anomeric centers of these monosaccharides were supported by the relatively large $^3J_{H-1,H-2(ax)}$ values of their anomeric protons (7.8–10.2 Hz). In the HMBC spectrum of **4** (Fig. 3), correlation peaks were observed between δ_H 5.19 (H-1 of Glc''') and δ_C 70.4 (C-6 of Glc'''), δ_H 5.10 (H-1 of Glc''') and δ_C 73.1 (C-4 of Dgn'''), δ_H 4.84 (H-1 of Dgn''') and δ_C 82.6 (C-4 of Ole'''), δ_H 4.74 (H-1 of Ole''') and δ_C 83.2 (C-4 of Dgt''), δ_H 5.39 (H-1 of Dgt'') and δ_C 82.9 (C-4 of Dgt'), and between δ_H 5.37 (H-1 of Dgt') and δ_C 74.9 (C-3 of aglycone). Thus, the structure of **4** was characterized as strophanthidin 3-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-diginopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-oleandropyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-digitoxopyranosyl-(1 $\rightarrow$ 4)- β -D-digitoxopyranoside.

In our quest to identify compounds with preferential cytotoxicity toward malignant tumor cells, we used HSC-2, HSC-3, and HSC-4 human oral squamous cell carcinoma and HL-60 human promyelocytic leukemia cells, as well as human non-malignant HGF gingival fibroblasts, HPLF periodontal ligament fibroblasts, and HPC pulp cells. The results for these assays are summarized in Table 4. Compounds **2**, **4**, and **5** showed potent cytotoxic activity against HSC-2, HSC-3, HSC-4, and HL-60 cells and displayed selective cytotoxicity toward malignant cells. The average CC₅₀ values of these compounds against malignant cells were lower than those of positive controls. It is notable that among the 5 α -cardenolides (**1–3**) only monoglucoside (**2**) was cytotoxic toward the tumor cell lines. Experiments were performed to determine the mechanism of cytotoxicity induced by compounds **2**, **4**,

Fig. 4. Morphological Observations of Representative Fields of HL-60 and HSC-2 Cells Stained with DAPI to Evaluate Fragmented and Condensed Nuclear Chromatins after Treatment with **2**, **4**, **5**, or Etoposide

and **5**, which showed potent cytotoxic activities against malignant cells. As is evident from the micrographs, HL-60 and HSC-2 cell death caused by **2**, **4**, and **5** (Fig. 4) was partially mediated through the induction of apoptosis: nuclear chromatin condensation and cell shrinkage were observed. The typical biochemical hallmarks of apoptosis are degradation of DNA by endogenous DNases and activation of caspase-3; however, the typical laddering of DNA fragmentation was not detected when HL-60 and HSC-2 cells were treated with **2** (0.5–8.0 μM), **4** (0.05–0.8 μM), and **5** (0.01–0.16 μM) for 6 h (data not shown). Similarly, caspase-3 was not activated when HL-60 and HSC-2 cells were treated with **2**, **4**, and **5** under the above conditions (data not shown). Our findings demonstrated that **2**, **4**, and **5** may induce caspase-3 independent apoptotic cell death in HL-60 and HSC-2 cells.

Experimental

Optical rotations were measured using a JASCO P-1030 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer, and UV spectra on a JASCO V-630 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^1H -NMR, Karlsruhe, Germany) and a Bruker AV-600 (600 MHz for ^1H -NMR) spectrometer using standard Bruker pulse programs. Chemical shifts are given as δ -value with reference to tetramethylsilane (TMS) as an internal standard. ESI-TOF-MS data were obtained on a Waters-Micromass LCT (Manchester, U.K.) mass spectrometer. Porous-polymer polystyrene resin (Diaion HP-20, Mitsubishi-Chemical, Tokyo, Japan), silica gel (300 mesh, Fuji-Silysia Chemical, Aichi, Japan), and ODS silica gel (75 μm , Nacalai-Tesque, Kyoto, Japan) were used for column chromatography. TLC was carried out on precoated Silica gel 60 F₂₅₄ (0.25 mm, Merck, Darmstadt, Germany) and RP-18 F_{254S} (0.25 mm thick, Merck) plates, and spots were visualized by spraying with 10% H_2SO_4 aq. solution followed by heating. HPLC was performed by using a system comprised of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh) or a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne injection port. The following reagents

were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, U.S.A.); fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, U.S.A.); RPMI1640 medium, RNase A, proteinase K, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, U.S.A.). All other chemicals used were of biochemical reagent grade.

Plant Material The seeds of *A. aestivalis* were obtained from a wholesale firm in Richters, Ontario, Canada in 2000. A voucher specimen was deposited in our laboratory (Voucher No. AA-05-001, Department of Medicinal Pharmacognosy).

Extraction and Isolation The seeds of *A. aestivalis* (4.0 kg) were extracted with hot MeOH (6 L). The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (180 g) was passed through a Diaion HP-20 column (20% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc). The MeOH eluate portion (fraction C, 60 g) was chromatographed on silica gel eluted with CHCl_3 -MeOH gradients (19:1, 9:1, 4:1, 2:1), and finally with MeOH alone, to afford nine fractions (C-i-C-ix). Fraction C-ii was subjected to column chromatography on ODS silica gel and eluted with MeCN-H₂O (1:2; 2:3; 1:1), and finally MeOH alone, to give 13 subfractions (C-ii-a-C-ii-m). Fraction C-ii-a was submitted to column chromatography on silica gel eluted with CHCl_3 -MeOH-H₂O (90:10:0, 60:10:1) and ODS silica gel with MeOH-H₂O (1:1) and MeCN-H₂O (2:7) to give **1** (9.7 mg). Column chromatography of fraction C-iv on ODS silica gel eluted with MeCN-H₂O (2:3) followed by MeOH alone gave eight subfractions (C-iv-a-C-iv-h). Fraction C-iv-a was chromatographed on silica gel eluted with CHCl_3 -MeOH-H₂O (40:10:1, 20:10:1, 7:4:1) to give seven fractions (C-iv-a-1-C-iv-a-7). Fraction C-iv-a-2 was subjected to silica gel column chromatography eluted with CHCl_3 -MeOH-H₂O (40:10:1) and ODS silica gel column chromatography with MeOH-H₂O (2:3) and MeCN-H₂O (1:4) to give **2** (42.1 mg) and **5** (20.3 mg). Fraction C-iv-a-7 was chromatographed on silica gel eluted with CHCl_3 -MeOH-H₂O (40:10:1, 50:10:1) and ODS silica gel with MeCN-H₂O (1:2) to yield **4** (22.9 mg). Fraction C-v was subjected to a silica gel column eluted with CHCl_3 -MeOH-H₂O (7:4:1) and an ODS silica

gel column with MeCN–H₂O (2:5) and MeOH–H₂O (1:2, 2:5) to give **3** (11.1 mg).

Compound 1: Amorphous solid. $[\alpha]_D^{26} +60.1$ ($c=0.10$, MeOH). HR-ESI-TOF-MS m/z : 429.2253 $[M+Na]^+$ (Calcd for C₂₃H₃₄NaO₆: 429.2253). UV [MeOH, nm (log ϵ): 234 (3.45). IR $\nu_{\max}$ (film) cm⁻¹: 3369 (OH), 1739 (C=O). ¹H-NMR data (500 MHz, C₅D₅N): see Table 1. ¹³C-NMR (125 MHz, C₅D₅N): see Table 2.

Compound 2: Amorphous solid. $[\alpha]_D^{26} +68.3$ ($c=0.10$, MeOH). HR-ESI-TOF-MS m/z : 591.2780 $[M+Na]^+$ (Calcd for C₂₉H₄₄NaO₁₁: 591.2781). UV [MeOH, nm (log ϵ): 234 (3.59). IR $\nu_{\max}$ (film) cm⁻¹: 3398 (OH), 1742 (C=O). ¹H-NMR data (500 MHz, C₅D₅N): see Table 1. ¹³C-NMR (125 MHz, C₅D₅N): see Table 2.

Enzymatic Hydrolysis of 2 Compound **2** (5.0 mg) was dissolved in AcOH–NaOAc buffer (pH 4.3, 2 mL) with naringinase (Sigma-Aldrich, 15.0 mg) and incubated at room temperature for 48 h. The crude reaction mixture was applied to a silica gel column eluted with CHCl₃–MeOH–H₂O (7:4:1) followed by MeOH to yield **1** (2.0 mg) and a sugar fraction (2.3 mg). The sugar fraction was analyzed by HPLC under the following conditions: column, Shodex Sugar SC1011 (8.0 mm

i.d.×300 mm, 5 μ m, Showa-Denko); solvent, H₂O; flow rate, 1.0 mL/min; column temperature, 80°C; detection, RI and OR. Identification of D-glucose was carried out by comparison of its retention time and optical rotation with those of an authentic sample. t_R (min): 7.19 (D-glucose, positive polarity).

Compound 3: Amorphous solid. $[\alpha]_D^{25} +61.5$ ($c=0.10$, MeOH). HR-ESI-TOF-MS m/z : 753.3320 $[M+Na]^+$ (Calcd for C₃₅H₅₄NaO₁₆: 753.3310). UV [MeOH, nm (log ϵ): 230 (3.69). IR $\nu_{\max}$ (film) cm⁻¹: 3393 (OH), 1735 (C=O). ¹H-NMR data (500 MHz, C₅D₅N): see Table 1. ¹³C-NMR (125 MHz, C₅D₅N): see Table 2.

Enzymatic Hydrolysis of 3 Compound **3** (3.6 mg) was treated with β -D-glucosidase (Sigma-Aldrich, 15.0 mg) in AcOH–NaOAc buffer (pH 5.0, 10 mL) at room temperature for 40 h. The reaction mixture was applied to a silica gel column eluted with CHCl₃–MeOH (7:4:1) followed by MeOH to yield **2** (1.5 mg) and a sugar fraction (0.8 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of that of **2** showed the presence of D-glucose.

Compound 4: Amorphous solid. $[\alpha]_D^{23} +23.9$ ($c=0.10$, MeOH). HR-ESI-TOF-MS m/z : 1299.6085 $[M+Na]^+$ (Calcd for C₆₁H₉₆NaO₂₈: 1299.5986). UV [MeOH, nm (log ϵ): 224 (3.99). IR $\nu_{\max}$ (film) cm⁻¹: 3393 (OH), 1737 (C=O). ¹H-NMR data (500 MHz, C₅D₅N): see Table 3. ¹³C-NMR (125 MHz, C₅D₅N): see Table 3.

Acid Hydrolysis of 4 A solution of **4** (4.0 mg) in 0.025 M H₂SO₄ (dioxane–H₂O, 1:1, 2.0 mL) was heated at 95°C for 1 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93SB (Organo, Tokyo, Japan) column and chromatographed on silica gel eluted with CHCl₃–MeOH–H₂O (40:10:1) to yield strophanthidin (0.2 mg) and a sugar fraction (2.5 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of **2** showed the presence of D-glucose, D-oleandrose, D-diginose, and D-digitoxose; t_R (min) 7.19 (D-glucose, positive polarity), 7.67 (D-oleandrose, negative polarity), 9.09 (D-digitoxose, positive polarity), and 9.10 (D-diginose, positive polarity).

Assay for Cytotoxic Activity The methodology of the assays using HSC-2, HSC-3, HSC-4, HL-60, HGF, HPC, and HPLF cells has been described previously.²¹⁾ Normal human oral cells (HGF, HPC, and HPLF) were prepared from the extracted tooth of a 12-year-old girl, according to the guideline of the Intramural Board of Ethics Committee (No. A0808) after obtaining informed consent. In brief, cells were incubated at 37°C for 48 h in DMEM medium supplemented with 10% heat-inactivated FBS, except for HL-60 cells which were cultured in RPMI1640 containing 10% FBS. Cell viability was assessed using the MTT method for all except HL-60 cells in which case the trypan blue exclusion procedure was used.

Assay for DNA Fragmentation HSC-2 cells (1.2×10^5) were inoculated onto 6-well plates and incubated for 48 h to allow complete adherence to the plate. HL-60 cells (5×10^5) were inoculated onto 24-well plates, and used immediately. Cells were cultured for 6 h with test compounds, harvested, washed once with phosphate-buffered saline (PBS) (–), and lysed. The lysate was incubated with RNase A and proteinase K, and then mixed with NaI solution, followed by EtOH. After centrifugation, the precipitate was washed with 70% EtOH and dissolved in Tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer. The sample was then applied to 2% agarose gel

Table 2. ¹³C-NMR Data for **1–3** in C₅D₅N

Positions	1	2	3
1	30.5	30.0	30.0
2	31.6	27.6	27.6
3	67.5	75.5	75.5
4	43.6	41.1	41.1
5	74.1	74.0	74.0
6	37.2	37.0	37.0
7	23.7	23.5	23.6
8	41.2	41.0	41.1
9	39.2	39.1	39.1
10	40.3	40.3	40.3
11	21.0	20.9	20.9
12	33.8	33.7	33.7
13	51.9	51.8	51.9
14	87.8	87.7	87.7
15	31.6	31.4	31.5
16	37.4	37.3	37.3
17	86.7	86.6	86.7
18	13.1	13.0	13.1
19	16.9	16.7	16.8
20	173.2	173.2	173.2
21	73.4	73.4	73.4
22	116.7	116.6	116.7
23	174.0	174.1	174.1
Glc 1'		102.7	102.3
2'		75.3	74.9
3'		78.6	76.9
4'		71.6	81.3
5'		78.3	76.3
6'		62.6	62.0
Glc 1''			105.0
2''			74.8
3''			78.2
4''			71.5
5''			78.5
6''			62.4

Table 3. ^1H - and ^{13}C -NMR Data for **4** in $\text{C}_5\text{D}_5\text{N}$

Positions	δ_{H}	J (Hz)	δ_{C}	Positions	δ_{H}	J (Hz)	δ_{C}
1 ax	2.57 ddd	14.6, 14.6, 3.2	18.5	Dgt 1''	5.39 dd	9.3, 1.2	99.8
1 eq	1.89 m			2'' ax	1.94 br t	9.3	38.7
2 ax	1.66 m		25.6	2'' eq	2.40 br d	12.0	
2 eq	2.18 br dd	14.1, 2.6		3''	4.61 m		67.4
3	4.33 br s	$W_{1/2}=8.6$	74.9	4''	3.47 dd	9.6, 3.0	83.2
4 ax	2.17 m		36.2	5''	4.29 m		68.8
4 eq	1.70 m			6''	1.37 d	6.2	18.5
5	—		74.0				
6 ax	2.29 m		36.9	Ole 1'''	4.74 dd	9.7, 1.2	101.4
6 eq	1.77 m			2''' ax	1.65 ddd	12.2, 9.7, 8.8	37.0
7 ax	1.42 m		24.8	2''' eq	2.43 ddd	12.2, 4.9, 1.2	
7 eq	2.45 m			3'''	3.49 m		79.2
8	2.28 m		41.9	4'''	3.38 dd	8.8, 8.8	82.6
9	1.75 m		39.5	5'''	3.58 m		71.5
10	—		55.3	6'''	1.30 d	6.2	18.7
11 ax	1.37 m		22.6	OMe	3.44 s		56.8
11 eq	1.56 m						
12 ax	1.34 m		39.5	Dgn 1'''	4.84 br d	10.2	101.1
12 eq	1.41 m			2''' ax	2.30 q-like	12.8	33.1
13	—		49.8	2''' eq	2.14 br d	12.8	
14	—		84.4	3'''	3.39 m		80.0
15 a	2.06 m		32.1	4'''	4.33 br s		73.1
15 b	1.83 m			5'''	3.54 m		71.0
16 a	2.06 m		27.2	6'''	1.67 d	6.4	18.1
16 b	1.95 m			OMe	3.39 s		56.3
17	2.78 br d	8.7	51.1				
18	1.00 s		16.0	Glc 1''''	5.10 d	7.8	104.6
19	10.40 s		208.5	2''''	3.94 dd	8.8, 7.8	75.7
20	—		175.7	3''''	4.16 dd	8.9, 8.8	78.3
21 a	5.29 dd	18.0, 1.3	73.7	4''''	4.00 dd	9.3, 8.9	71.8
21 b	5.03 dd	18.0, 1.3		5''''	4.08 m		77.6
22	6.13 br s		117.8	6'''' a	4.84 br d	11.8	70.4
23	—		174.5	6'''' b	4.31 dd	11.8, 6.7	
Dgt 1'	5.37 dd	9.4, 1.3	97.6	Glc 1''''	5.19 d	7.8	105.6
2' ax	1.92 br t	9.4	38.7	2''''	4.05 dd	8.5, 7.8	75.2
2' eq	2.32 br d	11.6		3''''	4.26 dd	9.1, 8.5	78.5
3'	4.62 m		67.4	4''''	4.24 dd	9.1, 9.1	71.7
4'	3.51 dd	9.5, 2.6	82.9	5''''	3.96 m		78.5
5'	4.26 m		68.6	6'''' a	4.54 dd	11.7, 2.3	62.7
6'	1.39 d	6.2	18.5	6'''' b	4.38 dd	11.7, 5.3	

Table 4. Cytotoxic Activities of **1–5**, Peplomycin, 5-FU, Melphalan, and Etoposide against Malignant and Normal Cells^{a)}

Compound	Human tumor cells, CC_{50}^b (μM)					Human normal cells, CC_{50} (μM)		
	HSC-2	HSC-3	HSC-4	HL-60	ave ^{c)}	HGF	HPC	HPLF
1	>160	>160	>160	>160	>160	>160	>160	>160
2	0.97	2.8	2.4	0.84	1.75	18	13	12
3	>160	>160	>160	>160	>160	110	>160	142
4	0.11	0.55	0.52	0.086	0.32	5.8	>160	6.0
5	0.013	0.062	0.028	0.012	0.029	0.28	>160	0.43
Peplomycin	5.9	21.3	24.8	n.d. ^{d)}		222	177	143
5-FU	42	89	>94	>502		>640	>338	>550
Melphalan	8.7	25	32	1.4	16.8	>160	>160	>160
Etoposide	24	n.d.	n.d.	0.3		n.d.	>100	n.d.

^{a)} Cells were treated for 48 h with various concentrations of each sample. ^{b)} The CC_{50} figure is the concentration of compound required to kill 50% of the cells and is the average of three independent determinations. ^{c)} Average CC_{50} value of the compound toward HSC-2, HSC-3, HSC-4, and HL-60 neoplastic cells. ^{d)} Not determined.

electrophoresis in Tris-Borate-EDTA (TBE) buffer.²¹⁾ DNA molecular marker (Bayou Biolabs, Harahan, LA, U.S.A.) and DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m²/min, 1 min) were run in parallel as positive controls.²²⁾ After staining with ethidium bromide, DNA was visualized by UV irradiation and photographed.²¹⁾

Assay for Caspase Activation HSC-2 cells (3×10^5) were inoculated onto an 85-mm dish and incubated for 48 h to allow complete adherence. HL-60 cells (1×10^6) were inoculated onto a 6-well plate and used immediately. The cells were further incubated for 3 h in fresh medium with or without (control) the sample. Cells were washed with PBS (–) and lysed in the lysis solution. The supernatant was collected after standing the lysate on ice for 10 min and centrifugation for 5 min at $21000 \times g$. Lysate (50 μ L, equivalent to 200 μ g protein) was mixed with 50 μ L lysis solution containing substrates for caspase-3 (DEVD-pNA (*p*-nitroanilide)) (Kamiya Biomedical, Seattle, WA, U.S.A.). After incubation for 4 h at 37°C, the absorbance of the liberated chromophore pNA was measured at 405 nm using a microplate reader.²¹⁾

References

- 1) Watanabe K., Mimaki Y., Sakuma C., Sashida Y., *J. Nat. Prod.*, **66**, 879–882 (2003).
- 2) Mimaki Y., Nadaoka I., Yasue M., Ohtake Y., Ikeda M., Watanabe K., Sashida Y., *J. Nat. Prod.*, **69**, 829–832 (2006).
- 3) Watanabe K., Mimaki Y., Sakagami H., Sashida Y., *Chem. Pharm. Bull.*, **50**, 121–125 (2002).
- 4) Watanabe K., Mimaki Y., Sakagami H., Sashida Y., *J. Nat. Prod.*, **66**, 236–241 (2003).
- 5) Mimaki Y., Yokosuka A., Hamanaka M., Sakuma C., Yamori T., Sashida Y., *J. Nat. Prod.*, **67**, 1511–1516 (2004).
- 6) Mimaki Y., Yokosuka A., Kuroda M., Hamanaka M., Sakuma C., Sashida Y., *J. Nat. Prod.*, **64**, 1226–1229 (2001).
- 7) Mimaki Y., Kuroda M., Asano T., Sashida Y., *J. Nat. Prod.*, **62**, 1279–1283 (1999).
- 8) Kuroda M., Uchida S., Watanabe K., Mimaki Y., *Phytochemistry*, **70**, 288–293 (2009).
- 9) Sun H., Zhang X.-T., Wang L., Zhang X.-Q., Wang Y., Chen S.-B., Xiao P.-G., Ye W.-C., *Helv. Chim. Acta*, **91**, 1961–1966 (2008).
- 10) Zhang X.-T., Zhang L.-H., Ye W.-C., Zhang X.-L., Yin Z.-Q., Zhao S.-X., Yao X.-S., *Chem. Pharm. Bull.*, **54**, 107–110 (2006).
- 11) Yoshimitsu H., Nishida M., Okawa M., Nohara T., *Chem. Pharm. Bull.*, **55**, 488–491 (2007).
- 12) Yoshimitsu H., Nishida M., Nohara T., *Chem. Pharm. Bull.*, **55**, 789–792 (2007).
- 13) Braca A., Prieto J. M., De Tommasi N., Tomè F., Morelli I., *Phytochemistry*, **65**, 2921–2928 (2004).
- 14) Abe F., Mori Y., Yamauchi T., Sakai Y., *Chem. Pharm. Bull.*, **36**, 3811–3815 (1988).
- 15) Kopp B., Krenn L., Kubelka E., Kubelka W., *Phytochemistry*, **31**, 3195–3198 (1992).
- 16) Yokosuka A., Mimaki Y., Sakuma C., Sashida Y., *Steroids*, **70**, 257–265 (2005).
- 17) Yokosuka A., Kawakami S., Haraguchi M., Mimaki Y., *Tetrahedron*, **64**, 1474–1481 (2008).
- 18) Warashina T., Noro T., *Phytochemistry*, **53**, 485–498 (2000).
- 19) Bai H., Li W., Koike K., Satou T., Chen Y., Nikaido T., *Tetrahedron*, **61**, 5797–5811 (2005).
- 20) Abe F., Yamauchi T., *Chem. Pharm. Bull.*, **26**, 3023–3027 (1978).
- 21) Sekine T., Takahashi J., Nishishiro M., Arai A., Wakabayashi H., Kurihara T., Kobayashi M., Hashimoto K., Kikuchi H., Katayama T., Kanda Y., Kunii S., Motohashi N., Sakagami H., *Anticancer Res.*, **27** (1A), 133–143 (2007).
- 22) Yanagisawa-Shiota F., Sakagami H., Kuribayashi N., Iida M., Sakagami T., Takeda M., *Anticancer Res.*, **15**, 259–265 (1995).