Bioorganic & Medicinal Chemistry Letters 20 (2010) 4927–4931

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Structure–activity relationship of lupane-triterpene glycosides from *Acanthopanax koreanum* on spleen lymphocyte IL-2 and IFN-γ

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ARTICLE INFO

Article history:
Received 11 April 2010
Revised 4 June 2010
Accepted 8 June 2010
Available online 15 June 2010

Keywords:
Acanthopanax koreanum
Araliaceae
Lupane-triterpene glycoside
Acankoreosides M–O
IL-2
IFN-γ

ABSTRACT

Phytochemical investigation resulted in the isolation of three new lupane-triterpene glycosides acankoreosides M–O (**1**, **2** and **8**) together with eight known lupane-triterpene glycosides (**3**–**7**, **9**–**11**) from the leaves of *Acanthopanax koreanum* (Araliaceae). Their chemical structures were elucidated by mass, 1D and 2D NMR spectroscopy. The effect of eleven lupane-triterpene glycosides on Con A-induced splenolytic production of IL-2 and IFN-γ were measured as markers of acquired immune responses. Compounds **4**, **5**, **7**, and **11** (5, 25, and 100 μM) significantly increased IFN-γ and IL-2 release in spleen cells.

Both IL-2 and IFN-γ play an important role in the immune-regulation in the body. IL-2 is normally produced by the body during an immune response.[1,2] IL-2 is indispensable for the development of T-cell immunologic memory, one of the unique characteristics of the immune system, and also vital during T cell development in the thymus for the maturation of a unique subset of T cells that is termed regulatory T cells.[3] Thus, IL-2 is required to discriminate between self and none-self, another one of the unique characteristics of the immune system.[1,2] In addition, IL-2 is able to promote production of immunoglobulins made by B cells and induce the differentiation and proliferation of natural killer cells.[4] IFN-γ mediates a number of modulator signals at several points in the pathway of the progression of B-lymphocyte responses.[5,6] Concanavalin A (Con A), a lymphocyte mitogen, induced splenolytic production of IL-2 and IFN-γ were measured as markers of acquired immune responses.[7]

Acanthopanax koreanum Nakai is deciduous scrub of the Araliaceae family and an endemic species in Korea. Its root and stem barks have been traditionally used by practitioners of oriental medicine as a tonic, as a prophylactic, and to treat rheumatism, paralysis, hepatitis, and diabetes.[8,9] Previous phytochemical and biological investigations of *A. koreanum* have yielded a number of triterpenes.[10–14]

In the preliminary data, the methanol extracts of leaves and stems of *A. koreanum* showed the immune enhancement activity on Con A-induced splenolytic production of IL-2 and IFN-γ. In order to clarify the active compounds for immune responses, three new lupane-type glycosides (**1**, **2**, and **8**) and eight compounds [acankoreoside I (**3**), acankoreoside A (**4**), acankoreoside D (**5**), acankoreoside F (**6**), and acantrifoside A (**7**), acankoreosides J (**9**), acankoreoside K (**10**), and acankoreoside L (**11**)] (see Fig. 1) previously isolated from the leaves of *A. koreanum*,[15,16] were tested their immune enhancement activity.

By using combined chromatographic separations, three new lupane-triterpene glycosides were isolated from the methanol extract of *A. koreanum* leaves.[17] Compound **1** was obtained as a white amorphous powder. Its basic ion peak at *m/z* 989.5 $[M+H]^+$ was observed in the positive-ion ESI-MS, and HRTOFMS analysis revealed the molecular formula to be $C_{48}H_{76}O_{21}$, with a cluster ion peak at *m/z* 989.4989 $[M+H]^+$ (calcd for $C_{48}H_{77}O_{21}$: 989.4957). The ^1H NMR spectrum of **1** (in methanol-d_4) showed the following signals: four tertiary methyl groups at δ_H 0.96, 1.08, 1.10, and 1.18 (each 3H, s); one secondary methyl group at δ_H 1.27, assigned to H-6″ of the rhamnose; and three anomeric protons were at δ_H 4.40, 4.85, and 5.46, suggesting the appearance of

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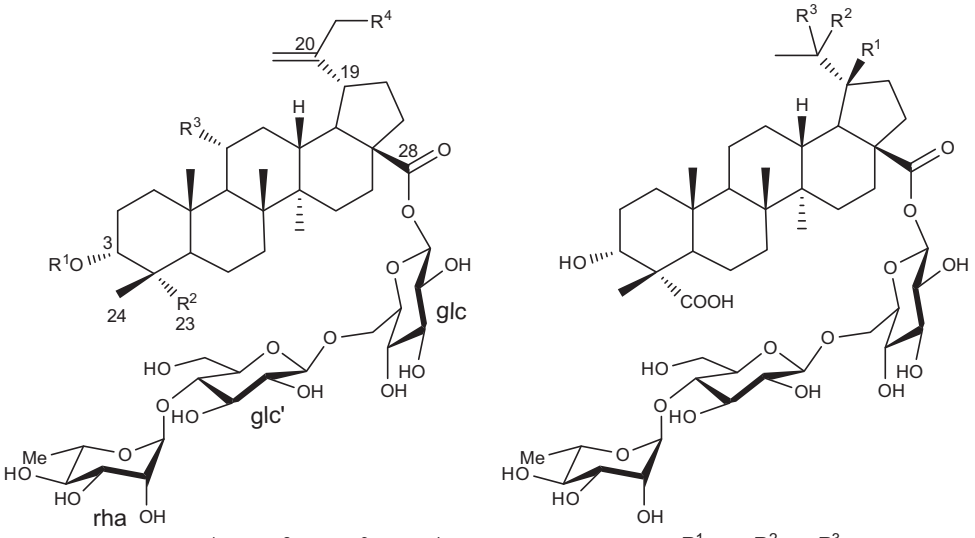
doi:10.1016/j.bmcl.2010.06.044



	R^1	R^2	R^3	R^4
1	H	COOH	OH	OH
2	Glc	CH_3	OH	OH
3	H	CHO	OH	OH
4	H	COOH	H	H
5	H	CHO	OH	H
6	H	COOH	H	OH
7	H	CH_3	OH	H

	R^1	R^2	R^3
8	OH	H	CH_3
9	H	=O	-
10	H	OH	CH_2OH
11	H	H	$CH(OCH_3)_2$

Figure 1. Structure of triterpenes (**1**–**11**) isolated from leaves of *A. koreanum*.

three sugar units. The ^{13}C NMR and DEPT spectra revealed 48 carbon signals, of which, 30 signals were assigned to a triterpenoid sapogenol moiety and 18 signals belong to three monosaccharide moieties. The aglycone of **1** was recognized to be a lupane-type triterpene by ^1H and ^{13}C NMR analysis (see Table 1), with the typical olefinic carbons at δ_C 155.9 and 107.7, four quaternary methyl carbons at δ_C 15.1, 17.5, 18.1, and 18.2, two oxymethine carbons at δ_C 71.1 and 73.8, one oxymethylene carbon at δ_C 65.5, and two carboxyl signals at δ_C 176.3 and δ 180.6. Assignment of the α-hydroxyl group at C-3 was performed by comparing its spectral data with literature values.[10–14] The chemical shifts of C-1 (35.5), C-3 (δ_C 73.8), and C-4 (δ_C 53.0) in the aglycone further confirmed the *axial* configuration of the 3-hydroxyl group by comparing with the corresponding data of the 3β-hydroxy-lup-20(29)-ene-23,28-dioic acid[18] [δ_C values for C-1 (39.0), C-3 (δ_C 84.4), and C-4 (δ_C 43.0)]. Moreover, the chemical shifts of C-11 (δ_C 71.1) and C-23 (δ_C 180.6) were also similar to those of 3α,11α-dihydroxy-lup-20(29)-ene-23,28-dioic acid[19] suggesting that the α-hydroxyl group was at C-11 and the carboxylic group was at C-23. In the HMBC spectrum, the methyl group H-24 (δ_H 1.18) correlated with carbons C-3 (δ_C 73.8), C-4 (δ_C 53.0), and C-23 (δ_C 180.6). In the ROESY spectrum, cross peaks between H-24 (δ_H 1.18) and H-25 (δ_H 1.10) as well as H-3 (δ_H 3.67) indicated that the methyl group at C-4 was *axial*, which in turn suggested that the carboxylic group at C-4 was α-positioned. Furthermore, the HMBC cross peaks from H-29 (δ_H 4.95 and 5.01) to C-19 (δ_C 43.7), C-20 (δ_C 155.9), and C-30 (δ_C 65.5) confirmed that the double bond was at C-20/C-29 and the other hydroxyl group was at C-30. Acid hydrolysis of **1** provided the monosaccharide components of L-rhamnose and D-glucose (identified as TMS derivatives by GC method).[20] Moreover, the HMBC correlations between the inner glc H-1 (δ 5.46) and C-28 of the aglycone (δ 176.3), between outer glc H-1′ (δ 4.40) and inner glc C-6 (δ 69.6), between rha H-1″ (δ 4.85) and glc C-4′ (δ 79.6) were observed. This evidence suggested the sequence of sugar linkages of **1**. Consequently, the structure of **1** was determined as 3α,11α,30-trihydroxylup-20(29)-en-23,28-dioic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**1**), named acankoreoside M.

Compound **2** was also obtained as a white amorphous powder. Its basic ion peak at *m*/*z* 1121.6 $[M+H]^+$ was observed in the positive-ion ESI-MS, and HRTOFMS analysis revealed the molecular formula to be $C_{54}H_{88}O_{24}$, with a cluster ion peak at *m*/*z* 1121.5824 $[M+H]^+$ (calcd for $C_{54}H_{89}O_{24}$: 1121.5744). The ^1H NMR spectrum of **2** (in methanol-d_4) showed the following signals: five tertiary methyl groups at δ_H 0.88, 0.95, 0.96, 1.07, and 1.08 (each 3H, s); one secondary methyl group at δ_H 1.27, assigned to H-6″ of the rhamnose; and four anomeric protons were at δ_H 4.27, 4.40, 4.84, and 5.45, suggesting the appearance of four sugar units. The ^{13}C NMR and DEPT spectra revealed 54 carbon signals, of which, 30 signals were assigned to a triterpenoid sapogenol moiety and 24 signals belong to four sugar moieties. The ^1H and ^{13}C NMR data of **2** (Table 1) were similar to those of acankoreoside D,[11] except difference of isopropenyl group in E ring. On the other hand, the HMBC correlations were observed between methylene protons H-29 (δ_H 4.94 and 5.00) and C-19 (δ_C 43.8), C-20 (δ_C 156.0), and C-30 (δ_C 65.6), as well as between H-30 (δ_H 4.08) and C-19 (δ_C 43.8), C-20 (δ_C 156.0), and C-29 (δ_C 107.6), confirming the double bond was at C-20/C-29 and the hydroxyl group was at C-30. The sugar linked to C-3 confirmed by HMBC correlations between H-3 (δ_H 3.41) and C-1‴ (δ_C 101.7) as well as between anomeric proton H-1‴ (δ_H 4.27) and C-3 (δ_C 82.4). Assignment of the *O*-β-D-glucopyranosyl moiety group at C-3 was performed as α configuration by comparing its spectral data [C-1 (δ_C 36.7), C-3 (δ_C 82.4), and C-5 (δ_C 51.0)] in the aglycone and those of the 3-*O*-β-D-glucopyranosyl betulinic acid 28-*O*-α-L-arabinopyranosyl(1→6)-β-D-glucopyranosyl ester (cussosaponin A)[21] [δ_C values for C-1 (39.0), C-3 (δ_C 88.7), and C-5 (δ_C 55.9)]. The HMBC correlations between the inner glc H-1 (δ 5.45) and C-28 of the aglycone (δ 176.4), between outer glc H-1′ (δ 4.40) and inner glc C-6 (δ 69.5), between rha H-1″ (δ 4.84) and glc C-4′ (δ 79.7) were observed. Moreover, the NMR data of

Table 1
The ^{1}H and ^{13}C NMR data of compounds **1**, **2**, and **8**

Pos.	**1**		**2**		**8**	
	δ_C[a,b]	δ_H[a,c] mult. (*J* in Hz)	δ_C[a,b]	δ_H[a,c] mult. (*J* in Hz)	δ_C[a,b]	δ_H[a,c] mult. (*J* in Hz)
Aglycone						
1	35.5	1.40	36.7	2.24 (dd, 3.5, 13.5)	34.5	1.43
2	26.4	1.92	19.2	1.36	26.1	1.39
3	73.8	3.67	82.4	3.41	73.3	3.58
4	53.0	—	38.5	—	52.1	—
5	45.4	1.91	51.0	1.30	47.4	1.86
6	22.6	1.47	22.2	1.62, 1.77	22.2	1.46
7	36.1	2.34	36.4	2.30	35.5	1.36
8	43.5	—	43.5	—	43.1	—
9	56.7	1.60 (d, 10.5)	56.3	1.55 (d, 10.5)	52.0	1.51 (d, 10.5)
10	40.0	—	40.4	—	38.2	—
11	71.1	3.42	71.2	3.42	22.2	1.51
12	39.4	1.31	39.5	1.29	26.1	1.29
13	38.2	2.42	38.3	2.42	39.1	2.42
14	44.3	—	44.0	—	44.3	—
15	30.8	2.21	30.9	1.50	30.9	1.29
16	32.9	2.37 (t, 9.5)	32.9	2.37 (t, 9.5)	34.5	1.39 (t, 9.5)
17	57.9	—	58.0	—	59.4	—
18	50.9	1.82	51.0	1.83	50.4	1.65
19	43.7	2.90 (dt, 4.5, 11.0)	43.8	2.88 (dt, 4.5, 11.0)	85.4	—
20	155.9	—	156.0	—	36.8	1.48
21	33.6	2.26	33.6	2.07	34.5	1.39
22	37.4	1.55	37.4	1.51	37.2	1.83
		1.98		1.97		
23	180.6		29.8	0.96 (s)	185.2	
24	18.2	1.18 (s)	23.3	0.88 (s)	18.6	1.08 (s)
25	17.5	1.10 (s)	17.3	1.07 (s)	17.4	0.90 (s)
26	18.1	0.96 (s)	18.0	0.95 (s)	17.1	0.95 (s)
27	15.1	1.08 (s)	15.2	1.08 (s)	15.5	1.03 (s)
28	176.3	—	176.4	—	176.7	—
29	107.7	4.95 (br s)	107.6	4.94 (br s)	21.9	1.16 (d, 6.0)
		5.01 (br s)		5.00 (br s)		
30	65.5	4.09	65.6	4.08 s	27.0	1.31 (d, 6.0)
C-28 O-Glc						
1	95.3	5.46 (d, 8.0)	95.4	5.45 (d, 8.0)	95.6	5.45 (d, 8.0)
2	74.0	3.33	74.1	3.31	74.1	3.33
3	78.2	3.43	78.3	3.43	78.2	3.43
4	71.0	3.41	71.1	3.41	71.2	3.42
5	78.3	3.43	78.4	3.43	78.5	3.43
6	69.6	3.80	69.5	3.80	69.8	3.80
		4.10 (d, 10.5)		4.11 (d, 10.5)		4.11 (d, 10.5)
Glc′ (1→6) Glc						
1′	104.5	4.40 (d, 8.0)	104.5	4.40 (d, 8.0)	104.8	4.40 (d, 8.0)
2′	75.4	3.25	75.4	3.20	75.5	3.25
3′	76.7	3.46	76.9	3.46	76.9	3.46
4′	79.6	3.54	79.7	3.54	79.8	3.53
5′	77.0	3.30	77.1	3.44	77.1	3.44
6′	62.0	3.66	62.1	3.66	62.1	3.66
		3.81 (d, 6.5)		3.81 (d, 6.5)		3.81 (d, 6.5)
Rha (1→4) Glc′						
1″	103.0	4.85 (br s)	103.1	4.84 (br s)	103.1	4.84 (br s)
2″	72.5	3.64 (dd, 3.5, 9.5)	72.3	3.62 (dd, 3.5, 9.5)	72.6	3.62 (dd, 3.5, 9.5)
3″	72.3	3.84	72.6	3.82	72.4	3.82
4″	73.6	3.41 (t, 9.5)	73.9	3.40 (t, 9.5)	73.3	3.40 (t, 9.5)
5″	70.7	3.95 (dd, 6.0, 9.5)	70.8	3.97 (dd, 6.0, 9.5)	70.9	3.96 (dd, 6.0, 9.5)
	18.0	1.27 (d, 6.0)	18.0	1.27 (d, 6.0)	18.0	1.27 (d, 6.0)
C-3 O-Glc″						
1‴			101.7	4.27 (d, 8.0)		
2‴			75.5	3.26		
3‴			78.6	3.36		
4‴			72.1	3.28 (t, 9.5)		
5‴			77.9	3.21		
6‴			63.2	3.66		
				3.85 (d, 6.5)		

[a] Measured in CD_3OD.
[b] 125 MHz.
[c] 500 MHz, Assignments were done by HMQC, HMBC, ^{1}H–^{1}H COSY and ROESY experiments, Glc: D-glucopyranosyl, Rha: L-rhamnopyranosyl.

the sugar moiety were superimposable on those of compound **1** and sugar moieties were also determined as L-rhamnose and D-glucose (identified as TMS derivatives by GC method).[20] This evidence suggested the sequence of sugar linkages of **2** was

28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl. Consequently, the structure of **2** was determined as 3-*O*-β-D-glucopyranosyl 3α,11α,30-trihydroxylup-20(29)-en-23,28-dioic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**2**), named acankoreoside N.

Compound **8** was also obtained as a white amorphous powder. Its basic ion peak at *m*/*z* 975.5 $[M+H]^+$ was observed in the positive-ion ESI-MS, and HRTOFMS analysis revealed the molecular formula to be $C_{48}H_{78}O_{20}$, with a cluster ion peak at *m*/*z* 975.5162 $[M+H]^+$ (calcd for $C_{48}H_{79}O_{20}$: 975.5165). The ^{1}H NMR spectrum of **8** (in methanol-d_4) showed the following signals: four tertiary methyl groups at δ_H 0.90, 0.95, 1.03, and 1.08 (each 3H, s); three secondary methyl groups at δ_H 1.16, 1.27, and 1.31; and three anomeric protons were at δ_H 4.40, 4.84, and 5.45, suggesting the appearance of three sugar units. The ^{13}C NMR and DEPT spectra of **8** revealed 48 carbon signals, of which, 30 signals were assigned to a triterpenoid sapogenol moiety and 18 signals belong to three sugar moieties. The ^{1}H and ^{13}C NMR data of **8** (Table 1) were similar to those of acankoreoside A,[10] except difference of branch chain in E ring. The HMBC correlations were observed between two secondary methyl protons H-29 (δ_H 1.16), H-30 (δ_H 1.31) and C-19 (δ_C 85.4) and C-20 (δ_C 36.8), confirming two methyl groups was at C-20; and one hydroxyl group was at C-19. On the other hand, the ROE correlation between H-18α (δ_H 1.65) and H-29 (δ_H 1.16) confirmed hydroxyl group at C-19 was β. Moreover, the HMBC cross peaks from H-3 (δ_H 3.58) to C-1 (δ_C 34.5) and C-5 (δ_C 47.4), from H-5 (δ_H 1.86) to C-4 (δ_C 52.1), C-23 (δ_C 185.2), and C-24 (δ_C 18.6), from H-24 (δ_H 1.08) to C-3 (δ_C 73.3), C-4 (δ_C 52.1), C-5 (δ_C 47.4), and C-23 (δ_C 185.2) confirmed location of the hydroxyl and carboxyl groups at C-3 and C-4, respectively. Similar to **1**, 3-hydroxyl and 4-carboxyl groups were assigned as the α-positions. The HMBC correlations between the inner glc H-1 (δ 5.45) and C-28 of the aglycone (δ 176.7), between outer glc H-1′ (δ 4.40) and inner glc C-6 (δ 69.8), between rha H-1″ (δ 4.84) and glc C-4′ (δ 79.8) were observed. Moreover, the NMR data of the sugar moiety were superimposable on those of compound **1**, and sugar moieties were also determined as L-rhamnose and D-glucose (identified as TMS derivatives by GC method).[20] This evidence suggested the sequence of sugar linkages of **8**. Consequently, the structure of **8** was determined as 3α,19β-dihydroxylupane-23,28-dioic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**8**), named acankoreoside O. The lymphoproliferation test was conducted as previously described with minor modifications.[22] Spleen cells (1×10^6/ml) were cultured and incubated with three different concentrations of isolated compounds (5, 25, and 100 μM) or the positive control, Con A (1 μg/mL). The results were expressed as mean values of picograms per milliliter of IL-2 and IFN-γ produced with standard error in triplicate cultures comparing with IL-2 and IFN-γ standard.

The water extracts of *A. koreanum* stems increased to 6.6 times for IL-2 release, 3.1 times for IFN-γ release compare to negative control. Moreover, the water extracts of *A. koreanum* leaves increased to 3.2 times for IL-2 release, 1.5 times for IFN-γ release compare to negative control. The effect of methanol extract of *A. koreanum* leaves on both cytokine releases was greater than those of water extract of *A. koreanum* leaves. These results revealed that the immunomodulatory agents are present in both leaves and stems of *A. koreanum*. Furthermore, the genuine immune stimulating effects were assessed by IL-2 and IFN-γ release in splenocytes treated with lupane-triterpene glycosides (**1**–**11**) isolated from the leaves of *A. koreanum* (see Tables 2 and 3). In absence of both test compounds and Con A, IL-2 and IFN-γ were secreted at very low concentration (1.94 ± 0.09 and 13.23 ± 0.42 pg/mL, respectively). In contrast, with the presence of Con A, the IL-2 and IFN-γ were released 15.67 ± 3.78 and 158.53 ± 27.36 pg/mL, respectively. As compared with the blank and Con A, compounds **4**, **5**, **7**, and **11** (5, 25, and 100 μM) significant increased IFN-γ productions ranging from 4.20 ± 0.17 pg/mL to 6.07 ± 0.17 pg/mL in spleen cells. Compounds **4**, **5**, **7**, and **11** (5, 25, and 100 μM) also exhibited potent effect on IL-2 release within 27.57 ± 1.75 to 74.47 ± 4.13 pg/mL. In the structure–activity relationship of these lupane-triterpene glycosides, the functional groups as methyl, aldehydic, carboxyl at C-4, hydroxyl group at C-11, and double bond at C-20/C-29 did not affect on IL-2 and IFN-γ increasing activity. However, the presence of hydroxyl group at C-30 or absence of C-30 affected the immune stimulatory activity. The compounds **4**, **5**, **7**, and **11** having no hydroxyl group at C-30 showed significant increasing activity for both IL-2 and IFN-γ. These results revealed that the immunomodulatory effects of *A. koreanum* were due to the lupane-triterpene glycosides in the leaves and stems of this plant.

Table 2
The effect of compounds isolated from leaves of *A. koreanum* on INF-γ release

Compound	IFN-γ release (pg/mL)		
	5 μM	25 μM	100 μM
Blank		1.94 ± 0.09	
Con A (1 μg/mL)		15.67 ± 3.78*	
1	1.57 ± 0.61	2.45 ± 0.11	2.48 ± 0.54
2	1.50 ± 0.35	1.55 ± 0.40	1.92 ± 0.32
3	1.25 ± 0.26	4.18 ± 0.47*	2.59 ± 0.08
4	2.89 ± 0.38*	4.62 ± 0.09**	4.57 ± 0.14**
5	3.69 ± 0.22**	4.62 ± 0.09*	5.29 ± 0.08**
6	3.30 ± 1.06**	4.00 ± 0.57**	4.20 ± 0.17**
7	4.55 ± 0.49**	5.23 ± 0.30**	5.41 ± 0.20**
8	1.81 ± 0.27	1.68 ± 0.14	2.44 ± 0.16
9	2.75 ± 0.35*	3.78 ± 0.57**	4.36 ± 0.08**
10	2.78 ± 0.92	3.2 ± 0.27	3.66 ± 0.33
11	3.53 ± 2.34*	3.71 ± 2.22*	6.07 ± 0.17**

Data are expressed as mean ± S.D. Statistically significant compared with blank.
* p <0.05.
** p <0.01.

Table 3
The effect of compounds isolated from leaves of *A. koreanum* on IL-2 release

Compound	IL-2 release (pg/mL)		
	5 μM	25 μM	100 μM
Blank		13.23 ± 0.42	
Con A (1 μg/mL)		158.53 ± 27.36*	
1	15.27 ± 0.53	16.92 ± 5.55	17.26 ± 5.3
2	17.11 ± 0.83	13.66 ± 0.65	19.06 ± 0.08
3	10.07 ± 2.21	12.91 ± 3.47	14.76 ± 6.55
4	17.04 ± 5.67*	24.87 ± 3.22*	29.78 ± 0.25**
5	19.26 ± 10.45*	25.32 ± 5.57*	41.34 ± 1.40**
6	16.09 ± 3.11*	23.47 ± 1.47**	27.57 ± 1.75**
7	20.07 ± 1.66*	25.57 ± 4.76*	50.66 ± 4.54**
8	13.89 ± 2.50	11.82 ± 5.18	16.30 ± 7.21
9	15.78 ± 6.95	16.14 ± 4.45	28.27 ± 2.84*
10	16.69 ± 0.34	20.24 ± 2.13	21.57 ± 2.56
11	20.68 ± 0.85**	45.06 ± 0.65**	74.47 ± 4.13**

Data are expressed as mean ± S.D. Statistically significant compared with blank.
* p <0.05.
** p <0.01.

Acknowledgments

This study was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815), Republic of Korea. The authors would like to thank Korean Basic Science Institute (KBSI) for taking NMR and MS experiments.

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17. The dried leaves of *A. koreanum* (4.0 kg) were extracted with hot MeOH (three times, 50 °C, 5 L each) to yield the methanol extract (80 g), which was then suspended in water (2 L) and extracted in turn with ethyl acetate (3 × 2 L) to give the ethyl acetate (AK1, 30 g), and water (AK2, 50 g) extracts. The AK2 extract (50 g) was chromatographed on a Diaion HP-20P column (Mitshubishi Chem. Ind. Co., Japan) eluting with water containing increasing concentrations of MeOH (100% H_2O, 25% MeOH, 50% MeOH, and 100% MeOH, 0.5 L each) to give five corresponding fractions, AK2A–AK2D. Fraction AK2D (10 g) was chromatographed on an YMC RP-18 column (4 × 30 cm) eluting with acetone/water (1:1, v/v, 1.5 L) to yield three sub-fractions, AK2D1–AK2D3. Sub-fraction AK2D1 (1.5 g) was further chromatographed on a silica gel column (2 × 40 cm) eluting with chloroform/methanol/water (3:1:0.1, v/v/v, 1.5 L) to obtain compound **2** (white amorphous powder, 5 mg). Sub-fraction AK2D2 (1.3 g) was further chromatographed on a silica gel column (1.5 × 30 cm) eluting with dichloromethane/methanol/water (35:10:1, v/v/v, 1.5 L) to obtain compounds **1** (white amorphous powder, 8 mg) and **8** (white amorphous powder, 7 mg).
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20. Each compound (2.0 mg) was dissolved in 1.0 N HCl (dioxane/H_2O, 1:1, v/v, 1.0 mL) and then heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 mL of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. The reaction mixture was heated at 60 °C for 2 h, and 0.1 mL of trimethylsilylimidazole solution was added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 mL, each), and the organic layer was analyzed by gas liquid chromatography (GC): Column: column SPB-1 (0.25 mm × 30 m); detector FID, column temp 210 °C, injector temperature 270 °C, detector temperature 300 °C, carrier gas He (2.0 mL/min). The retention times of persilylated glucose and rhamnose were founded to be 14.11 and 4.50 min, respectively, when compared with the standard solutions prepared by the same reaction from the standard monosaccharides. (The retention times of persilylated D-glucose, L-glucose, and L-rhamnose were 14.11, 14.26, and 4.50 min, respectively).
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