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Assamicin I and II, Novel Triterpenoid Saponins with Insulin-Like Activity from *Aesculus assamica* Griff.

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Abstract—Two novel triterpenoid saponins with insulin-like activity, termed assamicin I and II, were isolated from the roots of *Aesculus assamica* Griff. and their structures were characterized as **1** and **2**, respectively. They inhibited release of free fatty acids from epinephrine-treated rat adipocytes and enhanced glucose uptake into 3T3-L1 adipocytes.

In the course of our screening search for new bioactive natural products among Chinese plants distributed in Yunnan Province, we found that the ethanol extract of the roots of *Aesculus assamica* Griff. showed strong insulin-like activity against both rat and 3T3-L1 adipocytes. This finding prompted us to isolate and characterize the active principle involved in the plant, which led to the isolation of two novel triterpenoid saponins, termed assamicin I and II. In this paper, we preliminarily describe the structural elucidation and biological activity of the saponins.

The ethanol extract of the roots of *A. assamica* Griff. (1126.7 mg) was fractionated over a Sep-Pak C_{18} cartridge to obtain a crude saponin fraction. The 80% MeOH eluate from the cartridge (598.0 mg) was further purified by reversed-phase HPLC with a Capcell-Pak C_{18} column under a basic condition (30–80% CH_3CN in 10 mM $AcONH_4/NH_4OH$, pH 8.9), followed by that under an acidic condition (30–80% CH_3CN in 0.1% TFA). Two active components, **1** (7.8 mg) and **2** (2.4 mg), were successfully separated from a number of other saponin congeners involved in the plant by the HPLC.

Assamicin I (**1**) was obtained as a white powder and its molecular formula was determined as $C_{55}H_{86}O_{23}$ from the HR-FABMS and NMR spectra.[1] In the ^{13}C NMR spectrum of **1**, three carbonyl carbon signals were observed. By analysis of the COSY, HMQC and HMBC spectra of **1**, two of the three signals (δ 169.7 and 171.9) were assigned as carbonyl carbons involved in an angeloyl group and an acetyl group, respectively. Further analysis of the 2D NMR spectra revealed that **1** contains a protoaescigenin skeleton, and the angeloyl and acetyl groups form esters with hydroxyl groups at C-21 and C-28 of the triterpenoid skeleton. This was confirmed by comparison of the NMR data of **1** with those of isoescin Ib (**3**),[2] a triterpenoid saponin with the same aglycon as that of **1**. Structural elucidation of the sugar moiety of **1**, which has 18 carbon atoms, was also done by NMR analysis. The presence of three pyranose residues was shown by COSY correlation, and detailed analysis of the $^3J_{H,H}$ values among vicinal protons of each ring revealed the presence of rhamnose and galactose residues and the conformation of the third pyranose ring.[1] The third ring was identified as a glucuronic acid residue by the long-range coupling observed in the

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Table 1. ^{13}C NMR assignments of **1** and **2**[a]

C no.	δ		C no.	δ		C no.	δ	
	1	**2**		**1**	**2**		**1**	**2**
1	39.8	39.8	3-*O*-β-Glucopyranosiduronic acid moiety			21-*O*-Angeloyl		
2	27.8	27.8	1′	106.2	106.1	1‴′	169.7	
3	92.0	92.0	2′	78.6	79.0	2‴′	130.7	
4	45.1	45.1	3′	85.5	84.7	3‴′	137.3	
5	57.1	57.1	4′	72.9	73.6[b]	4‴′	17.0	
6	19.6	19.7	5′	79.2	79.0	5‴′	22.2	
7	34.4	34.4	6′	173.4	172.0	21-*O*-6-Deoxy-β-glucopyranosyl moiety		
8	41.1	41.2	2′-*O*-β-Galactopyranosyl moiety			1‴′		107.0
9	48.0	48.0	1″	105.1	105.1	2‴′		74.4
10	37.6	37.6	2″	74.4	73.8[b]	3‴′		76.7
11	25.3	25.3	3″	76.5	76.5	4‴′		75.0
12	124.0	125.0	4″	71.9	71.9	5‴′		71.5
13	143.9	144.1	5″	78.1	78.0	6‴′		19.0
14	43.0	43.0	6″	62.7	62.7	3‴′-*O*-Angeloyl		
15	35.9	36.0	3′-*O*-α-Rhamnopyranosyl moiety			1‴″		168.6
16	68.8	69.1	1‴	104.9	104.7	2‴″		129.5
17	48.3	47.9	2‴	73.7	73.7	3‴″		138.9
18	41.7	41.7	3‴	74.1	74.1	4‴″		17.1
19	48.5	48.8	4‴	75.1	75.2	5‴″		21.9
20	37.3	38.3	5‴	71.6	71.4	4‴′-*O*-Angeloyl		
21	82.4	93.1	6‴	19.6	19.7	1‴‴″		168.0
22	72.2	72.7	28-*O*-Acetyl			2‴‴″		128.9
23	23.5	23.6	1‴′	171.9	172.0	3‴‴″		140.3
24	64.5	64.5	2‴′	21.9	21.8	4‴‴″		17.1
25	17.0	17.0				5‴‴″		22.0
26	18.0	18.0						
27	28.6	28.7						
28	67.6	67.7						
29	31.0	31.0						
30	21.4	21.3						

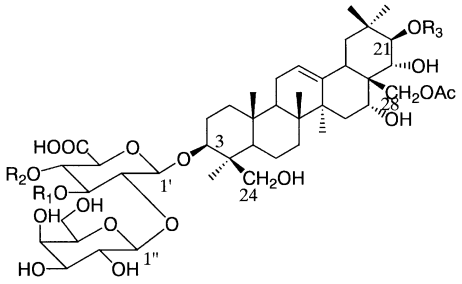
[a]Spectra were obtained in pyridine-d_5 on a JEOL GX-500.
[b]May be interchanged.

HMBC spectrum between H-5 of the ring and the carbonyl carbon (δ 173.4). The *J* value at each C-1 of rhamnose ($^2J_{C\text{-}1,H\text{-}1} = 176$ Hz), galactose ($^3J_{H\text{-}1,H\text{-}2} = 7.5$ Hz) and glucuronic acid ($^3J_{H\text{-}1,H\text{-}2} = 8.0$ Hz) residues indicated that they have α, β and β configurations, respectively. The connections among the three pyranose residues and the aglycon moiety, which contain all 55 carbon atoms in **1**, were analyzed by HMBC correlation, and the glycosylation linkages of rhamnose to glucuronic acid (1→3), galactose to glucuronic acid (1→2) and glucuronic acid to aglycon (C-1′–C-3) were clarified from the long-range couplings observed between H-1 of rhamnose and C-3 of glucuronic acid, H-1 of galactose and C-2 of glucuronic acid, and H-1 of glucuronic acid and C-3 of the aglycon. Since the molecular formula of **1** was satisfied when no other ester or ether linkage was present in **1**, the structure of assamicin was determined as 21-*O*-angeloyl-28-*O*-acetyl-protoaescigenin 3-*O*-[β-galactopyranosyl (1→2)][α-rhamnopyranosyl (1→3)]-β-glucopyranosiduronic acid (**1**, Table 2).

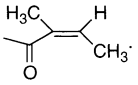
The molecular formula of assamicin II (**2**) was determined as $C_{66}H_{102}O_{28}$ from the HR-FABMS and NMR spectra.[1] Analysis of the NMR spectra of **2** showed that **2** consisted of the following moieties: a protoaescigenin skeleton, an acetyl group, two angeloyl groups, a 6-deoxyglucose residue and a trisaccharide residue the same as that involved in **1**. HMBC correlation revealed that hydroxyl groups at C-28 and C-3 of the triterpenoid moiety were acetylated and glycosylated with the trisaccharide residue similarly to the case of **1**. Furthermore, it showed that the two angeloyl groups form esters with hydroxyl groups at C-3 and C-4 of the 6-deoxyglucose residue, and C-21 of the triterpenoid skeleton was *O*-glycosylated with the 6-deoxyglucose residue. The $^3J_{H\text{-}1,H\text{-}2}$ value at C-1 of the 6-deoxyglucose (8.0 Hz) indicated β configuration of the residue. Thus, the structure of assamicin II was assigned as 21-*O*-(3,4-di-*O*-angeloyl-6-deoxy-β-glucopyranosyl)-28-*O*-acetyl-protoaescigenin 3-*O*-[β-galactopyranosyl (1→2)][α-rhamnopyranosyl (1→3)]-β-glucopyranosiduronic acid (**2**, Table 2). The assignments of carbons in the NMR spectra of **1** and **2** are summarized in Table 1.

Insulin-like activity of assamicin I and II was tested by the assay system with rat or 3T3-L1 adipocytes. It is known that insulin inhibits release of free fatty acids from epinephrine-treated rat adipocytes.[3] Assamicin I and II almost completely inhibited the release at concentrations of 100 and 25 μg/mL, respectively. On the other hand, they enhanced glucose uptake into 3T3-L1 adypocytes as insulin does.[4] When the cells were incubated in a medium containing [^{3}H]-2-deoxyglucose, the uptake of the radioisotope into the cells was enhanced 2.5- and 3.5-fold by addition of assamicin I and II, respectively, at the concentration of 25 μg/mL than in the case of the control without the sample. Assamicin II

Table 2.



		R_1	R_2	R_3
Assamicin I	**1**	α-Rhamnopyranosyl	H	Angeloyl[a]
Assamicin II	**2**	α-Rhamnopyranosyl	H	3,4-di-*O*-Angeloyl-6-deoxy-β-glucopyranosyl
Isoescin Ib	**3**	H	β-Glucopyranosyl	Angeloyl

[a]Angeloyl = 

showed stronger activity than assamicin I in both assay systems tested.

Saponins contained in *Aesculus* sp. have been known to show some important biological activities. Recently, the structures of triterpenoid saponins isolated from *A. indica* L.,[5,6] *A. hippocastanum* L.[2,7] and *A. chinensis* Bge. (Hippocastanaceae)[8,9] have been determined. Assamicin I and II are the first *Aesculus* saponins whose insulin-like activity on some adipocytes has been shown. Work to clarify the structures and biological activity of other saponin constituents involved in *A. assamica* Griff. is now in progress.

References and Notes

1. **1**: HR-FABMS (positive, glycerol matrix) *m*/*z* 1115.5623 $(M+H)^+$ (calcd for 1114.5560); ^{1}H NMR δ (pyridine-d_5, 500 MHz) 0.63 (H-25), 0.90 (H-26), 1.10 (H-29), 1.19 (H-23), 1.28 (H-30), 1.82 (H-27), 1.97 (H-2‴), 2.82 (H-18), 3.08 (H-19), 3.21 (d, *J* = 11 Hz, H-24), 3.35 (H-3), 4.21 (d, *J* = 11 Hz, H-24), 4.24 (H-28), 4.74 (H-16), 5.41 (H-12), 6.50 (d, *J* = 9.5 Hz, H-21), glucopyranosiduronic acid moiety, 4.83 (d, *J* = 8 Hz, H-1′), 4.41 (H-2′), 4.35 (H-3′), 4.47 (H-4′), 4.61 (d, *J* = 9 Hz, H-5′), [in CD_3OD (500 MHz), 4.47 (d, *J* = 8 Hz, H-1′), 3.79 (dd, *J* = 8, 9 Hz, H-2′), 3.69 (t, *J* = 9 Hz, H-3′), 3.54 (t, *J* = 9 Hz, H-4′), 3.58 (d, *J* = 9 Hz, H-5′)], galactopyranosyl moiety, 5.28 (d, *J* = 7.5 Hz, H-1″), 4.37 (H-2″), 3.91 (dd, *J* = 3.5, 10 Hz, H-3″), 4.46 (H-4″), 3.86 (br t, *J* = 6 Hz, H-5″), 4.46 (H-6″a), 4.35 (H-6″b), [in CD_3OD (500 MHz), 4.56 (d, *J* = 7.5 Hz, H-1″), 3.51 (dd, *J* = 7.5, 10 Hz, H-2″), 3.44 (dd, *J* = 3.5, 10 Hz, H-3″), 3.77 (br d, *J* = 3.5 Hz, H-4″), 3.50 (H-5″), 3.77 (dd, *J* = 6, 12 Hz, H-6″a), 3.71 (dd, *J* = 5, 12 Hz, H-6″b)], rhamnopyranosyl moiety, 6.16 (br s, H-1‴), 4.95 (br s, H-2‴), 4.55 (dd, *J* = 3, 9 Hz, H-3‴), 4.36 (H-4‴), 5.02 (H-5‴), 1.68 (d, *J* = 6.5 Hz, H-6‴), [in CD_3OD (500 MHz), 5.04 (d, *J* = 1.5 Hz, H-1‴), 3.99 (dd, *J* = 1.5, 3 Hz, H-2‴), 3.66 (dd, *J* = 3, 9 Hz, H-3‴), 3.37 (t, *J* = 9 Hz, H-4‴), 4.05 (dq, *J* = 6, 9 Hz, H-5‴), 1.23 (d, *J* = 6 Hz, H-6‴)], angeloyl, 1.96 (H-5⁗), 2.09 (d, *J* = 7.5 Hz, H-4⁗), 5.88 (H-3⁗); ^{13}C NMR: Table 1.
2: HR-FABMS (positive, glycerol matrix) *m*/*z* 1343.6653 $(M+H)^+$ (calcd for 1342.6558); ^{1}H NMR δ (pyridine-d_5, 500 MHz) 0.61 (H-25), 0.90 (H-26), 1.17 (H-23), 1.30 (H-30), 1.47 (H-29), 1.83 (H-27), 2.04 (H-2⁗), 2.79 (H-18), 3.10 (H-19), 3.17 (d, *J* = 6.0 Hz, H-24), 3.37 (H-3), 4.21 (d, *J* = 6.0 Hz, H-24), 4.27 (d, *J* = 10 Hz, H-28), 4.39 (d, *J* = 9.5 Hz, H-22), 4.45 (d, *J* = 10 Hz, H-28), 4.81 (d, *J* = 9 Hz, H-21), 4.85 (H-16), 5.40 (H-12), glucopyranosiduronic acid moiety, 4.78 (d, *J* = 7.5 Hz, H-1′), 4.40 (H-2′), 4.32 (H-3′), 4.33 (t, *J* = 9 Hz, H-4′), 4.44 (H-5′), [in CD_3OD (600 MHz), 4.47 (d, *J* = 8 Hz, H-1′), 3.79 (H-2′), 3.69 (t, *J* = 9 Hz, H-3′), 3.54 (H-4′), 3.58 (d, *J* = 9 Hz, H-5′)], galactopyranosyl moiety, 5.26 (d, *J* = 7.5 Hz, H-1″), 4.35 (H-2″), 3.90 (dd, *J* = 3.5, 10 Hz, H-3″), 4.45 (H-4″), 3.86 (br t, *J* = 6 Hz, H-5″), 4.46 (dd, *J* = 5, 11 Hz, H-6″a), 4.35 (H-6″b), [in CD_3OD (600 MHz), 4.56 (d, *J* = 7 Hz, H-1″), 3.51 (H-2″), 3.44 (dd, *J* = 3.5, 10 Hz, H-3″), 3.77 (H-4″), 3.50 (H-5″), 3.77 (H-6″a), 3.71 (H-6″b)], rhamnopyranosyl moiety, 6.22 (br s, H-1‴), 4.93 (br s, H-2‴), 4.57 (dd, *J* = 3, 9 Hz, H-3‴), 4.36 (t, *J* = 9 Hz, H-4‴), 5.10 (H-5‴), 1.71 (d, *J* = 6.5 Hz, H-6‴), [in CD_3OD (600 MHz), 5.04 (br. s, H-1‴), 3.99 (dd, *J* = 1.5, 3 Hz, H-2‴), 3.66 (dd, *J* = 3, 9 Hz, H-3‴), 3.38 (t, *J* = 9 Hz, H-4‴), 4.04 (dq, *J* = 6, 9 Hz, H-5‴), 1.23 (d, *J* = 6 Hz, H-6‴)], 6-deoxy-β-glucopyranosyl moiety, 4.91 (d, *J* = 8 Hz, H-1⁗), 4.11 (H-2⁗), 5.78 (t, *J* = 9.5 Hz, H-3⁗), 5.27 (t, *J* = 9.5 Hz, H-4⁗), 3.65 (dq, *J* = 6.0, 9.5 Hz, H-5⁗), 1.19 (d, *J* = 6 Hz, H-6⁗), [in CD_3OD (600 MHz), 4.50 (d, *J* = 8 Hz, H-1⁗), 3.55 (H-2⁗), 5.24 (t, *J* = 9.5 Hz, H-3⁗), 4.86 (H-4⁗), 3.77 (H-5⁗), 1.22 (d, *J* = 6 Hz, H-6⁗)], 3⁗-*O*-angeloyl, 1.85 (H-5⁗), 1.93 (H-4⁗), 5.86 (H-3⁗), 5.96 (H-3‴′), 4⁗-*O*-angeloyl, 1.88 (H-5‴′), 1.96 (H-4‴′), 5.96 (H-3‴′); ^{13}C NMR: Table 1.
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