Asernestioside C, a New Minor Saponin from the Roots of *Astragalus ernestii* COMB.; First Example of Negative Nuclear Overhauser Effect in the Saponins

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A minor saponin, asernestioside C (1), was isolated from the roots of *Astragalus ernestii* COMB. (Leguminosae) and its structure was determined from the two-dimensional nuclear magnetic resonance spectra and nuclear Overhauser effect (NOE) difference spectra. In this case, negative NOE was observed between the 3-H and the anomeric proton of 3-*O*-sugar moiety and between the 24-H and the 26-H_3. Examples of negative NOE were also found in some other saponins.

Keywords Asernestioside C; *Astragalus ernestii*; Astragali Radix; saponin; Asernestioside C; cycloastragenol; 2-D NMR; negative NOE

Dried roots of *Astragalus membranaceus* BUNGE and other *Astragalus* spp. (Leguminosae) are used as a crude drug "Huang qi" (Astragali Radix), which is prescribed in several Chinese traditional medicines as an antiperspirant, a diuretic, or a tonic.[1] Among *Astragalus* species, *A. membranaceus* BUNGE and *A. sieversianus* PALL were investigated chemically by several groups of authors, and lipids,[2] flavonoids,[3] γ-aminobutyric acid,[4] L-canavanine,[5] polysaccarides,[6] and saponins[7,8] have so far been characterized.

In the course of our study on the constituents of Chinese *Astragalus* plants, we have isolated three new saponins named asernestioside A (**2**), B (**3**), and C (**1**) from the roots of *A. ernestii* COMB. and determined the structures of the major constituents, **2** and **3**,[9] based on the chemical and spectral evidence. In this paper we wish to report the isolation and structure determination of the minor constituent, asernestioside C (**1**), by means of proton, carbon, and two-dimensional nuclear magnetic resonance (^{1}H-, ^{13}C-, and 2-D NMR) spectroscopies without a derivatization or degradation.

The 95% ethanolic extract from the dried roots of *A. ernestii* was treated with methanol and the methanol-soluble fraction was partitioned between butanol and water. The crude saponin obtained from the butanol-soluble portion was further separated by a combination of reversed-phase column chromatograhy and silica gel column chromatography to give a small amount of asernestioside C (**1**) together with asernestioside A (**2**)[9] and B (**3**).[9]

Asernestioside C (**1**), mp 204—207 °C, showed $[\alpha]_D$ −13.2° (MeOH) and its molecular formula was determined to be $C_{49}H_{80}O_{19}$ by fast-atom bombardment mass spectroscopy (FAB-MS) measurement ($M^+ + 1$, *m/z* 973). The infrared (IR) spectrum of **1** (in KBr) exhibited a strong hydroxyl absorption band at 3375 cm^{-1} and an ester-carbonyl absorption band at 1732 cm^{-1}. In the ^{1}H-NMR spectrum (in pyridine-d_5), it showed signals due to cyclopropane-methylene protons at δ 0.26 and 0.56 ppm

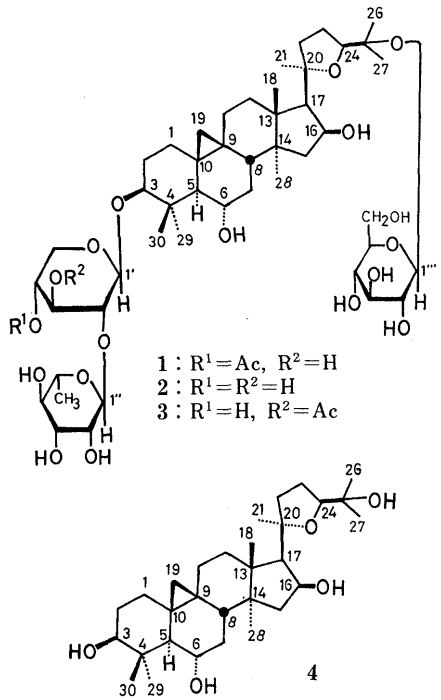

Chart 1

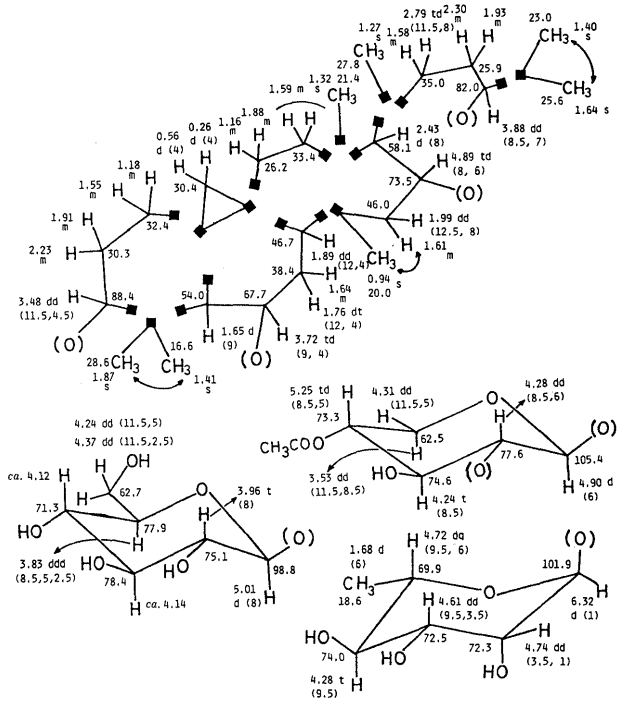

Fig. 1. Partial Structures of Asernestioside C (**1**) Deduced from the ^{1}H- and ^{13}C-NMR Data

: long-range coupling observed in ^{1}H–^{1}H COSY.

TABLE I. ^{1}H-NMR Chemical Shifts (in ppm) and Coupling Constants (in Hz, in parentheses) of Asernestioside C and Cycloastragenol in C_5D_5N

	Asernestioside C (1)	Cycloastragenol (4)	Sugar part of 1	
1-Hα	1.55 m	1.64 ddd (13, 12, 4.5)	Xylose	
1-Hβ	1.18 m	1.25 ddd (13, 4.5, 3)	1′-H	4.90 d (6)
2-Hα	2.23 m	2.055 dtd (12, 4.5, 3)	2′-H	4.28 dd (8.5, 6)
2-Hβ	1.91 m	1.96 tdd (12, 11.5, 4.5)	3′-H	4.24 t (8.5)
3-H	3.48 dd (11.5, 4.5)	3.66 dd (11.5, 4.5)	4′-H	5.25 td (8.5, 5)
5-H	1.65 d (9)	1.72 d (9.5)	5′-H_2	3.53 dd (11.5, 8.5)
6-H	3.72 td (9, 4)	3.80 td (9.5, 4)		4.31 dd (11.5, 5)
7-Hα	1.64	1.65 td (12, 9.5)	Rhamnose	
7-Hβ	1.76 dt (12, 4)	1.83 dt (12, 4)	1″-H	6.32 d (1)
8-H	1.89 dd (12, 4)	1.96 dd (12, 4)	2″-H	4.74 dd (3.5, 1)
11-Hα	1.88 m	1.98 dt (10, 3.5)	3″-H	4.61 dd (9.5, 3.5)
11-Hβ	1.16 m	1.23 td (10, 4.5)	4″-H	4.28 t (9.5)
12-H_2	1.59 m	1.63 m 1.67 m	5″-H	4.72 dq (9.5, 6)
15-H_2	1.61 m	1.78 dd (12.5, 7.5)	6″-H_3	1.68 d (6)
	1.99 dd (12.5, 8)	2.13 dd (12.5, 7.5)	Glucose	
16-H	4.89 td (8, 6)	5.02 qd (7.5, 2)	1‴-H	5.01 d (8)
17-H	2.43 d (8)	2.55 d (7.5)	2‴-H	3.96 t (8)
18-H_3	1.32 s	1.45 s	3‴-H	*ca.* 4.14
19-H_2	0.26 d (4)	0.35 d (4)	4‴-H	*ca.* 4.12
	0.56 d (4)	0.62 d (4)	5‴-H	3.83 ddd (8.5, 5, 2.5)
21-H_3	1.27 s	1.33 s	6‴-H_2	4.24 dd (11.5, 5)
22-H_2	1.58 m	1.69 ddd (11.5, 9, 2.5)		4.37 dd (11.5, 2.5)
	2.79 td (11.5, 8)	3.12 td (11.5, 9)	CH_3CO	1.91 s
23-H_2	1.93 m	2.06 dq (11.5, 9)		
	2.30 m	2.32 tdd (11.5, 5.5, 2.5)		
24-H	3.88 dd (8.5, 7)	3.89 dd (9, 5.5)		
26-H_3	1.40[a] s	1.30[a] s		
27-H_3	1.64[a] s	1.58[a] s		
28-H_3	0.94 s	1.03 s		
29-H_3	1.87 s	1.89 s		
30-H_3	1.41[b] s	1.37[b] s		

a) The signal at the high-field side was assigned arbitrarily to 26-H_3 and the other to 27-H_3. *b*) Assignment was done based on NOE experiments with 19-H (Fig. 3).

(each d, J=4 Hz, 19-H_2), seven tertiary methyls at δ 0.94, 1.27, 1.32, 1.40, 1.41, 1.64, and 1.87 (28-, 21-, 18-, 26-, 30-, 27-, and 29-methyl, respectively), and an acetyl methyl at δ 1.91 along with signals due to oxymethine and/or oxymethylene protons at around δ 3.4—6.4 ppm (Table I).

The ^{1}H-NMR signals of **1** could be analyzed reasonably by the use of ^{1}H–^{1}H shift correlation spectroscopy (^{1}H–^{1}H COSY) coupled with ^{1}H–^{13}C COSY, which indicated the presence of the partial structures shown in Fig. 1. Also it was suggested from the ^{1}H- and ^{13}C-NMR data that the sugar moieties in **1** may be β-xylopyranose, α-rhamnopyranose, and β-glucopyranose (Tables I and II) and that the double triplet at δ 5.25 (J=8.5, 5 Hz) due to a proton geminal to the ester group is ascribed to the 4′-proton of xylopyranose moiety. Therefore, the acetyl group should be linked to the 4′-position of the xylose residue.

Next, we measured the long-range ^{1}H–^{13}C COSY in order to clarify the connectivities of these partial structures. As shown in Fig. 2, the methyl signals at δ 0.94 ppm (28-H_3) showed long-range correlations with the carbons at δ 45.1 (s, C-13), 46.0 (s, C-14 and t, C-15), and 46.7 ppm (d, C-8) and the methyl signal at δ 1.32 ppm (18-H_3) showed long-range correlations with the carbons at δ 33.4 (t, C-12), 45.1 (s, C-13), 46.0 (s, C-14), and 58.1 ppm (d, C-17), indicating that C-12, C-14, C-17, and C-18 were connected with C-13, and C-8, C-13, C-15, and C-28 were connected with C-14. Also, the methyl signal at δ 1.27 ppm (21-H_3) showed long-range correlations with the carbons at δ 35.0 (t, C-22), 58.1 (d, C-17), and 87.2 ppm (s, C-20), indicating that C-17, C-21, and C-22 were connected with C-20. On the other hand, the methyl signal at δ 1.40 (26-H_3) and 1.64 ppm (27-H_3) showed correlations with the carbons at δ 25.6 (q, C-27), 78.5 (s, C-25), and 82.0 ppm (s, C-24) and at δ 23.0 (q, C-26), 78.5 (s, C-25), and 82.0 ppm (d, C-24), respectively, suggesting that C-26 and C-27 were connected with C-25, and C-25 with C-24. Furthermore, the methyl signals at δ 1.87 (29-H_3) and 1.41 ppm (30-H_3) showed correlations with the carbons at δ 28.6 (q, C-29), 42.6 (s, C-4), and 54.0 ppm (d, C-5) and at δ 16.6 (q, C-30), 42.6 (s, C-4), and 54.0 ppm (d, C-5), respectively, indicating the connectivities of C-4 and C-29, C-30, and C-5. These data and the other long-range correlations shown in Table II led us to suppose that **1** is a triglycoside of cycloastragenol (**4**). This was supported by the close similarity of its ^{13}C-NMR spectrum with that of **4** (Table II).[10]

The glycosidation sites of **1** were suggested by the ^{13}C-NMR spectrum compared with that of **4**. As shown in Table II, the signals of the oxygenated carbons C-3 and C-25 appeared at lower field and the carbons C-26 and C-27 at higher field than the corresponding signals of the aglycone (**4**), while the oxymethine carbons C-6 and C-16 appeared at almost the same chemical shifts. Therefore, the sugar moieties must be located at the C-3 and C-25 positions. On the other hand, the anomeric carbon of the β-glucopyranose residue resonated at higher field as compared with that of methyl β-glucopyranoside.[11] It has been reported that the anomeric carbon signal of tertiary alcoholic β-glucosides appears at significantly higher field (δ about 99) than those of primary (δ about 104) and secondary (δ about 102) alcoholic β-glucosides.[12] Thus, the glucopyranose must be attached to the C-25 position of the aglycone. As to the xylose and rhamnose moieties, the carbon C-2′ of xylopyranose showed the glycosidation shift (3 ppm), while none of the carbons of rhamnopyranose showed a glycosidation shift. Therefore, it followed that xylopyranose was attached to the 3-hydroxyl group of the aglycone and rhamnopyranose to the 2′-hydroxyl group of xylopyranose.

In order to confirm these conclusions, nuclear

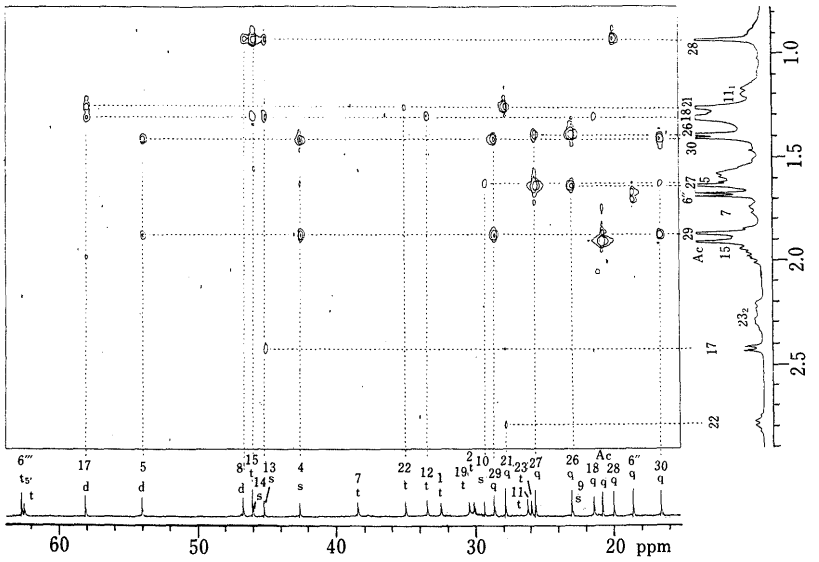

Fig. 2. Long-Range ^{1}H–^{13}C Shift-Correlated Spectrum of Asernestioside C (**1**) in the Upfield Region ($J = 10$ Hz; Sample, 15 mg; 35 h Run)

TABLE II. ^{13}C Chemical Shifts (in ppm) and Long-Range-Coupled ^{1}H Signals of Asernestioside C and Cycloastragenol in C_5D_5N[a]

	Asernestioside C (**1**)				Cycloastragenol (**4**)				Sugar part of **1**			Methyl glycoside[b]	
	δ		^{1}H L-r. coupled ($^{3}J_{C-H}$; $^{2}J_{C-H}$)		δ		^{1}H L-r. coupled ($^{3}J_{C-H}$; $^{2}J_{C-H}$)					α	β
C-1	32.4	t			32.8	t	19		Xylose				
C-2	30.3	t			31.4	t			C-1′	105.4	d	101.5	106.0
C-3	88.4	d			78.3	d	29, 30		C-2′	77.6	d	73.7	74.6
C-4	42.6	s		5, 29, 30	42.4	s		5, 29, 30	C-3′	74.6	d	75.5	78.1
C-5	54.0	d	29, 30		53.9	d	1, 29, 30		C-4′	73.3	d	71.4	70.9
C-6	67.7	d			68.3	d		5	C-5′	62.5	t	63.1	67.0
C-7	38.4	t			38.8	t		8					
C-8	46.7	d	28		47.2	d	15, 19, 28	7	Rhamnose				
C-9	20.8	s			20.9	s	7	8	C-1″	101.9	d	102.6	102.6
C-10	29.9	s		2	29.9	s	8	1, 5	C-2″	72.3	d	72.1	72.1
									C-3″	72.5	d	72.7	75.3
C-11	26.2	t			26.3	t			C-4″	74.0	d	73.8	73.7
C-12	33.4	t	18		33.4	t	17, 18		C-5″	69.9	d	69.5	73.4
C-13	45.1[c]	s	28	17, 18	45.0[c]	s	15, 28	17, 18	C-6″	18.6	q	18.6	18.5
C-14	46.0[c]	s	18	28	46.2[c]	s	18	8, 28					
C-15	46.0	t	28		46.7	t	28		Glucose				
C-16	73.5	d			73.4	d			C-1‴	98.8	d	101.3	105.5
C-17	58.1	d	15, 18, 21		58.4	d	15, 18, 21		C-2‴	75.1	d	73.7	74.9
C-18	21.4	q			21.6	q	17		C-3‴	78.4	d	75.3	78.2
C-19	30.4	t			31.0	t	5		C-4‴	71.3	d	72.0	71.4
C-20	87.2	s		17, 21	87.2	s		17, 21, 22	C-5‴	77.9	d	74.0	78.2
									C-6‴	62.7	t	62.7	62.6
C-21	27.8	q	22		28.6	q	17, 22						
C-22	35.0	t	21		34.9	t	17, 21		Acetyl group				
C-23	25.9	t			26.4	t			$\underline{C}H_3CO$	20.8	q		
C-24	82.0	d	26, 27		81.7	d	26, 27						
C-25	78.5	s		26, 27	71.2	s		26, 27	$CH_3\underline{C}O$	170.5	s		
C-26	23.0[d]	q	27		27.2[d]	q	27						
C-27	25.6[d]	q	26		28.2[d]	q	26						
C-28	20.0	q			20.2	q	8, 15α, β						
C-29	28.6	q	30		29.4	q	30						
C-30	16.6	q	5, 29		16.1	q	5, 29						

a) Carbon signals affected by glycosylation or acetylation are underlined. *b*) Taken from the literature (ref. 11). *c*) Assignments were done by comparisons with those of 24-methylenecycloartenol (ref. 13). *d*) These signals correspond to the 26- and 27-H_3 signals, respectively.

Overhauser effect (NOE) difference spectra were measured. Irradiation of both the 26-methyl and 27-methyl protons caused an increase of the intensity of 1‴-H of the glucopyranose residue, supporting the location of glucopyranose at the C-25 position (Fig. 3). It should be noted here that irradiation of the 26-methyl protons concomitantly caused a decrease of the intensity of 24-H. Also, it was observed that irradiation of 3-H caused a decrease of the intensity of 1′-H and *vice versa* (Fig. 3). These phenomena may be ascribed to the negative NOE as reported in the case of

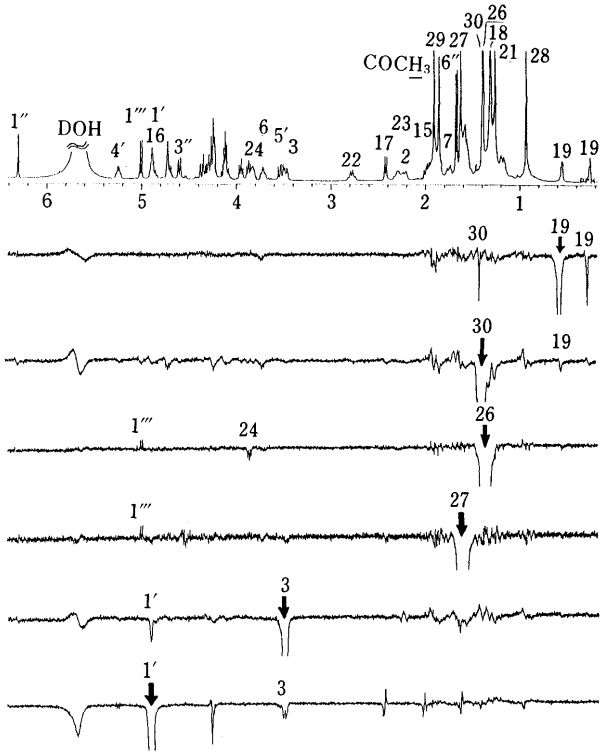

Fig. 3. NOE Difference Spectra of Asernestioside C (**1**) in C_5D_5N at 25 °C

some peptides[14] and a flavonoid glucoside.[15]

Based on the foregoing evidence, and by assuming that xylose, rhamnose, and glucose residue have the D, L, and D absolute configuration, respectively, the structure of asernestioside C (**1**) was concluded to be 25-*O*-*β*-D-glucopyranosyl cycloastragenol 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)]-(4′-*O*-acetyl)-*β*-D-xylopyranoside.

Then we measured the NOE difference spectra of some other saponins, such as astragalosides I (**5**), II (**6**), III (**7**), IV (**8**), V (**9**), and VI (**10**),[16] saikosaponins a (**11**) and c (**12**), and ginsenoside R_{b1} (**13**), to examine whether negative NOE is common in saponins or not. As shown in Fig. 4 and Table III, negative NOE was observed between the proton at the C-3 position and the anomeric proton of the 3-*O*-glucoside residue in all the compounds examined. On irradiation of the 21-methyl protons of **13**, negative NOE was also observed at the anomeric proton of the 20-*O*-glucoside residue, while positive NOE was observed on irradiation of 6-H of **5**, **6**, and **8**. Our present result is the first example of negative NOE in the saponin field[17] and it will be useful for the structure elucidation of saponins.

Experimental

The melting point was measured on a micromelting point apparatus and is uncorrected. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX400 spectrometer in C_5D_5N with tetramethylsilane as an internal

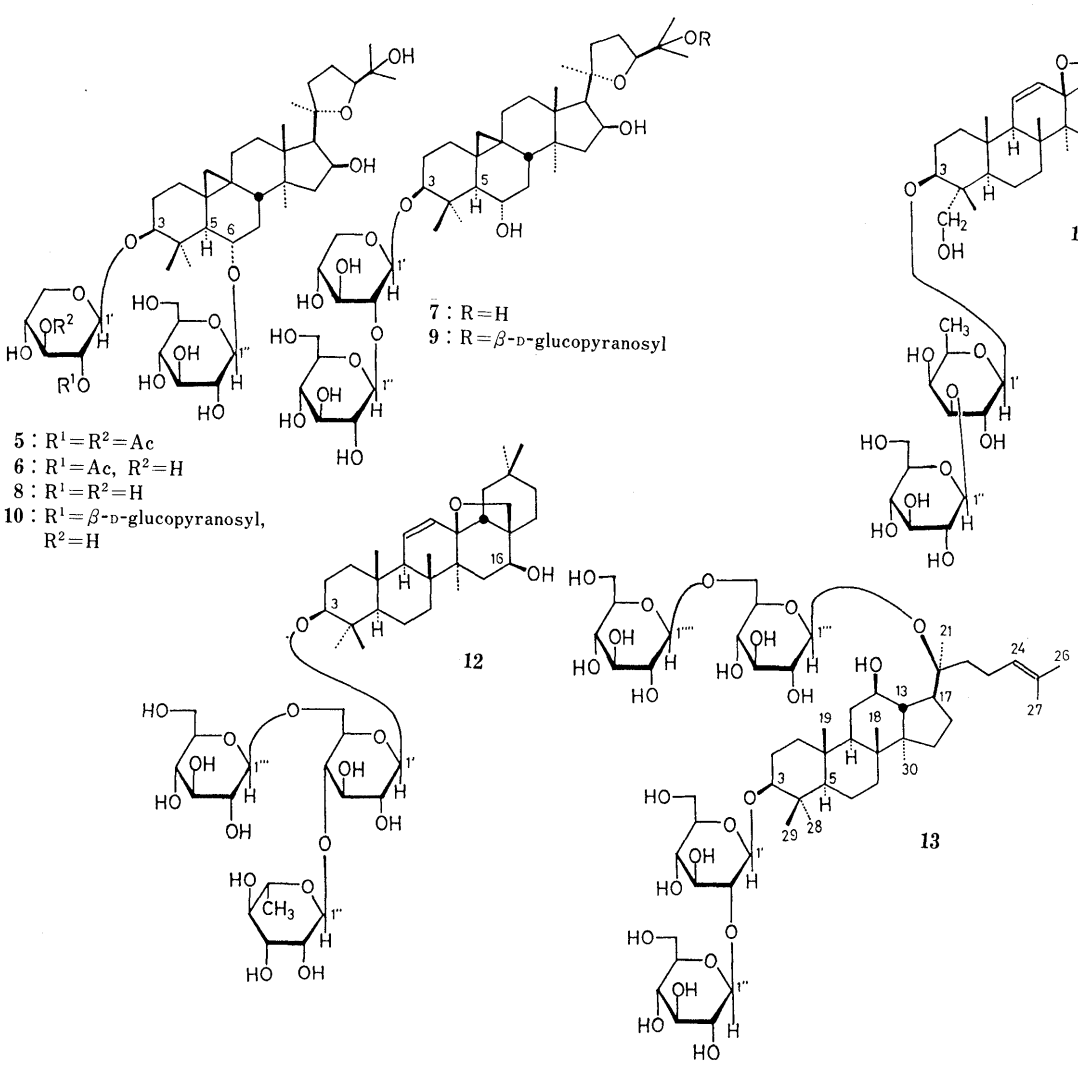

Chart 2

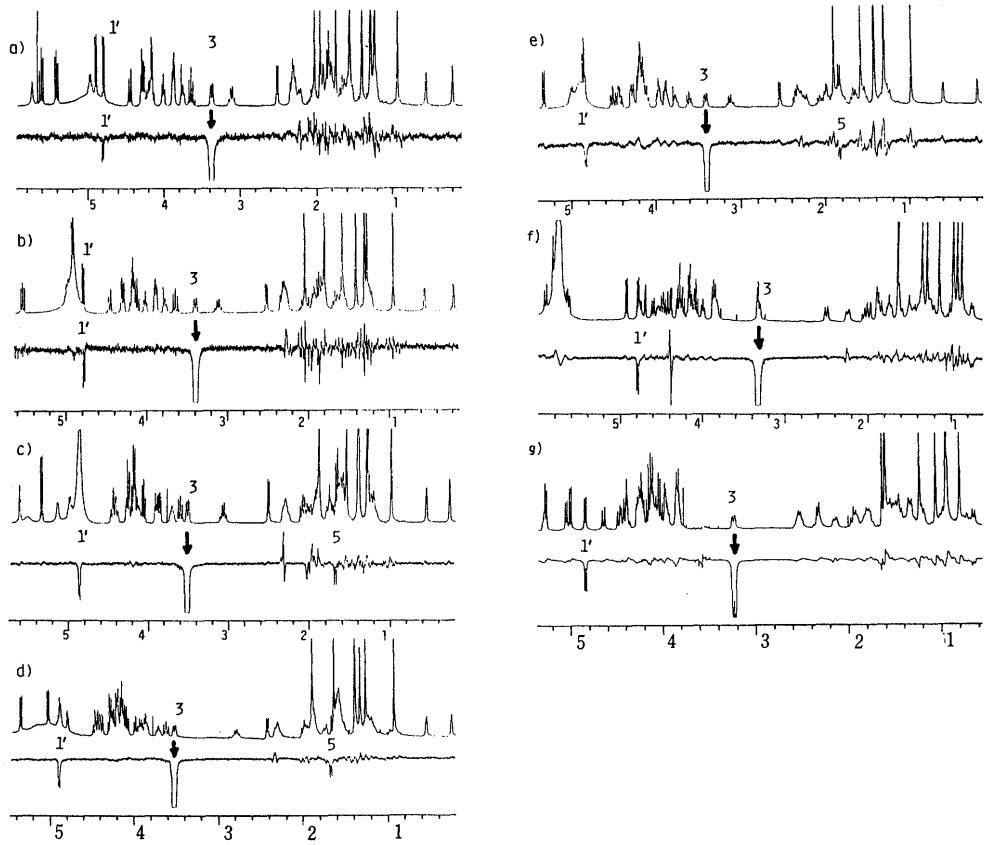

Fig. 4. NOE Difference Spectra of Some Saponins in C_5D_5N at 25 °C

Normal (Upper) and Difference (Lower) Spectra of a) Astragaloside I (**5**), b) Astragaloside II (**6**), c) Astragaloside III (**7**), d) Astragaloside V (**9**), e) Astragaloside VI (**10**), f) Saikosaponin c (**12**), and g) Ginsenoside R_{b1} (**13**)

TABLE III. NOE Observed in NOE Difference Spectra of Some Saponins[a)]

Proton(s) irradiated	Proton(s) which showed NOE[b)]								
	5	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
3	1′ (−)	1′ (−)	5 (−) 1′ (−)	1′ (−)	5 (−) 1′.(−)	5 (−) 1′ (−)	c)	1′ (−)	1′ (−)
6	1″ (+)	1″ (+)		1″ (+)					
18									13 (−) 19 (−)
19									18 (−) 29 (−)
21									1‴ (−)
28									3 (−) 5 (−) 29 (−) 1′ (−)
29									19 (−)
30									17 (−)
1′	3′ (−)	3 (−) 3′ (−)	3 (−) 3′ (+) 2″ (+)	3 (−)	3 (−)	3 (−)	3 (−) 5″ (−)	3 (−) 3′ (−) 5′ (−)	3 (−) 3′ (−) 5′ (−)
1″	3″ (+)	2″ (+) 3″ (+) 6″ (+)	2′ (+)	3″ (+)	d)	d)	3″ (−)	2′ (−) 3′ (−) 4′ (−) 2″ (−)	2′ (−)
1‴					d)			d)	17 (−) 3‴ (−) 5‴ (−)

a) Measured in C_5D_5N at 25 °C. *b*) (+) and (−) indicate positive and negative NOE, respectively. *c*) NOE experiment was not done because of the overlapping of the 3-H and 3″-H signals. *d*) Significant NOE was not observed.

standard, and chemical shifts are recorded in δ values. Multiplicities of ^{13}C-NMR signals were determined by the distortionless enhancement by polarization transfer (DEPT) method. The 2-D NMR spectra were measured by the use of the JEOL standard pulse sequences (^{1}H–^{1}H COSY, VCOSYN, 45° mixing pulse; ^{1}H–^{13}C COSY, VBDCHSHF, J=140 Hz; long-range ^{1}H–^{13}C COSY, VCHSHF, J=10 Hz) and the collected data

were processed with the standard JEOL software. NOE difference spectra were also measured by the use of the JEOL standard pulse sequence (DIFNOE2) with 5 s irradiation.

Extraction and Separation Dried roots (8 kg) of *Astragalus ernestii* COMB. were cut into small pieces and percolated with 95% EtOH. The extract was concentrated *in vacuo* to a syrup. Ten volumes of MeOH was added to this syrup and the insoluble material was filtered off. The filtrate was concentrated *in vacuo* and the residue was dissolved again in ten volumes of water and extracted with BuOH. The BuOH extract was concentrated *in vacuo* to give a syrup (300 g), which was then subjected to silica gel (2.4 kg) column chromatography with $CHCl_3$–MeOH–H_2O (7:3:1, lower layer) to give a mixture of crude saponins (98 g). This mixture was subjected to reversed-phase column chromatography (Waters, Bondapak C_{18}, 500 g) and eluted with H_2O, H_2O–MeOH (9:1, 8:2, 6:4, 4:6, and 2:8), and MeOH. The fractions eluted with H_2O–MeOH (6:4 and 4:6) were combined and further separated by repeated silica gel column chromatography. Elution with $CHCl_3$–MeOH–H_2O (7:3:1, lower layer) gave asernestioside C (**1**, 45 mg) along with asernestiosides A (**2**, 408 mg)[9] and B (**3**, 1.5 g).[9]

Asernestioside C (1) Colorless fine crystals (from MeOH), mp 204—207 °C, $[\alpha]_D$ −13.22° (c=0.32, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3375 (OH), 1732 (CO), 1623, 1369, 1241, 1039. ^{1}H- and ^{13}C-NMR: see Tables I and II. FAB-MS m/z: 973 $[M+H]^+$.

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