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Recurvosides A - E, New Polyhydroxylated Steroidal Saponins from *Nolina recurvata* Stems

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Abstract: A series of five new polyhydroxylated steroidal saponins, named recurvosides A - E, were isolated from the stems of *Nolina recurvata* (Agavaceae). Their structures were determined through detailed interpretation of various spectra, including two-dimensional (2D) NMR, and by acid-catalized hydrolysis. Among them, recurvoside E was revealed to be very unique in structure being a trisdesmoside with a β-D-fructofuranose as the monosaccharide component attached to the C-21 hydroxyl group of the aglycone. The isolated saponins were evaluated for inhibitory activity on cAMP phosphodiesterase as a primary screening test to find new biologically active compounds.

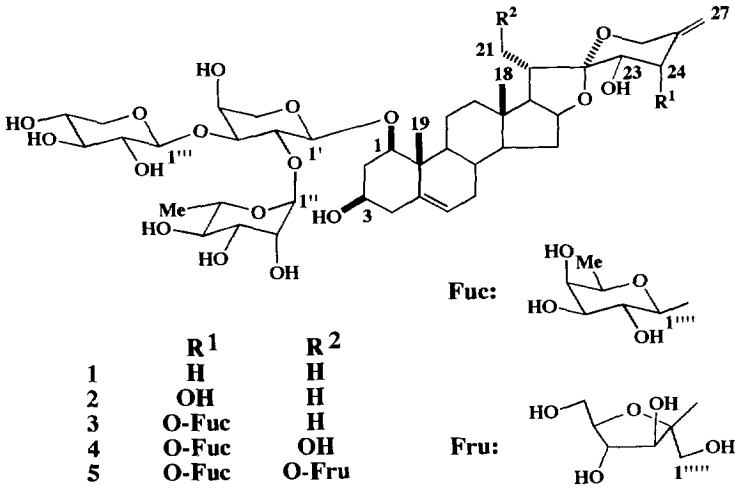
Introduction

We have been engaging in systematic studies on the steroidal constituents of plants of the family Liliaceae to identify new bioactive substances by means of cAMP phosphodiesterase inhibition test as the primary screening.[1] Our attention has been now turn out to the steroidal compounds of plants of the family Agavaceae since most of the Agavaceae plants had been classified to Liliaceae or Amanyllidaceae and the species belonging to the representative genera, *Agave* and *Yucca* in Agavaceae are known as rich sources of steroidal saponins.[2]

Nolina recurvata (Agavaceae) is indigenous to Mexico and grows a large stem. Several flavonoids were isolated from *N. recurvata*[3] but no steroidal compound appears to have been detected in the plant up to the present. Our phytochemical screening of *N. recurvata* stems has resulted in the isolation of a series of five new polyhydroxylated steroidal saponins, named recurvosides A (**1**) - E (**5**). This paper reports the structural elucidation of the new saponins based on detailed interpretation of various spectra, including two-dimensional (2D) NMR spectroscopies and acid-catalized hydrolysis. The inhibitory activity exhibited by the saponins on cAMP phosphodiesterase was also investigated.

Results and Discussion

The 1-butanol-soluble fraction of the methanolic extract of *N. recurvata* yielded recurvoside A (**1**) (0.0064% fresh weight), B (**2**) (0.008%), C (**3**) (0.0098%), D (**4**) (0.075%) and E (**5**) (0.001%) after a series of chromatographic separations.



	R1	R2
1	H	H
2	OH	H
3	O-Fuc	H
4	O-Fuc	OH
5	O-Fuc	O-Fru

Recurvoside A (**1**) was assigned the molecular formula $C_{43}H_{66}O_{17}$ by the ^{13}C NMR data, negative-ion FABMS (*m/z* 853 [M - H]$^-$) and elemental analysis. The glycosidic nature of **1** was shown by the IR absorptions at 3420 and 1035 cm^{-1}. The ^1H NMR spectrum of **1** in pyridine-d_5 exhibited two three-proton singlets at δ 1.37 and 1.03, and a three-proton doublet at δ 1.11 (J = 7.0 Hz), which were recognized as typical steroid methyls. Furthermore, signals for three anomeric protons at δ 6.30 (br s), 4.97 (d, J = 7.5 Hz) and 4.73 (d, J = 7.2 Hz), exomethylene protons at δ 4.83 and 4.76 (each br s) and an olefinic proton at δ 5.56 (br d, J = 5.5 Hz), completed distinctive features of the structure of **1**. Attempted acid hydrolysis of **1** with 1M hydrochloric acid in dioxane - H_2O (1 : 1) led to production of L-arabinose, L-rhamnose and D-xylose in a ratio of 1 : 1 : 1 together with unidentifed artifactual sapogenols; no genuine aglycone could be obtained. Treatment of **1** with acetic anhydride in pyridine introduced nine acetyl groups, being consistent with the presence of nine primary or secondary hydroxyl groups. The ^{13}C NMR spectrum displayed 43 resonance lines, 16 of which could be due to the monosaccharide components and 27 due to the aglycone part. One distinctive quaternary carbon signal at δ 111.8 obviously indicated a ketal carbon. The above data led to the hypothesis that **1** was a steroid of spirostanol with three monosaccharides.

Inspection of the ^1H-^1H COSY spectrum combined with the HOHAHA spectrum, followed by the HMQC spectrum, which were measured in pyridine-d_5 - methanol-d_4 (11 : 1) to eliminate the resonances of exchangeable protons and optimize spectral despersion, allowed to identify the ^1H-^1H spin-networks and assign the corresponding one-bond coupled ^{13}C signals. The ^1H-^1H networks, including an exomethylene and two methyl groups, isolated by quaternary carbons, were connected through the use of the HMBC spectrum

optimized for the $^{n}J_{C\text{-}H}$ of 8 Hz, leading to assemble the aglycone part of **1** as spirosta-5,25(27)-diene bearing oxygen atoms at C-1, -3 and -23.

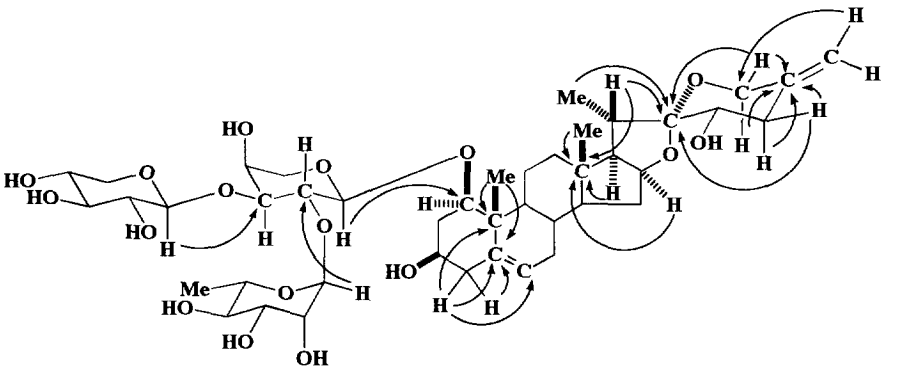


Fig. 1. HMBC Correlations of **1**.

The C-1β and C-3β orientations of the oxygen atoms were confirmed by the ^{1}H NMR parameters of the 1-H and 3-H protons ($^{3}J_{1\text{-H}\ (\delta\ 3.74)\ -\ 2ax\text{-H}\ (\delta\ 2.27)}$ = 12.0 Hz, $^{3}J_{1\text{-H}\ -\ 2eq\text{-H}\ (\delta\ 2.63)}$ = 4.0 Hz and $W_{1/2\ (3\text{-H: }\delta\ 3.79)}$ = 25.0 Hz). The resonance at δ 3.83 assignable to 23-H was split into doublet of doublets with the *J* values of 12.6 and 5.2 Hz, and showed clear NOE correlations with both 20-H (δ 2.94, m) and 21-Me (δ 1.08, d, *J* = 7.0 Hz) in the phase-sensitive NOESY spectrum, indicating the C-23*S* configuration as well as usual C-22α configuration. The NOEs between 24_{eq}-H (δ 2.76, dd, *J* = 12.6, 5.2 Hz) and 27a-H (δ 4.81, br s), 27b-H (δ 4.84, br s) and 26b-H (δ 3.96, d, *J* = 12.5 Hz), and 26a-H (δ 4.38, d, *J* = 12.5 Hz) and 16-H (δ 4.58, q-like, *J* = 8.5 Hz), confirmed the F-ring conformation to be nearly chair-form.

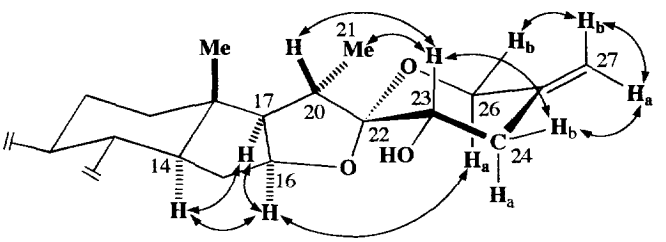


Fig. 2. NOE Correlations of **1**.

The presence of an α-L-arabinopyranosyl ($^{4}C_{1}$), an α-L-rhamnopyranosyl ($^{1}C_{4}$) and a β-D-xylopyranosyl ($^{4}C_{1}$) units in **1** was readily revealed by tracing out the sequential ^{1}H-^{1}H coupling systems through the ^{1}H-^{1}H COSY spectrum, starting from the anomeric proton signals. The sequence of the monosaccharide and its linkage site to the aglycone were subsequently confirmed by the observation of $^{3}J_{C\text{-}H}$ correlations from each anomeric proton traversing the glycosidic bond to carbon of another substituted

monosaccharide or aglycone in the HMBC spectrum. The anomeric proton signals at δ 6.19, 4.90 and 4.67 assigned to rhamnose, xylose and arabinose were correlated to the three-bond coupled ^{13}C signals at δ 74.2 (C-2 of arabinose), 84.7 (C-3 of arabinose) and 84.0 (C-1 of aglycone), respectively. The structure of the trisaccharide, 2,3-branched arabinose embracing rhamnose at C-2 and xylose at C-3, and its linkage to C-1 of the aglycone, was thus given. The data presented above led to construct the full structure of **1** as (23*S*)-spirosta-5,25(27)-diene-1β,3β,23-triol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside}.

Recurvoside B (**2**), $C_{43}H_{66}O_{18}$, has one more oxygen atom than does **1**. The NMR data of **2** showed that it possessed an identical saccharide structure to **1**, but slightly differed from it in terms of the aglycone structure. The ^{1}H-^{1}H COSY, HOHAHA and HMQC spectra accounted for the introduction of an additional hydroxyl group at C-24. The 23-H proton (δ 3.86) clearly showed NOE correlations with 20-H (δ 2.91, m) and 21-Me (δ 1.07, d, J = 7.0 Hz), indicating the C-23*S* configuration. The 24-H proton (δ 4.63) was coupled to 23-H by 4.0 Hz, and showed NOEs with 23-H and 27a-H (δ 5.09, d, J = 0.9 Hz), which was consistent with the C-24*S* configuration. The NOEs between 27b-H (δ 5.00, d, J = 0.9 Hz) and 26b-H (δ 3.99, d, J = 12.2 Hz), and 26a-H (δ 4.77, d, J = 12.2 Hz) and 16-H (δ 4.60, q-like, J = 8.6 Hz) indicated the F-ring conformation to be nearly chair-form. The structure of **2** was formulated as (23*S*,24*S*)-spirosta-5,25(27)-diene-1β,3β,23,24-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside}.

Recurvoside C (**3**), $C_{49}H_{76}O_{22}$, was a much polar constituent than **1** and **2**. The ^{1}H NMR spectrum of **3** showed four anomeric proton signals at δ 6.29 (br s), 5.14 (d, J = 7.9 Hz), 4.96 (d, J = 7.4 Hz) and 4.71 (d, J = 7.3 Hz) as well as three steroid methyls at δ 1.40 (s), 1.07 (d, J = 7.0 Hz) and 0.93 (s) and exomethylene at δ 5.23 and 5.08 coupled each other (J = 1.0 Hz). Acid hydrolysis of **3** gave L-arabinose, L-rhamnose, D-xylose and D-fucose as the carbohydrate compounds. On comparison of the whole ^{13}C NMR spectrum of **3** with that of **2**, a set of additional signals corresponding to a terminal β-D-fucopyranosyl appeared, and the signals due to C-24 of the aglycone was displaced downfield by 8.8 ppm to be observed at δ 83.0, confirming that the C-24 hydroxyl group was the glycosylated position to which the additional D-fucose was linked. Partial hydrolysis of **3** with 0.2M hydrochloric acid gave **2** and D-fucose. The structure of **3** was shown to be (23*S*,24*S*)-spirosta-5,25(27)-diene-1β,3β,23,24-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside} 24-*O*-β-D-fucopyranoside.

Recurvoside D (**4**), $C_{49}H_{76}O_{23}$, has one more oxygen atom than does **3**. In the ^{13}C NMR spectrum of **4**, the signal due to the C-21 methyl, which was observed at δ 14.7 in **3**, was displaced signal due to hydroxymethyl carbon at δ 62.4, accompanied by downfield shift of C-20 by 8.8 ppm and upfield shift of C-17 by 3.6 ppm. All other signals were almost superimposable between **3** and **4**. Thus, **4** was proven to be the hydroxy derivative of **3** at C-21, that is, (23*S*,24*S*)-spirosta-5,25(27)-diene-1β,3β,21,23,24-pentol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside} 24-*O*-β-D-fucopyranoside.

Recurvoside E (**5**), $C_{55}H_{86}O_{28}$, was immediately suggested to have the same aglycone as **4** by the ^{1}H and ^{13}C NMR spectra. On acid hydrolysis, **5** liberated L-arabinose, L-rhamnose, D-xylose, D-fucose and D-fructose. The signals from a 2,3-branched α-L-arabinopyranosyl, a terminal α-L-rhamnopyranosyl, a terminal β-D-xylopyranosyl and a terminal β-D-fucopyranosyl units were could be observed in the ^{13}C NMR of **5** as in those of **3** and **4**. Furthermore, six signals, δ 62.7 (CH_2), 105.3 (C), 79.2 (CH), 77.1 (CH), 84.2 (CH) and

Table 1. ^{13}C NMR Spectral Data for Compounds **1** - **5** in Pyridine-d_5

	C	**1**	**2**	**3**	**4**	**5**
	1	83.7	83.7	83.7	83.7	83.7
	2	37.4	37.5	37.5	37.4	37.4
	3	68.3	68.2	68.2	68.3	68.2
	4	43.9	43.8	43.9	43.9	43.8
	5	139.6	139.5	139.7	139.8	139.6
	6	124.7	124.7	124.6	124.7	124.6
	7	32.1	32.0	31.9	32.0	31.8
	8	33.1	33.0	33.0	33.2	33.1
	9	50.4	50.4	50.4	50.3	50.1
	10	42.9	42.9	42.9	43.0	42.8
	11	24.1	24.1	24.0	24.0	23.9
	12	40.6	40.6	40.5	40.3	39.9
	13	40.8	40.7	40.8	41.0	40.7
	14	56.9	56.9	56.8	56.9	56.7
	15	32.4	32.3	32.3	32.6	32.4
	16	82.0	83.3	82.2	82.2	81.8
	17	62.5	61.4	61.5	57.9	57.4
	18	16.9	16.8	16.8	16.9	16.7
	19	15.0	15.0	15.1	15.1	15.0
	20	35.8	37.1	37.5	46.3	43.5
	21	14.6	14.6	14.7	62.4	62.2
	22	111.8	112.7	111.8	111.7	111.3
	23	68.6	69.6	70.4	72.0	72.0
	24	38.9	74.2	83.0	83.5	83.2
	25	144.4	146.4	144.0	143.9	143.4
	26	64.3	60.8	61.5	61.6	61.5
	27	109.3	112.3	113.7	113.7	114.1
Ara	1'	100.5	100.5	100.5	100.4	100.5
	2'	74.3	74.2	74.3	74.3	74.2
	3'	84.4	84.5	84.4	84.4	84.4
	4'	69.6	69.6	69.5	69.6	69.5
	5'	67.1	67.1	67.1	67.1	67.1
Rha	1"	101.9	101.8	101.8	101.8	101.8
	2"	72.5	72.5	72.5	72.5	72.5
	3"	72.6	72.6	72.6	72.6	72.5
	4"	74.3	74.2	74.2	74.2	74.2
	5"	69.6	69.6	69.5	69.5	69.7
	6"	19.1	19.1	19.1	19.1	19.2
Xyl	1"'	106.5	106.5	106.4	106.4	106.5
	2"'	74.7	74.6	74.6	74.6	74.6
	3"'	78.3	78.3	78.2	78.2	78.2
	4"'	71.0	71.0	71.0	71.0	71.0
	5"'	66.9	67.0	66.9	66.9	67.1
Fuc	1""			106.3	106.3	106.1
	2""			73.1	73.2	73.1
	3""			75.4	75.4	75.4
	4""			72.8	72.9	72.8
	5""			71.6	71.6	71.5
	6""			17.2	17.3	17.2
Fru	1""'					62.7
	2""'					105.3
	3""'					79.2
	4""'					77.1
	5""'					84.2
	6""'					64.3

Table 2. ^{1}H and ^{13}C NMR Chemical Shift Assignments of the Aglycone Moiety of Compounds **1** and **5** in Pyridine-d_5 - Methanol-d_4 (11 : 1)

	1			**5**		
position	^{1}H	J (Hz)	^{13}C	^{1}H	J (Hz)	^{13}C
1	3.74 dd	12.0, 4.0	84.0	3.71 dd	11.9, 3.6	83.9
2 a	2.63		37.4	2.62		37.3
b	2.27 q-like	12.0		2.26 q-like	11.9	
3	3.79 m	25.0 [a]	68.3	3.77 m	25.0 [a]	68.3
4 a	2.61 dd	12.7, 12.2	43.7	2.62 dd	11.9, 11.9	43.7
b	2.52 dd	12.7, 4.3		2.52 dd	11.9, 4.4	
5			139.5			139.6
6	5.56 br d		125.0	5.56		124.9
7 a	1.86		32.1	1.75		31.9
b	1.50			1.40		
8	1.52		33.1	1.40		33.2
9	1.47		50.5	1.40		50.3
10			43.0			42.9
11 a	2.87		24.1	2.73		24.0
b	1.57			1.40		
12 a	1.57		40.7	1.30		40.1
b	1.29			1.09		
13			40.9			40.8
14	1.14		57.0	1.00		56.9
15 a	2.01		32.4	1.76		32.5
b	1.50			1.40		
16	4.58 q-like	8.5	82.1	4.52 q-like	8.0	83.3
17	1.80 dd	8.5, 7.1	62.6	1.54 dd	8.0, 6.4	57.4
18	1.00 s		16.9	0.91 s		16.8
19	1.34 s		15.0	1.33 s		15.1
20	2.94 m		35.9	3.20 m		43.4
21 a	1.08 d	7.0	14.6	4.17		62.1
b				3.86		
22			111.8			111.4
23	3.83 dd	12.6, 5.2	68.5	4.51		71.9
24 a	2.86 dd	12.6, 12.6	38.8	4.51		81.9
b	2.76 dd	12.6, 5.2				
25			144.4			143.7
26 a	4.38 d	12.5	64.3	4.76 d	11.7	61.6
b	3.96 d	12.5		3.99 d	11.7	
27 a	4.84 br s		109.5	5.06 br s		114.2
b	4.81 br s					

[a] $W_{1/2}$

64.3 (CH_2) assignable to a terminal D-fructose appeared in **5**, and the shift values were well corresponded to those of methyl β-D-fructofuranoside; the ^{13}C shifts featured a remarkable difference between methyl α- and β-D-fructofuranosides.[4] Partial hydrolysis of **5** with 0.2M hydrochloric acid gave **4** and D-fructose. The issue of the linkage position of the fructose to the aglycone was difficult to establish by conventional spectroscopic comparison because the ^{13}C shifts due to the aglycone part were almost identical between **4** and **5**, with only difference being C-20, which was shifted upfield by 2.8 ppm in **5**, and no downfield shift by *O*-glycosylation could be detected at C-3, -21 and -23. This was solved by inspection of the HMBC spectrum. With all the ^{1}H and ^{13}C signals assigned by the ^{1}H-^{1}H COSY, HOHAHA and HMQC spectra, attention was focused on the

Table 3. ^{1}H and ^{13}C NMR Chemical Shift Assignments of the Saccharide Moieties of Compounds **1** and **5** in Pyridine-d_5 - Methanol-d_4 (1 1 : 1)

		1			**5**		
	position	^{1}H	J (Hz)	^{13}C	^{1}H	J (Hz)	^{13}C
Ara	1'	4.67 d	7.5	100.7	4.65 d	7.8	100.6
	2	4.54 dd	8.9, 7.5	74.2	4.52 dd	9.2, 7.8	74.2
	3	4.06 dd	8.9, 3.2	84.7	4.05 dd	9.2, 3.2	84.6
	4	4.37 br s		69.7	4.36 br s		69.7
	5a	4.21 dd	11.5, 2.1	67.0	4.17 br d	11.3	67.1
	b	3.69 br d	11.5		3.67 br d	11.3	
Rha	1"	6.19 br s		101.8	6.20 br s		101.8
	2	4.66 br d	3.4	72.4	4.67 br d	3.4	72.4
	3	4.46 dd	9.4, 3.4	72.3	4.46 dd	9.4, 3.4	72.3
	4	4.16 dd	9.5, 9.4	74.1	4.17 dd	9.4, 9.4	74.1
	5	4.69 dq	9.5, 6.1	69.5	4.69 dq	9.4, 6.1	69.5
	6	1.65 d	6.1	19.1	1.65 d	6.1	19.1
Xyl	1'''	4.90 d	7.5	106.5	4.90 d	7.5	106.5
	2	3.83 dd	8.4, 7.5	74.6	3.83 dd	8.6, 7.5	74.6
	3	3.97 dd	9.5, 8.4	78.1	3.97 dd	8.9, 8.6	78.1
	4	4.02 ddd	10.2, 9.5, 5.0	70.9	4.04 ddd	10.3, 8.9, 4.2	70.9
	5a	4.22 dd	11.0, 5.0	67.0	4.23 dd	11.4, 4.2	67.1
	b	3.61 dd	11.0, 10.2		3.61 dd	11.4, 10.3	
Fuc	1''''				5.01 d	7.8	106.0
	2				4.27 dd	9.3, 7.8	72.9
	3				3.99 dd	9.3, 3.4	75.2
	4				3.91 br d	3.4	72.8
	5				3.73 m		71.6
	6				1.43	6.4	17.2
Fru	1''''' a				4.17 d	11.7	62.3
	b				4.12 d	11.7	
	2						105.2
	3				5.02 d	8.2	78.9
	4				4.65 dd	8.2, 8.2	77.0
	5				4.50 ddd	8.2, 6.9, 3.2	84.1
	6 a				4.32 dd	12.1, 6.9	64.5
	b				4.28 dd	12.1, 3.2	

^{13}C signal due to C-2 of the fructose at δ 105.2, which showed a $^{3}J_{C\text{-}H}$ correlation with 21a-H at δ 4.17. The additional HMBC correlation from each anomeric proton across the glycosidic bond to another substituted monosaccharide or aglycone reinforced the respective linkage of the arabinose embracing rhamnose at C-2 and xylose at C-3, and the fucose to C-1 and C-24 of the aglycone. The structure of **5** was thus entierly secured to be (23*S*,24*S*)-spirosta-5,25(27)-diene-1β,3β,21,23,24-pentol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside} 21-*O*-β-D-fructofuranoside 24-*O*-β-D-fucopyranoside.

Fructose is a common monosaccharide, which is abundant in certain fruits and honey and consituent of sucorse and inulin. It is, however, very rare to be present as a component as plant glycoside, and to the best of our knowledge,[2,5] recurvoside E (**5**) is the first representative of a saponin embracing a fructose as the carbohydrate component among both the steroid and triterpenoid saponins reported up to now. It must be also

emphasized that recurvoside E (**5**) is very uniqe in structure being a trisdesmoside of the C-1, -3, -21, -23 and -24 hydroxylated spirostan.

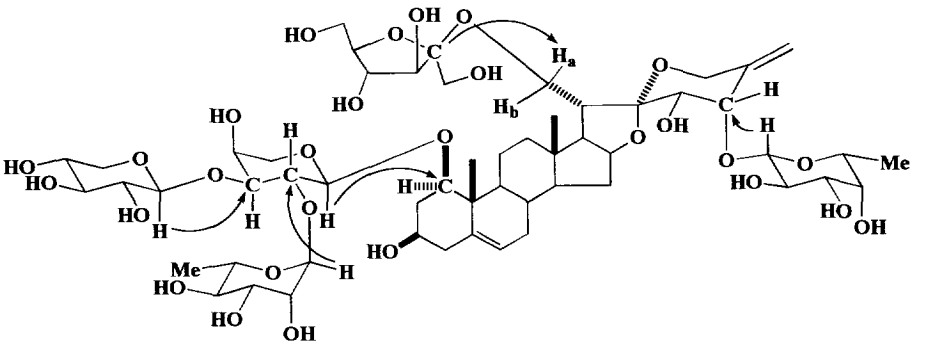


Fig. 3. HMBC Correlations of **5**.

The isolated saponins were examined for inhibitory activity on cAMP phosphodiesterase. This test provides a useful tool for screening of biologically active compounds present in natural sources.[6] Recurvoside A (**1**) exhibited medium inhibitory activity with IC_{50} value of 19.5×10^{-5} M and introduction of hydroxyl group to C-24 reduced the activity (**2**: 51.2×10^{-5} M). Modifications of **2** with the further polar substituents, formation of a β-D-fucopyaranosyl linkage to C-24 (**3**), followed by introduction of a hydroxyl group to C-21 (**4**) and substitution with a β-D-fructofuranose at C-21 (**5**) led to an enhancement of activity (**3**: 26.3×10^{-5} M; **4**: 17.7×10^{-5} M; **5**: 11.9×10^{-5} M).

Experimental

General: Optical rotations were measured using a JASCO DIP-360 automatic digital polarimeter. IR spectra were recorded on a Hitachi 260-30 spectrophotometer and MS on a VG AutoSpec E instrument. Elemental analysis was carried out using Perkin-Elmer 240B elemental analyzer. 1D NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz for ^{1}H-NMR) and 2D NMR on a Bruker AM-500 instrument (500 MHz for ^{1}H-NMR). Chemical shifts are given as δ-values with reference to tetramethylsilane (TMS), the internal standard. Silica-gel (Fuji-Silysia Chemical), Diaion HP-20 (Mitsubishi-Kasei), Sepahdex LH-20 (Pharmacia) and octadecylsilanized (ODS) silica-gel (Nacalai Tesque) were used for column chromatographies. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed using a Tosoh HPLC system (Tosoh: pump, CCPM; controller, CCP controller PX-8010; detector, RI-8010 or UV-8000) equipped with a KAPCELL PAK C_{18} column (SHISEIDO, 10 mm i.d. x 250 mm, ODS, 5 μm) for preparative HPLC and a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d. x 250 mm, ODS, 5 μm) or a TSK-gel Silica-60 column (Tosoh, 4.6 mm i.d. x 250 mm, silica-gel, 5μm) for analytical HPLC. The liquid scintillation counter used was an Aloka LSC-903 instrument. Beef heart phosphodiesterase was purchased from Boehringer. Snake venom nucleotidase and cyclic AMP were obtained from Sigma, and [^{3}H]-cAMP from the Radiochemical Center.

Extraction and isolation. Commercially available fresh stems of *N. recurvata* (2.5 kg) were cut into pieces and extracted with MeOH under reflux. The extract was concentrated almost to dryness under reduced pressure and the crude residue, after dilution with H_2O, was extracted with *n*-BuOH. The *n*-BuOH-soluble phase was fractionated on a silica-gel column, eluting with a gradient mixture of CH_2Cl_2 - MeOH (9 : 1; 4 : 1; 2 : 1), and finally with MeOH to give four fractions (I - IV). Fraction III contained a considerable amount of saccharides, the removal of which was performed by column chromatography on Diaion HP-20 with an increasing amount of MeOH in H_2O. The fractions eluted with 80% MeOH and 100% MeOH were combined and purified by silica-gel column chromatography with a mobile phase consisting of $CHCl_3$ - MeOH - H_2O (20 : 10 : 1) to give **3** (246 mg, 0.0098%) and **4** (1.89 g, 0.075%) as the pure compounds and a mixture of **1** and **2**. Preparative HPLC using MeOH - H_2O (4 : 1) as the solvent system was applied to the mixture to furnish **1** (161 mg, 0.0064%) and **2** (200 mg, 0.008%). Fraction IV was chromatographed on Diaion HP-20 with H_2O - MeOH, silica-gel with $CHCl_3$ - MeOH - H_2O (20 : 10 : 1), Sephadex LH-20 with MeOH, and on ODS silica-gel with MeOH - H_2O to yield **5** (26.2 mg, 0.001%).

Recurvoside A (1). An amorphous powder, $[\alpha]_D^{26}$ -62.9° (MeOH; *c* = 0.25). Anal. Calcd for $C_{43}H_{66}O_{17}\cdot H_2O$: C, 59.16; H, 7.85. Found: C, 59.02; H, 7.72. Negative-ion FABMS *m/z* 853 $[M - H]^-$; IR ν_{max} (KBr) cm^{-1}: 3420 (OH), 2900 (CH), 1445, 1365, 1245, 1130, 1035, 975; ^1H NMR (pyridine-d_5): δ 6.30 (1H, br s, 1"-H), 5.56 (1H, br d, *J* = 5.5 Hz, 6-H), 4.97 (1H, d, *J* = 7.5 Hz, 1'''-H), 4.83 and 4.76 (each 1H, br s, 27-H_2), 4.73 (1H, d, *J* = 7.2 Hz, 1'-H), 1.70 (3H, d, *J* = 6.1 Hz, 6"-Me), 1.37 (3H, s, 19-Me), 1.11 (3H, d, *J* = 7.0 Hz, 21-Me), 1.03 (3H, s, 18-Me).

Acetylation of 1. Compound **1** (30 mg) was acetylated with Ac_2O in pyridine and the crude acetate was chromatographed on silica-gel eluting with hexane - Me_2CO (3 : 2) to yield the corresponding nonaaceate (**1a**) (34.1 mg). Compound **1a**: an amorphous powder. IR ν_{max} (KBr) cm^{-1}: 2960 (CH), 1745 (C=O), 1435, 1365, 1235, 1135, 1040, 975; ^1H NMR (benzene-d_6): δ 5.73 (1H, dd, *J* = 10.2, 3.6 Hz, 3"-H), 5.65 (1H, dd, *J* = 3.6, 1.4 Hz, 2"-H), 5.62 (1H, d, *J* = 1.4 Hz, 1"-H), 5.54 (1H, dd, *J* = 8.5, 8.5 Hz, 3'''-H), 5.51 (1H, dd, *J* = 10.2, 10.2 Hz, 4"-H), 5.48 (1H, br d, *J* = 5.5 Hz, 6-H), 5.19 (1H, dd, *J* = 12.3, 5.6 Hz, 23-H), 5.18 (1H, dd, *J* = 8.5, 6.7 Hz, 2'''-H), 5.12 (1H, br s, 4'-H), 5.01 (1H, ddd, *J* = 8.5, 8.5, 5.1 Hz, 4'''-H), 4.91 (1H, m, 3-H), 4.78 (1H, d, *J* = 6.7 Hz, 1'''-H), 4.68 and 4.66 (each 1H, br s, 27-H_2), 4.67 (1H, dq, *J* = 10.2, 6.2 Hz, 5"-H), 4.49 (1H, q-like, *J* = 6.7 Hz, 16-H), 4.35 (1H, d, *J* = 12.2 Hz, 26a-H), 4.10 (1H, dd, *J* = 7.8, 7.6 Hz, 2'-H), 4.03 (1H, dd, *J* = 11.6, 5.1 Hz, 5a'''-H), 3.97 (1H, d, *J* = 7.6 Hz, 1'-H), 3.88 (1H, dd, *J* = 13.1, 1.9 Hz, 5'a-H), 3.83 (1H, d, *J* = 12.2 Hz, 26b-H), 3.56 (1H, dd, *J* = 12.1, 4.0 Hz, 1-H), 3.49 (1H, dd, *J* = 7.8, 3.7 Hz, 3'-H), 3.35 (1H, dd, *J* = 11.6, 8.5 Hz, 5'''b-H), 2.88 (1H, br d, *J* = 13.1 Hz, 5'b-H), 2.86 (1H, dd, *J* = 12.3, 12.3 Hz, 24a-H), 2.64 (1H, dd, *J* = 12.3, 5.6 Hz, 24b-H), 1.96, 1.94, 1.90, 1.80, 1.78 x 2, 1.73, 1.69 and 1.58 (each 3H, s, Ac x 9), 1.31 (3H, d, *J* = 6.2 Hz, 6"-Me), 1.23 (3H, s, 19-Me), 1.12 (3H, d, *J* = 6.9 Hz, 21-Me), 0.98 (3H, s, 18-Me).

Recurvoside B (2). An amorphous powder, $[\alpha]_D^{26}$ -60.0° (MeOH; *c* = 0.36). Anal. Calcd for $C_{43}H_{66}O_{18}\cdot H_2O$: C, 58.10; H, 7.71. Found: C, 57.94; H, 7.48. Negative-ion FABMS *m/z* 869 $[M - H]^-$; IR ν_{max} (KBr) cm^{-1}: 3420 (OH), 2905 (CH), 1445, 1370, 1245, 1135, 1040, 975; ^1H NMR (pyridine-d_5): δ 6.32

(1H, br s, 1''-H), 5.55 (1H, br d, J = 5.6 Hz, 6-H), 5.09 and 4.99 (each 1H, br s, 27-H_2), 4.98 (1H, d, J = 7.7 Hz, 1'''-H), 4.73 (1H, d, J = 7.3 Hz, 1'-H), 1.72 (3H, d, J = 6.2 Hz, 6''-Me), 1.38 (3H, s, 19-Me), 1.11 (3H, d, J = 7.0 Hz, 21-Me), 1.01 (3H, s, 18-Me).

Recurvoside C (3). An amorphous powder, $[\alpha]_D^{26}$ -51.2° (MeOH; c = 0.29). Anal. Calcd for $C_{49}H_{76}O_{22}\cdot H_2O$: C, 56.86; H, 7.60. Found: C, 56.45; H, 7.52. Negative-ion FABMS m/z 1015 $[M - H]^-$; IR ν_{max} (KBr) cm^{-1}: 3390 (OH), 2905 (CH), 1445, 1370, 1245, 1125, 1040, 975; ^1H NMR (pyridine-d_5): δ 6.29 (1H, br s, 1''-H), 5.57 (1H, br d, J = 5.4 Hz, 6-H), 5.23 and 5.08 (each 1H, d, J = 1.0 Hz, 27-H_2), 5.14 (1H, d, J = 7.9 Hz, 1''''-H), 4.96 (1H, d, J = 7.4 Hz, 1'''-H), 4.71 (1H, d, J = 7.3 Hz, 1'-H), 1.70 (3H, d, J = 6.1 Hz, 6''-Me), 1.47 (3H, d, J = 6.4 Hz, 6''''-Me), 1.40 (3H, s, 19-Me), 1.07 (3H, d, J = 7.0 Hz, 21-Me), 0.93 (3H, s, 18-Me).

Recurvoside D (4). Colorless needles recrystallized from $CHCl_3$ - MeOH, mp 222 - 226 °C, $[\alpha]_D^{26}$ -43.9° (MeOH; c = 0.36). Anal. Calcd for $C_{49}H_{76}O_{23}\cdot 2H_2O$: C, 55.05; H, 7.54. Found: C, 54.81; H, 7.41. Negative-ion FABMS m/z 1031 $[M - H]^-$; IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2920 (CH), 1445, 1375, 1250, 1135, 1045, 980; ^1H NMR (pyridine-d_5): δ 6.27 (1H, br s, 1''-H), 5.58 (1H, br d, J = 5.4 Hz, 6-H), 5.20 and 5.05 (each 1H, br s, 27-H_2), 5.15 (1H, d, J = 7.9 Hz, 1''''-H), 4.96 (1H, d, J = 7.4 Hz, 1'''-H), 4.70 (1H, d, J = 7.2 Hz, 1'-H), 1.66 (3H, d, J = 6.1 Hz, 6''-Me), 1.47 (3H, d, J = 6.4 Hz, 6''''-Me), 1.38 (3H, s, 19-Me), 1.01 (3H, s, 18-Me).

Recurvoside E (5). An amorphous powder, $[\alpha]_D^{26}$ -51.1° (MeOH; c = 0.38). Anal. Calcd for $C_{55}H_{86}O_{28}\cdot 5H_2O$: C, 51.40; H, 7.53. Found: C, 51.35; H, 7.05. Negative-ion FABMS m/z 1194 $[M]^-$; IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2925 (CH), 1370, 1255, 1125, 1040, 975; ^1H NMR (pyridine-d_5): δ 6.33 (1H, br s, 1''-H), 5.56 (1H, br d, J = 5.3 Hz, 6-H), 5.09 (1H, d, J = 7.9 Hz, 1''''-H), 5.07 (2H, s, 27-H_2), 4.97 (ovelapping with H_2O signal, 1'''-H), 4.69 (1H, d, J = 7.3 Hz, 1'-H), 1.70 (3H, d, J = 6.1 Hz, 6''-Me), 1.46 (3H, d, J = 6.4 Hz, 6''''-Me), 1.36 (3H, s, 19-Me), 1.92 (3H, s, 18-Me).

Acid hydrolysis of 1, 3 and 5. A solution of **1** (39.7 mg) in 1M HCl (dioxane - H_2O, 1 : 1, 8 ml) was refluxed for 1h under an Ar atmosphere. The reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU (Organo) column, and then transferred to a silica-gel column, eluting with $CHCl_3$ - MeOH (19 : 1) and then MeOH to give several unidentified artifactual sapogenols and a fraction containing monosaccharides. The fraction was applied to a Sep-Pak C_{18} cartridge (Waters) eluting with H_2O to give a mixture of monosaccharides (19.6 mg). Arabinose, rhamnose and xylose were identified as being present in the mixture by direct TLC comparison with authentic samples: arabinose, Rf 0.43; rhamnose, Rf 0.69; xylose, Rf 0.54 (*n*-BuOH - Me_2CO - H_2O, 4 : 5 : 1). To the sugar mixture (2 mg) in H_2O (1 ml), (-)-α-methylbenzylamine (5 mg) was added followed by $Na[BH_3CN]$ (8 mg) in EtOH (1 ml). The mixture was left standing for 3 h at 40 °C, then acetylated with Ac_2O (0.3 ml) in pyridine (0.3 ml). The reaction mixture was passed through a Sep-Pak C_{18} cartridge, eluting with a gradient mixture of MeCN - H_2O (1 : 4; 1 : 1; 9 : 1, each 10 ml). The MeCN - H_2O (9 : 1) fraction was then passed through a TOYOPAK IC-SP M cartridge (Tosoh), eluting with EtOH (10 ml), to give a mixture of the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the

monosaccharides, which was then analyzed by HPLC.[7] Derivatives of L-arabinose, L-rhamnose and D-xylose were detected. Following this procedure, **3** (20.1 mg) and **5** (5 mg) were subjected to acid hydrolysis. Compound **3** gave L-arabinose, L-rhamnose, D-xylose and D-fucose, and **5** gave L-arabinose, L-rhamnose, D-xylose, D-fucose and D-fructose. Fructose could be only detectable in the crude hydrolysate of **5** by direct TLC comparison with an authentic sapmle. The absolute configuration of the fructose was chosen in keeping with that mostly encountered among plants.

Partial hydrolysis of 3 and 5. A solution of **3** (80.4 mg) in 0.2M HCl (dioxane - H_2O, 1 : 1, 5 ml) was refluxed for 30 min under an Ar atmosphere. The reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU column, and chromatographed on silica-gel eluting with $CHCl_3$ - MeOH (3 : 1) and ODS silica-gel with MeOH - H_2O (7 : 3) to give **2** (8.2 mg) as a partial hydrolysate and D-fucose. Following this procedure, **5** (5.1 mg) was subjected to partial hydrolysis to give **4** (1 mg) and D-fructose.

Assay of cAMP phosphodiesterase activity. The phosphodiesterase activity was assayed by a modification of the method of Thompson and Brooker as described previously.[8] The assay was a two-step isotopic procedure. Tritium-labelled cAMP was hydrolyzed to 5'-AMP by phosphodiesterase, and the 5'-AMP was then further hydrolyzed to adenosine by snake venom nucleotidase. The hydrolysate was treated with an anion-exchange resin (Dowex AG1-X8; BIO-RAD) to adsorb all charged nucleotides, leaving [^{3}H]-adenosine as the only labelled compound to be counted.

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