Pergamon

0031-9422(95)00117-4

Phytochemistry, Vol. 39, No. 5, pp. 1103–1110, 1995

0031 9422/95 $9.50 + 0.00

STEROIDAL SAPONINS FROM THE TUBERS OF *DICHELOSTEMMA MULTIFLORUM* AND THEIR INHIBITORY ACTIVITY ON CYCLIC-AMP PHOSPHODIESTERASE

TOSHIHIRO INOUE, YOSHIHIRO MIMAKI,* YUTAKA SASHIDA,* TAMOTSU NIKAIDO† and TAICHI OHMOTO†

School of Pharmacy, Tokyo University of Pharmacy and Life Science (formerly, Tokyo College of Pharmacy), 1432-1, Horinouchi, Hachioji, Tokyo 192-03, Japan; †School of Pharmaceutical Sciences, Toho University, 2-2-1, Miyama, Funabashi, Chiba 274, Japan

(*Received in revised form* 21 *December* 1994)

Key Word Index—*Dichelostemma multiflorum*; Liliaceae; tubers; steroidal saponins; spirostanol saponins; c-AMP phosphodiesterase inhibition.

Abstract—Phytochemical examination of the tubers of *Dichelostemma multiflorum* led to the isolation of three new steroidal saponins together with two known saponins. The structures of the new compounds were determined by spectral data and a few chemical transformations to be (25*R*)-5α-spirostane-1β,3β-diol (brisbagenin) 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 3)-4-*O*-acetyl-α-L-arabinopyranoside}, brisbagenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-[α-L-rhamnopyranosyl-(1 → 3)]-4-*O*-acetyl-α-L-arabinopyranoside} and (22*S*,25*S*)-5α-spirostan-3β-ol 3-*O*-{*O*-β-D-galactopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside}. The known compounds were identified as desglucolanatigonin II and gitonin, with certain amounts of the corresponding C-25*S* isomers. Inhibitory activity of the isolated saponins and their derivatives on cAMP phosphodiesterase was evaluated to identify new compounds with medicinal potential.

INTRODUCTION

The genera *Triteleia*, *Ipheion* and *Dichelostemma* are taxonomically closely related, and were formerly included in the genus *Brodiaea* [1]. During our search for bioactive compounds of natural origin, we have isolated a number of new steroidal saponins, some of which exhibited potent inhibitory activity on cAMP phosphodiesterase, from the bulbs of *Ipheion uniflorum* [2], *Triteleia lactea* [3] and from the tubers of *Brodiaea californica* [4]. In continuation of this work, phytochemical screening has been made of the tubers of *Dichelostemma multiflorum*, a Liliaceaeous plant native to the Pacific coastline of the USA; no chemical work appears to have been done on the plant until now.

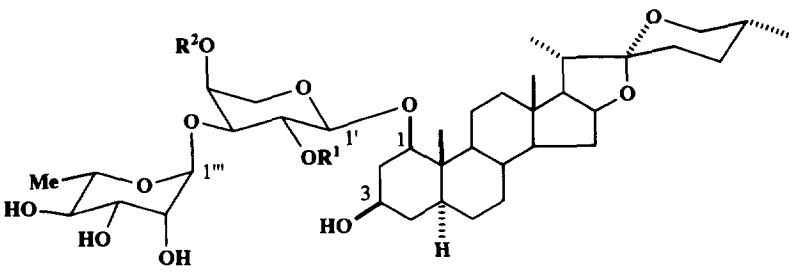
This paper reports the identification and structural determination of the five steroidal saponins, including three new compounds, isolated from the methanolic extract of the tubers of *D. multiflorum*. Furthermore, inhibitory activity of the isolated saponins and their derivatives on cAMP phosphodiesterase was evaluated. This test provides a useful tool for screening of biologically active compounds present in a natural source [5–8].

RESULTS AND DISCUSSION

The concentrated 1-butanol-soluble fraction of the methanolic extract of *D. multiflorum* tubers was repeatedly subjected to column chromatography on silica gel and octadecylsilanized (ODS) silica gel, and to reversed-phase HPLC to yield compounds **1** (114 mg), **2** (50 mg), **3** (140 mg), **4** (44 mg) and **5** (32 mg).

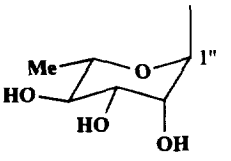
Compound **1** was obtained as a white amorphous powder, $[\alpha]_D$ −36.7° in a mixed solvent of chloroform and methanol (1:1). The molecular formula ($C_{40}H_{64}O_{13}$) was determined by the negative-ion FAB mass spectrum (*m/z* 752 $[M]^-$), ^{13}C NMR data (40 carbons) and elemental analysis. The presence of an acetyl group in the molecule was shown by IR (1740 cm^{-1}), ^1H NMR [δ1.99 (3H, *s*)] and ^{13}C NMR (δ170.6 and 20.9). The fragment ion peak at *m/z* 709 was assignable to an $[M - acetyl]^-$ ion. The ^1H NMR spectrum of **1** showed signals for four typical steroid methyls; two appeared as singlets at δ0.97 and δ0.87 and the other two as doublets at δ1.11 (6.9 Hz) and δ0.69 (J = 4.9 Hz). Two anomeric protons were also noted at δ5.96 (*br s*) and 4.78 (*d*, J = 7.4 Hz). The fundamental structure of **1**, based upon a steroid of a (25*R*)-spirostanol, was suggested by the ^{13}C NMR [δ109.2 (a quaternary carbon signal assignable to C-22 of spirostanols)] [9] and ^1H NMR spectra [δ3.58 (*dd*, J = 10.3, 2.6 Hz, 26eq-H) and δ3.50 (*dd*, J = 10.3, 10.3 Hz, 26ax-H)]. Acid hydrolysis of **1** with 1 M hydrochloric acid in dioxane-H_2O (1:1) gave an aglycone, identified as (25*R*)-5α-spirostane-1β,3β-diol (brisbagenin) (**1a**) [10] by the negative-ion FAB mass spectrum, ^1H and ^{13}C NMR spectral data, and L-arabinose and L-rhamnose in a ratio of 1:1 as the carbohydrate compounds. Alkaline treatment of **1** with 4% potassium hydroxide in ethanol,

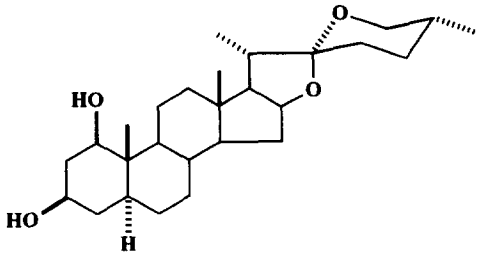
*Authors to whom correspondence should be addressed.



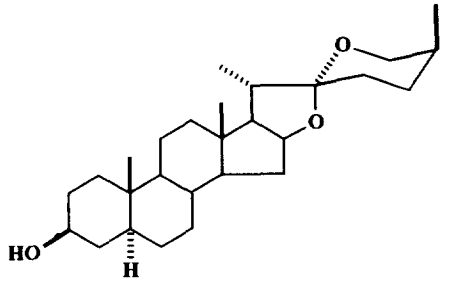
	R^1	R^2
1	H	Ac
1b	H	H
2	α-L-Rha*p*	Ac
2a	α-L-Rha*p*	H

α-L-Rha*p* :





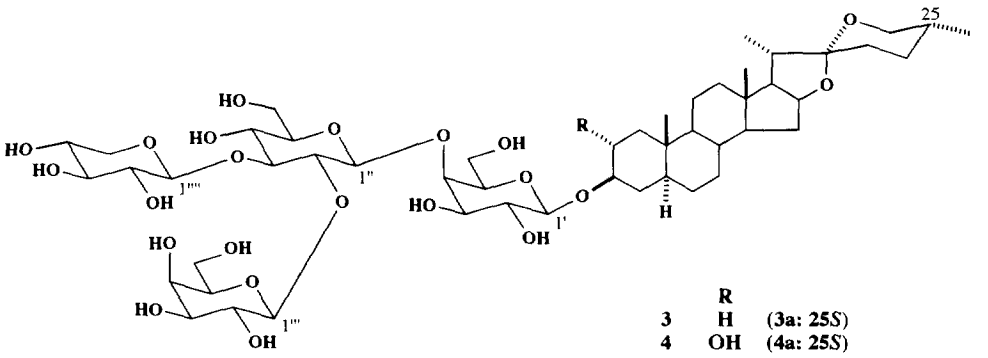
1a



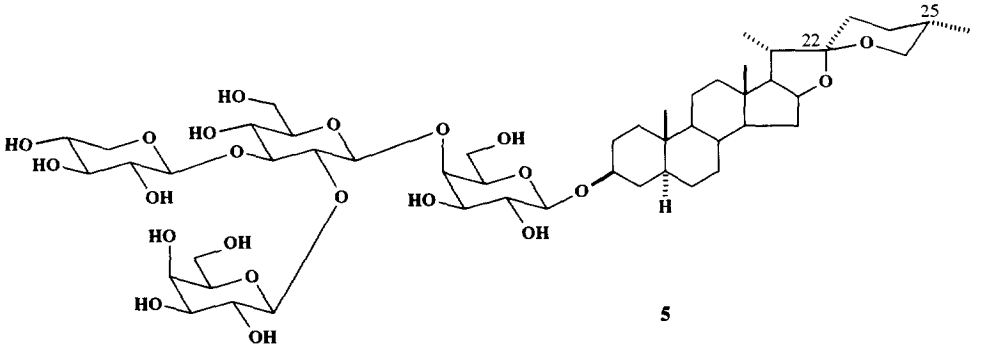
5a

cleaved the acetyl moiety **1** to yield a deaceryl derivative (**1b**). Acetylation of **1** with acetic anhydride in pyridine introduced an additional five acetyl groups (**1c**). Spectral analysis of **1** and its derivatives allowed the establishment of its structure.

The ^{13}C NMR signals of the saccharide moiety of **1b** were assigned by comparing them with those of reference methyl glycosides [9, 11], taking account of the downfield shift caused by *O*-glycosylation and the result of the acid hydrolysis, indicating the presence of a terminal α-L-rhamnopyranosyl unit (δ103.9, 72.4, 72.7, 74.2, 70.1 and 18.7) and a C-3 glycosylated α-L-arabinopyranosyl unit (δ101.8, 71.9, 80.7, 69.5 and 67.8). These data indicated the saccharide sequence to be *O*-α-L-rhamnopyranosyl-(1 → 3)-α-L-arabinopyranose. There are two possible linkage positions of the disaccharide to the aglycone: C-1 or C-3 hydroxyl group. By comparing the ^{13}C assignments of **1b** with that of **1a**, *O*-glycosylation shift could be detected at C-1 (+ 4.6 ppm). Furthermore, in the ^{1}H NMR spectrum of **1c** the signals due to H-3 of the aglycone as well as H-2 and H-4 of the arabinose and H-2 to H-4 of the rhamnose were shifted downfield by more than 1 ppm compared with those of **1b**, while the signals due to H-1 of the aglycone and H-3 of the arabinose remained almost unaffected (± 0.2 ppm). The structure of **1b** was formulated as brisbagenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 3)-α-L-arabinopyranoside}. The acetyl group was found to be located at the C-4



	R	
3	H	(3a: 25*S*)
4	OH	(4a: 25*S*)



5

hydroxy position of the arabinose by the following data. In the ^{13}C NMR spectrum of **1**, the signal due to the arabinose C-4 was shifted to a lower field by 2.7 ppm, and upfield the signals due to C-2 and C-4 shifted by 2.2 and 3.2 ppm, respectively, compared with those of **1b**. In addition, the signal at δ5.63 (*br s*) was assigned to H-4 of the arabinose in the ^1H NMR spectrum of **1**, which was shifted downfield by 1.23 ppm compared with that of **1b**. Thus, the full structure of **1** was determined to be brisbagenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 3)-4-*O*-acetyl-α-L-arabinopyranoside}.

Spectral features of **2** were similar to those of **1**, suggesting a steroidal glycoside structure of the same type, with an acetyl group. On comparison of the whole ^{13}C NMR spectrum of **2** with that of **1**, a set of additional signals, corresponding to a terminal α-L-rhamnopyranosyl unit, appeared (δ102.1, 72.5, 72.5, 73.6, 69.8 and 19.2). The signal due to C-2 of the arabinose was displaced downfield by 2.5 ppm and that due to C-1 moved to the upper field by 1.9 ppm, indicating that the C-2 position of the arabinose was the glycosylated position to which additional α-L-rhamnose was linked. Alkaline treatment of **2** gave a deacetyl derivative (**2a**). The position esterified with acetic acid in **2** was confirmed to be the C-4 hydroxyl group of the arabinose by comparing the ^1H NMR of **2** with that of **2a**. The H-4 proton of the arabinose of **2** resonated at δ5.53 (*br s*), which was shifted downfield by 1.07 ppm from that of **2a**. The structure of **2** was assigned as brisbagenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-[α-L-rhamnopyranosyl-(1 → 3)]-4-*O*-acetyl-α-L-arabinopyranoside}.

It has been reported that the C-2 glycosylated arabinopyranose attached to the aglycone is predominantly present as the $^1C_4$ conformation to reduce the steric hindrance between the C-2 glycosylated position and the aglycone in several saponins [12–14]. The arabinoses of the isolated saponins are considered to be present as the $^4C_1$ conformation because of the large $^3J_{H\text{-}1,H\text{-}2}$ values (> 7 Hz).

The steroidal aglycone (**1a**), brisbagenin, has been isolated from the saponified extract of *Cordyline cannifolia* leaves [10]. However, compounds **1** and **2** are the first steroidal saponins with brisbagenin as the aglycone; no other glycoside of brisbagenin has been reported.

Compounds **3** and **4** were identified as desglucolanatigonin II [15] and gitonin [15] by various spectral data. The ^1H and ^{13}C NMR spectra showed that **3** and **4** contained certain amounts of the corresponding C-25*s* isomers (**3a** and **4a**).

Compound **5** was shown to have same molecular formula as **3**, $C_{50}H_{82}O_{22}$, deduced from the negative-ion FAB mass spectrum (*m/z* 1033 $[M\text{-}H]^-$, ^{13}C NMR (50 carbons) and elemental analysis. It gave neotigogenin (**5a**), and D-galactose, D-glucose and D-xylose in a ratio of 2:1:1 on acid hydrolysis. The ^{13}C NMR assignments exhibited a close similarity to those of **3** and **3a**, except for the signals due to the E- and F-ring carbons, suggesting that the aglycone of **5** might be different in stereostructure from those of **3** and **3a** with respect to the E- and/or F-parts. The phase-sensitive NOESY spectrum provided certain information for the stereostructure assignments. All ^1H NMR signals were assigned by the ^1H–^1H COSY

Table 1. ^{13}C NMR spectral data for compounds **1**, **1a**, **1b**, **2**, **2a** and **3–5**

C	1	1a	1b	2	2a	3	4	5
1	81.6	77.1	81.7	82.0	82.4	37.1	45.6	37.2
2	37.7	44.1	37.8	37.2	37.4	30.0	70.4	30.0
3	67.6	67.9	67.7	67.8	67.8	77.6	84.4	77.6
4	39.6	39.7	39.7	39.6	39.7	34.9	34.2	34.9
5	43.0	43.0	43.1	43.1	43.2	44.7	44.7	44.7
6	28.8	29.1	28.9	28.8	28.9	28.9	28.1	28.9
7	32.4	32.6	32.5	32.4	32.5	32.4	32.2	32.5
8	36.5	36.2	36.4	36.7	36.5	35.2	34.6	35.0
9	54.9	55.7	55.1	54.8	55.0	54.4	54.4	54.5
10	41.4	42.1	41.5	41.3	41.5	35.8	36.9	35.8
11	23.8	24.8	23.7	24.0	23.7	21.3	21.4	21.1
12	40.9	41.0	40.9	40.9	40.8	40.2	40.0	40.5
13	40.4	40.5	40.3	40.5	40.3	40.8	40.8	41.3
14	56.8	56.9	56.8	56.9	56.9	56.4	56.3	55.6
15	32.4	32.5	32.5	32.4	32.5	32.1	32.1	33.1
16	81.1	81.1	81.1	81.1	81.1	81.1	81.1	80.8
17	63.3	63.3	63.3	63.3	63.2	62.9	63.0	62.7
18	16.9	16.8	16.8	17.0	16.8	16.6	16.6	16.7
19	8.2	7.6	8.2	8.7	8.8	12.3	13.4	12.7
20	42.0	42.1	42.0	42.0	42.0	42.0 (42.5)	42.0 (42.5)	42.1
21	15.0	15.0	14.9	14.9	15.0	15.0 (14.9)	15.0 (14.8)	17.0
22	109.2	109.3	109.2	109.2	109.3	109.2 (109.7)	109.2 (109.7)	110.5
23	31.8	31.9	31.8	31.8	31.8	31.8 (26.2)[c]	31.8 (26.2)[c]	28.2
24	29.3	29.3	29.3	29.3	29.3	29.3 (26.4)[c]	29.3 (26.4)[c]	28.1
25	30.6	30.6	30.6	30.6	30.6	30.6 (27.5)	30.6 (27.5)	30.7
26	66.8	66.9	66.8	66.8	66.8	66.9 (65.1)	66.9 (65.1)	69.6
27	17.3	17.3	17.3	17.3	17.3	17.3 (16.3)	17.3 (16.3)	17.3
1′	101.3		101.8	99.4	100.1	102.3	103.1	102.3
2′	71.9		71.9	74.4	74.2	73.1	72.4	73.1
3′	78.5		80.7	81.1	84.0	75.8	75.7	75.8
4′	72.2[c]		69.5	72.2	69.4	79.7	79.4	79.6
5′	64.5		67.8	63.7	67.5	75.4	75.6	75.4
6′						60.5	60.5	60.5
1″				102.1	101.9	105.6	105.3	105.6
2″				72.5	72.6	81.1	81.0	81.1
3″				72.5	72.6	85.7	86.0	85.6
4″				73.6	73.8	70.6	70.4	70.6
5″				69.8	69.7	77.6	77.6	77.6
6″				19.2	19.1	63.1	63.0	63.1
1‴	104.2		103.9	104.7	104.4	105.3	105.2	105.3
2‴	72.3[c]		72.4	72.4	72.4	73.8	73.7	73.8
3‴	72.7		72.7	72.6	72.6	74.0	74.4	74.0
4‴	73.9		74.2	74.0	74.1	70.4	70.4	70.4
5‴	70.5		70.1	70.8	70.3	77.3	77.2	77.3
6‴	18.6		18.7	18.5	18.5	62.5	62.6	62.6
1⁗						104.8	104.8	104.8
2⁗						75.0	75.0	75.0
3⁗						78.4	78.5	78.4
4⁗						70.7	70.7	70.7
5⁗						67.3	67.3	67.3
6⁗								
Ac	170.6			170.7				
	20.9			20.8				

[a]Spectra were measured in pyridine-d_5.
[b]Shifts for the C-25 isomers (**3a** and **4a**) of **3** and **4** were shown in the parentheses.
[c]Signals may be interchanged.

Table 2. ^{1}H and ^{13}C NMR chemical shift assignments of the aglycone moiety of **5** in pyridine-d_5–methanol-d_4 (10:1)

Position	^{1}H	*J* (Hz)	^{13}C
1ax	0.82 *ddd*	13.1, 13.1, 4.3	37.5
1eq	1.52		
2ax	1.62		30.2
2eq	2.02		
3	3.89		78.0
4ax	1.35 *br dd*	12.5, 12.5	35.1
4eq	1.79 *br d*	12.5	
5	0.91		35.0
6	1.13 (2H)		29.2
7ax	0.80		32.8
7eq	1.55		
8	1.40		35.3
9	0.54 *ddd*	11.7, 11.7, 3.8	55.9
10			36.1
11ax	1.99 *ddd*	13.1, 13.1, 2.8	21.6
11eq	1.38		
12ax	1.05 *ddd*	12.7, 12.7, 3.8	40.8
12eq	1.64		
13			41.6
14	0.94		56.5
15α	2.01		33.4
15β	1.43		
16	4.31 *q*-like	8.6	81.1
17	1.59 *dd*	8.6, 6.2	62.9
18	0.92 *s*		17.2
19	0.64 *s*		12.5
20	2.29 *dq*	6.2, 7.2	42.4
21	1.01 *d*	7.2	16.8
22			110.8
23ax	1.46 *ddd*	12.1, 12.1, 4.1	28.4
23eq	1.68 *br dd*	12.1, 3.0	
24a	1.52		28.3
24b	1.58		
25	1.63		31.0
26	3.67 (2H) *d*	8.3	69.9
27	0.70 *d*	6.4	17.5

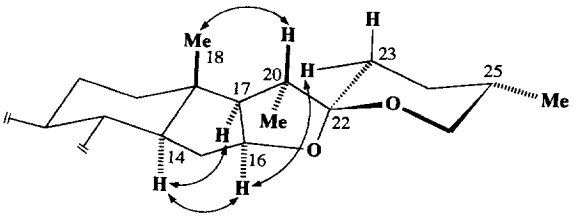


Fig. 1. NOE correlations of **5** in pyridine-d_5–methanol-d_4 (10:1).

Table 3. ^{1}H and ^{13}C NMR chemical shift assignments of the saccharide moieties of **5** in pyridine-d_5–methanol-d_4 (10:1)

Position	^{1}H	*J* (Hz)	^{13}C
1′	4.86 *d*	7.7	102.5
2′	4.27 *dd*	9.9, 7.7	73.1
3′	4.04 *dd*	9.9, 2.9	75.7
4′	4.51 *br d*	2.9	79.9
5′	4.00		75.5
6′	4.58 *dd*	11.1, 9.1	60.7
	4.13 *dd*	11.1, 5.1	
1″	5.10 *d*	8.0	105.6
2″	4.43 *dd*	9.2, 8.0	81.3
3″	4.09 *dd*	9.2, 9.2	86.0
4″	3.71 *dd*	9.2, 9.2	70.6
5″	3.82		78.0
6″	4.44		62.9
	3.96		
1‴	5.42 *d*	7.8	105.4
2‴	4.48 *dd*	9.8, 7.8	73.8
3‴	3.89 *dd*	9.8, 3.2	74.2
4‴	4.26		70.4
5‴	3.98		77.4
6‴	4.55 *dd*	11.5, 5.9	62.4
	4.35 *dd*	11.5, 5.2	
1⁗	5.04 *d*	7.4	105.0
2⁗	3.85 *dd*	8.9, 7.4	75.0
3⁗	3.90 *dd*	8.9, 8.9	78.4
4⁗	4.02		70.8
5⁗	4.16 *dd*	10.9, 5.4	67.4
	3.55 *dd*	10.9, 10.9	

spectrum before inspection of the NOESY spectrum (Table 2). The clear NOE correlations, H-14/H-17 and H-16, and H-20/Me-18 indicated the usual D/E *cis*-ring junction and C-20*s* configuration. The H-16 proton showed an intense NOE with 23_{eq}-H, giving evidence for C-22*s*. The C-25*s* configuration was corroborated by the fact that **5** was transformed into neotigogenin on acid treatment. The ^{1}H and ^{13}C NMR assignments of the tetrasaccharide moiety of **5** were established by interpretation on the ^{1}H–^{1}H COSY and HMQC spectra, as shown in Table 3. The two-dimensional NMR spectra were measured in a mixed solvent of pyridine-d_5 and methanol-d_4 (10:1) to minimize signal overlap and remove exchangeable protons. Thus the structure of **5** was formulated as (22*s*,25*s*)-5α-spirostan-3β-ol 3-*O*-{*O*-β-D-galactopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside}.

Opinions are divided on the classification of the genus *Dichelostemma* to the family Liliaceae or Amaryllidaceae because characteristics of both families, (flowers having superior ovaries and the umbellate inflorescence) are combined in the plant. In this study, we isolated five steroidal saponins, including three new compounds (**1**, **2** and **5**), and detected no alkaloid. From the viewpoint of secondary metabolite production, it seems proper that the genus *Dichelostemma* is classified to Liliaceae.

cAMP plays an important role as a 'second messenger' in various biological process, and the cAMP phosphodiesterase inhibition test provides a useful tool for the screening of biologically active compounds present in natural sources [5–8]. The isolated saponins and their derivatives were evaluated for inhibitory activity on

Table 4. Inhibitory activity on cAMP phosphodiesterase of the isolated saponins and their derivatives

Compound	IC_{50} ($\times 10^{-5}$ M)
1	20.6
1b	76.2
2	11.8
2a	10.0
3	12.3
4	11.4
5	15.4
Papaverine (positive control)	3.0

cAMP phosphodiesterase. The IC_{50} values are listed in Table 4. The brisbagenin disaccharide with an acetyl group at the C-4 hydroxyl group of the arabinose (**1**) showed an IC_{50} value of 20.6 ($\times 10^{-5}$ M) and the deacetyl derivative (**1a**) was less potent (76.2) than **1**, accounting for the contribution of the acetyl group to the activity, while the brisbagenin 2,3-branched trisaccharide with an acetyl group (**2**) was as potent as the deacetyl derivative (**2a**), both showing potent activity (**2**: 11.8; **2a**: 10.0). The saponins embracing a tetrasaccharide also exhibited considerable activity (**3**: 12.3; **4**: 11.4; **5**: 15.4).

EXPERIMENTAL

General. NMR (ppm, *J* Hz): 1D (Bruker AM-400) and 2D (Bruker AM-500). CC: silica gel (Fuji-Silysia Chemical) and ODS silica gel (Nacalai Tesque). TLC: precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck) and RP-18 $F_{254}S$ (0.25 mm thick, Merck). HPLC: a Tosoh HPLC system (pump, CCPM; controller, CCP controller PX-8010; detector, RI-8010 or UV-8000) equipped with a Kaseisorb LC ODS-120-5 column (Tokyo-Kasei-Kogyo, 10 mm i.d. × 250 mm, ODS, 5 μm) for prep. HPLC or with a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d. × 250 mm, ODS, 5 μm) for the analyt. HPLC.

Chemicals. Beef heart phosphodiesterase: Boehringer. Snake venom nucleotidase and cAMP: Sigma. [^{3}H]-cAMP: Radiochemical Center.

Plant material. D. multiflorum bulbs purchased from Heiwaen, Japan, were cultivated and the plant specimen is on file in our laboratory.

Extraction and isolation. Fresh bulbs of *D. multiflorum* (2.1 kg) were cut into pieces and exhaustively extracted with hot MeOH. The MeOH extract was concd under red. press. and the viscous concentrate partitioned between H_2O and *n*-BuOH. CC of the *n*-BuOH extract on silica gel and elution with a gradient mixt. of CH_2Cl_2–MeOH–H_2O (6:1:0; 4:1:0; 20:10:1; 7:4:1), and finally with MeOH, gave five frs (I–V).

Fr. II was purified by ODS silica gel CC with MeOH–H_2O (4:1) to give **1** (114 mg). Fr. IV was chromatographed on ODS silica gel with MeOH–H_2O (4:1) and silica gel with $CHCl_3$–MeOH–H_2O (35:10:1) to give **2** (50 mg). Chromatography of fr. V on ODS silica gel with MeOH–H_2O (4:1; 3:2) and silica gel with $CHCl_3$–MeOH–H_2O (40:10:1; 30:10:1; 20:10:1) to yield **3** (140 mg) and **4** (44 mg) as pure compounds, and **5** with a few impurities. Final purification of **5** was carried out by prep. HPLC with MeOH–H_2O (4:1; 7:3); **5** (32 mg).

Compound **1**. Amorphous powder, $[\alpha]_D^{28}$ −36.7° ($CHCl_3$–MeOH, 1:1; *c* 0.10). (Found: C, 58.88; H, 8.00. Calc. for $C_{40}H_{64}O_{13}\cdot 7/2H_2O$: C, 58.88; H, 8.77%. Negative-ion FAB-MS *m/z*: 752 $[M]^-$, 709 $[M - acetyl]^-$, 563 $[M - rhamnosyl - acetyl]^-$: IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 2930 (CH), 1740 (C=O), 1455, 1380, 1245, 1045, 1020, 980, 920, 900, 865, 835, 810, 755, 700. ^{1}H NMR (pyridine-d_5): δ 5.96 (1H, *br s*, H-1‴), 5.63 (1H, *br s*, H-4′), 4.78 (1H, *d*, *J* = 7.4 Hz, H-1′), 4.76 (1H, *br d*, *J* = 3.0 Hz, H-2‴), 4.54 (1H, *q*-like, *J* = 7.5 Hz, H-16), 4.52 (1H, *dd*, *J* = 9.2, 3.0 Hz, H-3‴), 4.38 (1H, *dd*, *J* = 9.2, 6.1 Hz, H-5‴), 4.35–4.23 (4H, overlapping, 2′-, 3′-, 5′a-, H-4‴), 3.92 (1H, *m*, H-3), 3.87 (1H, *dd*, *J* = 11.8, 4.2 Hz, H-1), 3.73 (1H, *br d*, *J* = 12.8 Hz, H-5′b), 3.58 (1H, *dd*, *J* = 10.3, 2.6 Hz, H-26eq), 3.50 (1H, *dd*, *J* = 10.3, 10.3 Hz, H-26ax), 1.99 (3H, *s*, Ac), 1.69 (3H, *d*, *J* = 6.1 Hz, 6‴-Me), 1.11 (3H, *d*, *J* = 6.9 Hz, 21-Me), 0.97 (3H, *s*, 19-Me), 0.87 (3H, *s*, 18-Me), 0.69 (3H, *d*, *J* = 4.9 Hz, 27-Me).

Acid hydrolysis of **1**. A soln of **1** (30 mg) in 1M HCl (dioxane–H_2O, 1:1) was heated at 100° for 3 hr under an Ar atmosphere. After cooling, the reaction mixt. 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; 1:1) to give an aglycone, identified as brisbagenin (**1a**) (15.3 mg), and a mixt. of monosaccharides (11 mg).

Compound **1a**: *amorphous powder.* Positive-ion FAB-MS *m/z*: 433 $[M + H]^+$. ^{1}H NMR (pyridine-d_5): δ 4.57 (1H, *q*-like, *J* = 6.9 Hz, H-16), 3.99 (1H, *m*, H-3), 3.73 (1H, *dd*, *J* = 11.3, 4.4 Hz, H-1), 3.59 (1H, *dd*, *J* = 10.5, 3.5 Hz, H-26eq), 3.52 (1H, *dd*, *J* = 10.5, 10.5 Hz, H-26ax), 1.14 (3H, *s*, 19-Me), 1.12 (3H, *d*, *J* = 6.9 Hz, 21-Me), 0.92 (3H, *s*, 18-Me), 0.70 (3H, *d*, *J* = 5.6 Hz, 27-Me). Arabinose and rhamnose were identified as being present in the mixt. by direct TLC comparison with authentic samples: arabinose, R_f 0.48; rhamnose, R_f 0.66 (*n*-BuOH–Me_2CO–H_2O, 4:5:1). The sugar fr. (2 mg) was diluted with H_2O (1 ml) and treated with (−)-α-methylbenzylamine (5 mg) and Na[BH_3CN] (8 mg) in EtOH (1 ml) at 40° for 4 hr, followed by acetylation with Ac_2O (0.3 ml) in pyridine (0.3 ml). The reaction mixt. was passed through a Sep-Pak C_{18} cartridge (Waters), eluting with H_2O–MeCN (4:1, 10 ml) and then with MeCN (10 ml). The MeCN eluate fr. was further passed through a Toyopak IC-SP M cartridge (Tosoh) with EtOH (10 ml) to give a mixt. of 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides, which was then analysed by HPLC [16, 17]. Derivatives of L-arabinose, and L-rhamnose were detected in a ratio of 1:1.

Alkaline hydrolysis of **1**. Compound **1** (10 mg) was treated with 4% KOH in EtOH (2 ml) and the reaction mixt. neutralized by passing it through an Amberlite

IR-120B (Organo) column. The eluate was subjected to Sep-Pak C_{18} cartridge, eluting with initially with H_2O–MeOH (4:1, 10 ml) and then with H_2O–MeOH (1:4, 10 ml) to yield the deacetyl derivative (**1b**) (8 mg).

Compound **1b**. Amorphous powder, $[\alpha]_D^{28}$ −64.7° ($CHCl_3$–MeOH, 1:1; *c* 0.10). Negative-ion FAB-MS *m/z*: 709 $[M-H]^-$, 563 $[M-$ rhamnosyl$]^-$. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2920 (CH), 1450, 1375, 1240, 1170, 1040, 975, 915, 895, 860, 830, 805, 780, 695. ^1H NMR (pyridine-d_5): δ6.09 (1H, *br s*, H-1‴), 4.78 (1H, *br d*, *J* = 3.0 Hz, H-2‴), 4.76 (1H, *d*, *J* = 7.6 Hz, H-1′), 4.61 (1H, *dd*, *J* = 9.5, 7.6 Hz, H-2′), 4.60 (1H, *dd*, *J* = 9.4, 3.0 Hz, H-3‴), 4.53 (1H, *q*-like, *J* = 7.6 Hz, H-16), 4.48 (1H, *dq*, *J* = 9.4, 6.2 Hz, H-5‴), 4.40 (1H, *br s*, H-4′), 4.31 (1H, *dd*, *J* = 9.4, 9.4 Hz, H-4‴), 4.28 (1H, *dd*, *J* = 12.5, 2.2 Hz, H-5′a), 4.19 (1H, *dd*, *J* = 9.5, 3.3 Hz, H-3′), 3.90 (1H, *m*, H-3), 3.89 (1H, *dd*, *J* = 11.0, 3.8 Hz, H-1), 3.70 (1H, *br d*, *J* = 12.5 Hz, H-5′b), 3.58 (1H, *dd*, *J* = 10.5, 3.0 Hz, H-26eq), 3.40 (1H, *dd*, *J* = 10.5, 10.5 Hz, H-26ax), 1.68 (3H, *d*, *J* = 6.2 Hz, 6‴-Me), 1.10 (3H, *d*, *J* = 6.9 Hz, 21-Me), 0.97 (3H, *s*, 19-Me), 0.83 (3H, *s*, 18-Me), 0.69 (3H, *d*, *J* = 5.2 Hz, 27-Me).

Acetylation of **1**. Compound **1** (25 mg) was acetylated with Ac_2O in pyridine and the crude acetate chromatographed on silica gel with hexane–Me_2CO (7:2) to yield the corresponding pentaacetate (**1c**) (23.7 mg).

Compound **1c**: *amorphous powder*. IR ν_{max}^{KBr} cm^{-1}: 2945 (CH), 1735 (C=O), 1445, 1365, 1235, 1220, 1175, 1135, 1115, 1080, 1045, 1020, 975, 955, 915, 895, 860, 800, 740, 695. ^1H NMR (pyridine-d_5): δ5.72 (1H, *dd*, *J* = 10.0, 7.8 Hz, H-2′), 5.71 (1H, *dd*, *J* = 10.0, 3.3 Hz, H-3‴), 5.67 (1H, *br d*, *J* = 3.3 Hz, H-2‴), 5.60 (1H, *dd*, *J* = 10.0, 10.0 Hz, H-4‴), 5.59 (1H, *br s*, H-4′), 5.44 (1H, *br s*, H-1‴), 4.89 (1H, *m*, H-3), 4.79 (1H, *d*, *J* = 7.8 Hz, H-1′), 4.53 (1H, *q*-like, *J* = 7.6 Hz, H-16), 4.47 (1H, *dq*, *J* = 10.0, 6.2 Hz, H-5‴), 4.39 (1H, *dd*, *J* = 10.0, 3.6 Hz, H-3′), 4.29 (1H, *dd*, *J* = 13.0, 1.0 Hz, H-5′a), 3.80 (1H, *br d*, *J* = 13.0 Hz, H-5′b), 3.76 (1H, *dd*, *J* = 10.7, 4.4 Hz, H-1), 3.58 (1H, *dd*, *J* = 10.4, 3.0 Hz, H-26eq), 3.50 (1H, *dd*, *J* = 10.4, 10.4 Hz, H-26ax), 2.27, 2.12, 2.04 × 2, 2.02, 1.97 (each 3H, *s*, Ac), 1.40 (3H, *d*, *J* = 6.2 Hz, 6‴-Me), 1.15 (3H, *d*, *J* = 6.8 Hz, 21-Me), 0.98 (3H, *s*, 19-Me), 0.88 (3H, *s*, 18-Me), 0.69 (3H, *d*, *J* = 5.0 Hz, 27-Me).

Compound **2**. Amorphous powder, $[\alpha]_D^{28}$ −51.3° ($CHCl_3$–MeOH, 1:1; *c* 0.10). (Found: C, 58.57; H, 7.95. Calc. for $C_{46}H_{74}O_{17}\cdot 5/2\,H_2O$: C, 58.52; H, 8.43%). Negative-ion FAB-MS *m/z*: 897 $[M-H]^-$, 855 $[M-$ acetyl$]^-$, 709 $[M-$ rhamnosyl × 2 − acetyl$]^-$, 563 $[M-$ rhamnosyl × 2 − acetyl$]^-$. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2935 (CH), 1745 (C=O), 1455, 1380, 1245, 1135, 1100, 1045, 1020, 980, 920, 900, 865, 840, 815, 760, 700. ^1H NMR (pyridine-d_5): δ5.95 (1H, *br s*, H-1‴), 5.55 (1H, *br s*, H-1″), 5.53 (1H, *br s*, H-4′), 4.80 (1H, *br d*, *J* = 3.0 Hz, H-2‴), 4.73 (1H, *m*, H-5″), 4.72 (1H, *d*, *J* = 7.6 Hz, H-1′), 4.59 (1H, *br d*, *J* = 3.0 Hz, H-2″), 4.54 (1H, *q*-like, *J*-7.6 Hz, H-16), 4.51 (2H, overlapping, H-3″, H-3‴), 4.40 (1H, *dd*, *J* = 9.1, 7.6 Hz, H-2′), 4.31–4.22 (3H, overlapping, 4″, 4‴, H-5‴), 4.30 (1H, *dd*, *J* = 12.7, 2.0 Hz, H-5′a), 4.13 (1H, *dd*, *J* = 9.1, 3.3 Hz, H-3′), 3.89 (1H, *m*, H-3), 3.84 (1H, *dd*, *J* = 10.9, 3.9 Hz, H-1), 3.65 (1H, *br d*, *J* = 12.7 Hz, H-5′b), 3.58 (1H, *dd*, *J* = 10.4, 2.7 Hz, H-26eq), 3.50 (1H, *dd*, *J* = 10.4, 10.4 Hz, H-26ax), 2.00 (3H, *s*, Ac), 1.75 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.65 (3H, *d*, *J* = 5.4 Hz, 6‴-Me), 1.21 (3H, *s*, 19-Me), 1.12 (3H, *d*, *J* = 7.0 Hz, 21-Me), 0.94 (3H, *s*, 18-Me), 0.69 (3H, *d*, *J* = 5.2 Hz, 27-Me).

Alkaline hydrolysis of **2**. Compound **2** (25 mg) was treated with 4% KOH in EtOH (5 ml) and the reaction mixt. neutralized by passing it through an Amberlite IR-120B column. The eluate was subjected to silica gel CC, eluting with $CHCl_3$–MeOH–H_2O (40:10:1) to yield the deacetyl derivative (**2a**) (20 mg).

Compound **2a**: Amorphous powder, $[\alpha]_D^{28}$ −70.0° ($CHCl_3$–MeOH, 1:1; *c* 0.10). Negative-ion FAB-MS *m/z*: 855 $[M-H]^-$, 709 $[M-$ rhamnosyl$]^-$, 563 $[M-$ rhamnosyl × 2$]^-$. IR ν_{max}^{KBr} cm^{-1}: 3395 (OH), 2915 (CH), 1445, 1375, 1255, 1235, 1135, 1040, 975, 915, 985, 855, 830, 805, 775, 690. ^1H NMR (pyridine-d_5): δ5.96 (1H, *br s*, H-1‴), 5.65 (1H, *br s*, H-1″), 4.84 (1H, *br d*, *J* = 2.0 Hz, H-2‴), 4.76 (1H, *d*, *J* = 7.3 Hz, H-1′), 4.71 (1H, *m*, H-5″), 4.70 (1H, *dd*, *J* = 9.4, 7.3 Hz, H-2′), 4.61 (2H, overlapping, 2″-, H-3″), 4.56–4.47 (3H, overlapping, 16-, 3‴-, H-5‴), 4.46 (1H, *br s*, H-4′), 4.31–4.20 (3H, overlapping, 5′a-, 4″-, H-4‴), 4.08 (1H, *dd*, *J* = 9.4, 3.2 Hz, H-3′), 3.90 (1H, *m*, H-3), 3.87 (1H, *dd*, *J* = 11.1, 3.7 Hz, H-1), 3.67 (1H, *br d*, *J* = 13.4 Hz, H-5′b), 3.58 (1H, *dd*, *J* = 10.5, 2.7 Hz, H-26eq), 3.49 (1H, *dd*, *J* = 10.5, 10.5 Hz, H-26ax), 1.72 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.63 (3H, *d*, *J* = 6.2 Hz, 6‴-Me), 1.22 (3H, *s*, 19-Me), 1.09 (3H, *d*, *J* = 7.0 Hz, 21-Me), 0.85 (3H, *s*, 18-Me), 0.68 (3H, *d*, *J* = 5.2 Hz, 27-Me).

Compound **3**. Amorphous powder, $[\alpha]_D^{28}$ −38.7° ($CHCl_3$–MeOH, 1:1; *c* 0.10). Negative-ion FAB-MS *m/z*: 1033 $[M-H]^-$. ^1H NMR (pyridine-d_5): δ5.47 (1H, *d*, *J* = 7.6 Hz, H-1‴), 5.18 (1H, *d*, *J* = 7.5 Hz, H-1″), 5.08 (1H, *d*, *J* = 6.8 Hz, H-1⁗), 4.91 (1H, *d*, *J* = 7.7 Hz, H-1′), 1.14 (3H, *d*, *J* = 6.6 Hz, 21-Me), 1.08 (*d*, *J* = 7.2 Hz, 27-Me of 25*S*-isomer), 0.82 (3H, *s*, 18-Me), 0.70 (*d*, *J* = 4.3 Hz, 27-Me of 25*R*-isomer), 0.63 (3H, *s*, 19-Me).

Compound **4**. Amorphous powder, $[\alpha]_D^{28}$ +0.7° ($CHCl_3$-MeOH, 1:1; *c* 0.10). Negative-ion FAB-MS *m/z*: 1049 $[M-H]^-$. ^1H NMR (pyridine-d_5): δ5.52 (1H, *d*, *J* = 7.8 Hz, H-1‴), 5.18 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.12 (1H, *d*, *J* = 7.6 Hz, H-1⁗), 4.95 (1H, *d*, *J* = 7.3 Hz, H-1′), 1.14 (*d*, *J* = 6.7 Hz, 21-Me of 25*S*-isomer), 1.13 (*d*, *J* = 6.7 Hz, 21-Me of 25*R*-isomer), 1.08 (*d*, *J* = 7.0 Hz, 27-Me of 25*S*-isomer), 0.80 (3H, *s*, 18-Me of 25*R*-isomer), 0.79 (*s*, 18-Me of 25*R*-isomer), 0.70 (*d*, *J* = 4.9 Hz, 27-Me of 25*R*-isomer), 0.69 (3H, *s*, 19-Me).

Compound **5**. Amorphous powder, $[\alpha]_D^{28}$ −7.3° ($CHCl_3$–MeOH, 1:1; *c* 0.10). (Found: C, 53.54; H, 7.56. Calc. for $C_{50}H_{82}O_{22}\cdot 5H_2O$: C, 53.37; H, 8.24%). Negative-ion FAB-MS *m/z*: 1033 $[M-H]^-$, 901 $[M-$ xylosyl$]^-$, 871 $[M-$ galactosyl$]^-$, 739 $[M-$ xylosyl − galactosyl$]^-$, 577 $[M-$ xylosyl − galactosyl − glucosyl$]^-$. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2925 (CH), 1455, 1360, 1255, 1155, 1060, 990, 915, 885, 870, 695. ^1H NMR (pyridine-d_5): δ5.47 (1H, *d*, *J* = 7.8 Hz, H-1‴), 5.18 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.07 (1H, *d*, *J* = 7.1 Hz, H-1⁗), 4.91 (1H, *d*, *J* = 7.7 Hz, H-1′), 1.02

(3H, *d*, J = 7.3 Hz, 21-Me), 0.94 (3H, *s*, 18-Me), 0.70 (3H, *d*, J = 6.2 Hz, 27-Me), 0.62 (3H, *s*, 19-Me).

Acid hydrolysis of **5**. Compound **5** (8 mg) was subjected to acid hydrolysis as in the case of **1**, which gave neotigogenin (**5a**) (3.2 mg), and D-galactoes, D-glucose and D-xylose (2:1:1).

Assay of cAMP phosphodiesterase activity. The phosphosphodiesterase activity was assayed by a modification of the method of Thompson and Brooker as described previously [6, 7]. The assay consisted of a two-step isotopic procedure. Tritium-labelled cAMP was hydrolysed to 5'-AMP by phosphodiesterase and the 5'-AMP was then further hydrolysed 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 and to leave [^{3}H]-adenosine as the only labelled compound to be counted.

Acknowledgements—We thank Dr Y. Shida, Mrs C. Sakuma, Mrs Y. Katoh and Mr H. Fukaya of the Central Analytical Centre of our College for the measurements of the FAB mass and 2D NMR spectra and elemental analysis.

REFERENCES

1. Bryan, J. E. (1989) in *Bulbs* Vol. 1, p. 102. Timber Press, Portland.
2. Nakamura, O., Mimaki, Y., Sashida, Y., Nikaido, T. and Ohmoto, T. (1994) *Chem. Pharm. Bull.* **42**, 1116.
3. Mimaki, Y., Nakamura, O., Sashida, Y., Nikaido, T. and Ohmoto, T. (1995) *Phytochemistry* **38**, 1279.
4. Nakamura, O., Mimaki, Y., Sashida, Y., Nikaido, T. and Ohmoto, T. (1994) *Chem. Letters* 805.
5. Nikaido, T., Ohmoto, T., Kinoshita, T., Sankawa, U., Nishibe, S. and Hisada, S. (1981) *Chem. Pharm. Bull.* **29**, 2586.
6. Nikaido, T., Ohmoto, T., Noguchi, H., Kinoshita, T., Saitoh, H. and Sankawa, U. (1981) *Planta Medica* **43**, 18.
7. Nikaido, T., Ohmoto, T., Sankawa, U., Tomimori, T., Miyaichi, Y. and Imoto, Y. (1988) *Chem. Pharm. Bull.* **36**, 654.
8. Sakurai, H., Nikaido, T., Ohmoto, T., Ikeya, Y. and Mitsuhashi, H. (1992) *Chem. Pharm. Bull.* **40**, 1191.
9. Agrawal, P. K., Jain, D. C., Gupta, R. K. and Thakur, R. S. (1985) *Phytochemistry* **24**, 2479.
10. Jewers, K., Burbage, M. B., Blunden, G. and Griffin, W. J. (1974) *Steroids* **24**, 203.
11. Agrawal, P. K. (1992) *Phytochemistry* **31**, 3307.
12. Ishii, H., Kitagawa, I., Matsushita, K., Shirakawa, K., Tori, K., Tozyo, T., Yoshikawa, M. and Yoshimura, Y. (1981) *Tetrahedron Letters* **22**, 1529.
13. Mizutani, K., Hayashi, A., Kasai, R., Tanaka, O., Yoshida, N. and Nakajima, T. (1984) *Carbohydr. Res.* **126**, 177.
14. Tanaka, O. (1985) *Yakugaku Zasshi* **105**, 323.
15. Tschesche, R., Seidel, L., Sharma, S. C. and Wulff, G. (1972) *Chem. Ber.* **105**, 3397.
16. Oshima, R. and Kumanotani, J. (1981) *Chem. Letters* 943.
17. Oshima, R., Yamauchi, Y. and Kumanotani, J. (1982) *Carbohydr. Res.* **107**, 169.