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# STEROIDAL GLYCOSIDES FROM *ALLIUM MACLEANII* AND *A. SENESCENS*, AND THEIR INHIBITORY ACTIVITY ON TUMOUR PROMOTER-INDUCED PHOSPHOLIPID METABOLISM OF HELA CELLS

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**Key Words Index**—*Allium macleanii*; *Allium senescens*; Liliaceae; cholestane trisdesmoside; steroidal saponins; HeLa cell; phospholipid metabolism inhibition; antitumour-promoter activity.

**Abstract**—A new polyhydroxylated cholestane trisdesmoside and a new spirostanol pentasaccharide, together with five known spirostanol saponins, were isolated from the bulbs of *Allium macleanii*, and two known spirostanol saponins were isolated from the bulbs of *A. senescens*. The identification and structural assignments of the steroidal glycosides were performed by spectroscopic analysis and hydrolysis. Furthermore, the isolated compounds were evaluated for inhibitory activity on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated $^{32}$P-incorporation into phospholipids of HeLa cells, which is recognized as an excellent primary screening test to identify new antitumour-promoter compounds.

## INTRODUCTION

Plants of the genus *Allium* have long been used both as a food and medicine [1] and are well known for their production of steroidal saponins, as well as sulphide compounds, as the main secondary metabolities. We have previously performed the phytochemical screening of *Allium giganteum* [2–4], *A. aflatunense* [3], *A. schubertii* [5, 6], *A. albopilosum* [7] and *A. ostrowskianum* [7], and isolated a considerable number of new steroidal saponins and cholestane glycosides.

As a continuation of our studies on the steroidal constituents of *Allium* plants with medicinal potential, we have analysed the bulbs of *A. macleanii* Baker and *A. senescens* Linnaeus, resulting in the isolation of a new polyhydroxylated cholestane trisdesmoside and a new spirostanol pentasaccharide, together with five known spirostanol saponins, from *A. macleanii*, and two known spirostanol saponins from *A. senescens*. In this paper, we describe the identification and structural determination of the isolated steroidal glycosides, and their inhibitory activity on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated $^{32}$P-incorporation into phospholipids of HeLa cells, which provides a useful tool for screening of compounds with antitumour-promoter activity [8–10].

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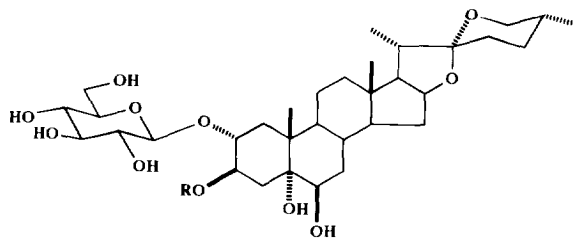
## RESULTS AND DISCUSSION

The 1-butanol-soluble phase of the methanolic bulb extract of *A. macleanii* gave seven steroidal glycosides (**1**–**7**) after a series of chromatographic separations.

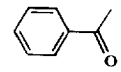
Compounds **1**–**5** are known steroidal saponins and the structures were identified as (25*R*)-5α-spirostane-2α,3β,5α,6β-tetrol 2-*O*-β-D-glucopyranoside [2], 3-*O*-benzoyl ester of **2** [2], (25*R*)-5α-spirostane-2α,3β,6β-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside} [3, 4, 6, 7], 4''''-*O*-benzoyl ester of **3** [6] and methyl ester of (25*R*)-5α-spirostane-2α,3β,6β-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[4-*O*-(*S*)-3-hydroxy-3-methylglutaryl-β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside} [6], respectively. The physical and spectral data of these compounds were consistent with literature values. Copies of the original spectra can be obtained from the author.

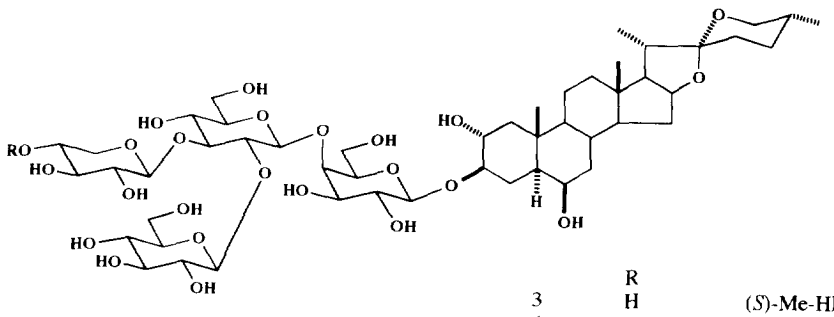
Compound **6** was obtained as a white amorphous powder. The negative-ion FAB-mass spectrum showed an $[M - H]^-$ ion at *m/z* 1149, which was consistent with the molecular formula, $C_{55}H_{90}O_{25}$. The IR, $^1$H NMR



| | R |
|---|---|
| 1 | H |
| 2 | Bz |

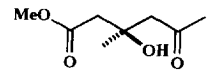
Bz:

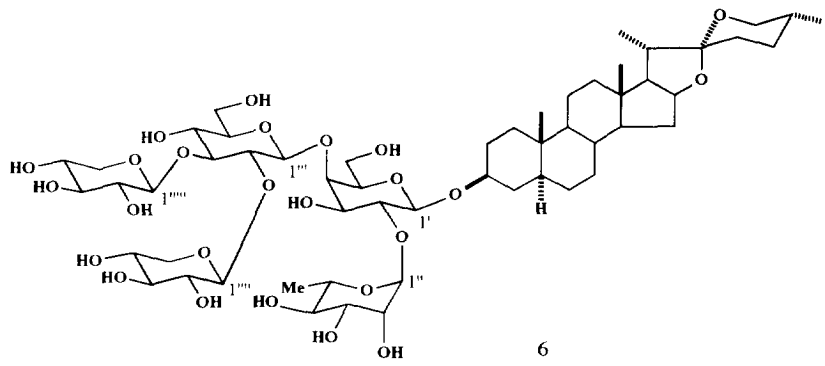




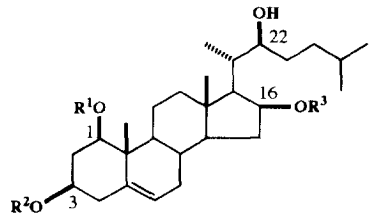
| | R |
|---|---|
| 3 | H |
| 4 | Bz |
| 5 | (*S*)-Me-HMG |

(*S*)-Me-HMG:





6



| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| 7 | α-L-Rha*p* | α-L-Rha*p* | β-D-Glc*p* |
| 7a | H | H | H |
| 7b | H | H | β-D-Glc*p* |

and $^{13}$C NMR spectra allowed the identification of **6** as (25*R*)-5α-spirostan (tigogenin) pentasaccharide. Furthermore, the presence of two terminal β-D-xylopyranosyl units, a terminal α-L-rhamnopyranosyl unit, a 2,3-branched β-D-glucopyranosyl unit and a 2,4-branched β-D-galactopyranosyl unit in the molecule was indicated by the $^{13}$C NMR spectrum of **6**, and all the carbon signals due to the sugar moiety were in good agreement with those of tribulosin, a neotigogenin pentasaccharide isolated from *Tribulus terrestris* [11]. Thus, compound

**6** was shown to be a C-25 isomer of tribulosin, namely tigogenin 3-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-β-D-xylopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside}. This compound has not been isolated previously.

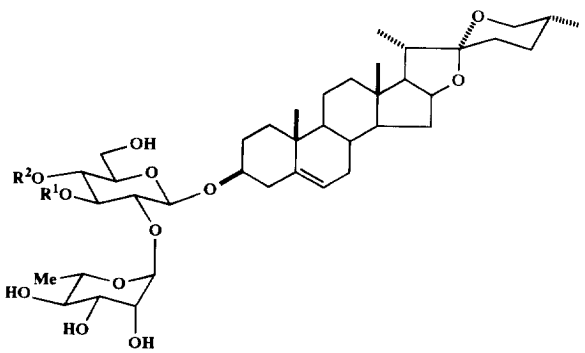
Compound **7** has a molecular formula of $C_{45}H_{76}O_{17}$ deduced from the negative-ion FAB-mass spectrum (*m/z* 887 $[M - H]^-$), $^{13}C$ NMR spectrum (45 carbon signals) and elemental analysis. The $^1H$ NMR spectrum showed three anomeric proton signals at δ5.56 (*br s*), 5.48 (*br s*) and 4.75 (*d*, *J* = 7.7 Hz), five secondary methyl proton signals at δ1.63 (*d*, *J* = 6.0 Hz), 1.60 (*d*, *J* = 5.4 Hz), 1.17 (*d*, *J* = 7.0 Hz), 0.94 (*d*, *J* = 6.1 Hz) and 0.93 (*d*, *J* = 6.1 Hz), two angular methyl proton signals at δ1.14 and 1.05 (each *s*), and an olefinic proton signal at δ5.46 (*br d*, *J* = 5.4 Hz). The signals at δ1.63 and 1.60 were due to the methyl groups of 6-deoxyhexoses. The above $^1H$ NMR data suggested that **7** was a cholestene trisaccharide. Acid hydrolysis of **7** with 1 M hydrochloric acid in dioxane–$H_2O$ (1 : 1) gave L-rhamnose and D-glucose in a ratio of 2 : 1, and an aglycone (**7a**), identified as (22 *S*)-cholest-5-ene-1β, 3β, 16β, 22-tetrol, i.e. the aglycone of schubertoside D [5, 7]. The $^{13}C$ NMR signals of the saccharide residue were assigned by comparing them with those of reference methyl glycosides [12, 13], confirming the presence of two terminal α-L-rhamnopyranosyl units (δ97.9, 72.9, 72.9, 73.7, 70.7 and 18.7; 99.7, 72.7, 72.8, 74.1, 70.0 and 18.5) and a terminal β-D-glucopyranosyl unit (δ106.9, 75.6, 78.2, 71.8, 78.7 and 63.0). Furthermore, glycosylation-induced downfield shifts were observed at the aglycone C-1, C-3 and C-16 hydroxy positions in **7** when the whole $^{13}C$ NMR signals were compared with those of **7a**, indicating that C-1, C-3 and C-16 of **7a** were the glycosylated positions to which either of the rhamnose and glucose was linked. Mild hydrolysis of **7** with 0.2 M hydrochloric acid gave a partial hydrolysate (**7b**). The $^1H$ NMR and $^{13}C$ NMR spectra of **7b** showed the existence of a β-D-glucopyranosyl unit in **7b**, and the downfield shift by *O*-glycosylation was observed at C-16 in comparison with the $^{13}C$ signals between **7b** and **7a**, leading to the identification of **7b** as (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol 16-*O*-β-D-glucopyranoside [7]. Accordingly, the structure of **7** was determined to be (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol 1,3-di-*O*,*O*′-α-L-rhamnopyranoside 16-*O*-β-D-glucopyranoside. It must be emphasized that **7** has a unique structure, being a trisdesmoside of a polyhydroxylated colestane.

Table 1. $^{13}C$ NMR spectral data (in pyridine-$d_5$) for compounds **7**, **7a** and **7b**

| C | 7 | 7a | 7b |
|---|---|---|---|
| 1 | 81.1 | 78.2 | 78.2 |
| 2 | 33.6 | 44.0 | 44.0 |
| 3 | 73.2 | 68.2 | 68.2 |
| 4 | 39.2 | 43.6 | 43.7 |
| 5 | 138.0 | 140.4 | 140.3 |
| 6 | 125.8 | 124.5 | 124.6 |
| 7 | 31.5 | 32.3 | 32.2 |
| 8 | 33.4 | 33.3 | 33.1 |
| 9 | 50.7 | 51.6 | 51.5 |
| 10 | 43.0 | 43.6 | 43.5 |
| 11 | 24.7 | 24.2 | 24.3 |
| 12 | 40.6 | 41.3 | 40.9 |
| 13 | 42.3 | 42.5 | 42.2 |
| 14 | 55.3 | 55.3 | 55.4 |
| 15 | 37.1 | 37.5 | 37.3 |
| 16 | 82.6 | 75.4 | 82.7 |
| 17 | 58.1 | 58.4 | 58.2 |
| 18 | 13.9 | 15.3 | 13.8[a] |
| 19 | 14.4 | 13.9[a] | 13.9[a] |
| 20 | 36.0 | 36.2 | 36.0 |
| 21 | 12.6 | 13.7[a] | 12.6 |
| 22 | 73.2 | 71.5 | 73.2 |
| 23 | 33.8 | 32.1 | 33.8 |
| 24 | 36.7 | 36.8 | 36.8 |
| 25 | 28.9 | 28.5 | 28.9 |
| 26 | 23.0[a] | 22.8[b] | 23.0[b] |
| 27 | 23.1[a] | 23.0[b] | 23.1[b] |
| 1′ | 97.9 | | |
| 2′ | 72.9[b] | | |
| 3′ | 72.9[b] | | |
| 4′ | 73.7 | | |
| 5′ | 70.7 | | |
| 6′ | 18.7[c] | | |
| 1″ | 99.7 | | |
| 2″ | 72.7[b] | | |
| 3″ | 72.8[b] | | |
| 4″ | 74.1 | | |
| 5″ | 70.0 | | |
| 6″ | 18.5[c] | | |
| 1‴ | 106.9 | | 107.0 |
| 2‴ | 75.6 | | 75.7 |
| 3‴ | 78.2[d] | | 78.2[c] |
| 4‴ | 71.8 | | 71.8 |
| 5‴ | 78.7[d] | | 78.8[c] |
| 6‴ | 63.0 | | 63.0 |

[a-d] Assignments interchangeable in each column.

*A. senescens* gave compounds **8** and **9**, and they were identified as (25*R*)-spirost-5-en-3β-ol (diosgenin) 3-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-[*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)]-β-D-gluco-




| | $R^1$ | $R^2$ |
|---|---|---|
| 8 | H | α-L-Rha*p*-(1→2)-α-L-Rha*p* |
| 9 | β-D-Glc*p* | H |

Table 2. Inhibitory effects of the isolated compounds on TPA-enhanced $^{32}P$-incorporation into phospholipids of HeLa cells[a]

| Compounds | Inhibition (%) 50 $\mu g\,ml^{-1}$ | Inhibition (%) 5 $\mu g\,ml^{-1}$ |
|---|---|---|
| **1** | 0 | –[c] |
| **2** | 7.2 | – |
| **3** | 42.5 | – |
| **4** | 61.4 | – |
| **5** | 17.5 | – |
| **6** | *[b] | 64.7 |
| **7** | 31.7 | – |
| **8** | 62.8 | – |
| **9** | * | 11.5 |

[a] Data, expressed as percentage of inhibition on TPA-enhanced $^{32}P$-incorporation, the deviations of which are within 5%.

[b] The samples exhibited cytotoxicity towards HeLa cells.

[c] Not measured.

pyanoside} [14] and diosgenin 3-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-β-D-glucopyranoside} [15], respectively.

The inhibitory effects of the isolated compounds on TPA-stimulated $^{32}P$-incorporation into phospholipids of HeLa cells are shown in Table 2. Compounds **3**, **4**, **7** and **8** exhibited considerable activity at a concentration of 50 $\mu g\,ml^{-1}$, which means they are as potent as laxogenin, a steroidal sapogenin isolated from *Allium bakeri* as an antitumour-promoter compound [16]. Compounds **6** and **9** were cytotoxic towards HeLa cells at 50 $\mu g\,ml^{-1}$, and at the lower concentration (5 $\mu g\,ml^{-1}$), they exhibited 64.7 and 11.5 % inhibition, respectively. Compound **6** is more potent than the steroidal saponins previously isolated by us [17], showing $IC_{50}$ values lower than approximately 4.5 μM.

## EXPERIMENTAL

*General.* Optical rotations were measured with a Jasco DIP-360 automatic digital polarimeter. IR spectra were recorded on a Hitachi 260-30 instrument and MS on a VG AutoSpec E machine. Elemental analysis was perfomed on a Perkin–Elmer 240B elemental analyzer. NMR spectra were recorded with a Bruker AM-400 spectrometer employing the standard Bruker software. Chemical shifts are given as δ values (ppm) with TMS as int. standard. Silica gel (Fuji-Silysia Chemical), Diaion HP-20 (Mitsubishi-Kasei), Sephadex LH-20 (Pharmacia) and octadecylsilanized (ODS) silica gel (Nacalai Tesque) were used for CC. TLC was carried out on pre-coated 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 with 10% $H_2SO_4$ followed by heating. HPLC was performed using a Tosoh HPLC system (Tosoh: pump, CCPM; controller, CCP controller PX-8010; detector, UV-8000) equipped with a Kaseisorb LC ODS-120-5 column (Tokyo-Kasei-Kogyo, 4.6 mm i.d. × 250 mm, ODS, 5 μm).

*Chemicals.* TPA was obtained from Pharmacia PL Biochemicals. Radioactive inorganic phosphate ($^{32}P$, carrier-free) was purchased from the Japan Radioisotope Associations. All other chemicals were of biochemical reagent grade.

*Plant materials.* The bulbs of *A. macleanii* and *A. senescens* were purchased from Heiwaen (Japan). The bulbs were cultivated and the plant specimens are on file in our laboratory.

*Extraction and isolation.* Fresh bulbs of *A. macleanii* (5.7 kg) were cut into pieces and extracted with hot MeOH. The MeOH extract, after removal of the solvent under red. pres. was partitioned between *n*-BuOH and $H_2O$. The *n*-BuOH-soluble phase was fractionated on a silica gel column using a mobile phase composed of $CH_2Cl_2$–MeOH with increasing amounts of MeOH (9:1, 4:1, 2:1), and finally with MeOH alone to give five frs (I–V). Fr. III was chromatographed on silica gel eluting with $CHCl_3$–MeOH (9:1) to give compound **2** (2.40 g). Fr. IV was shown by TLC analysis to contain several steroidal glycosides and abundant saccharides, from which the saccharides were removed by passing through a Diaion HP-20 column eluting with $H_2O$ gradually enriched with MeOH. The MeOH eluate fr. was repeatedly chromatographed on silica gel with $CHCl_3$–MeOH–$H_2O$ (20:10:1) and ODS silica gel with MeOH–$H_2O$ (4:1, 7:3, 3:2) to give compounds **1** (123 mg), **3** (668 mg), **4** (471 mg), **5** (62 mg), **6** (669 mg) and **7** (115 mg).

The *n*-BuOH-soluble fr. prepared from the bulbs of *A. senescens* (2.3 kg) by the same procedures as in the case of those of *A. macleanii* was subjected to Diaion HP-20 CC. The MeOH eluate fr. was chromatographed on silica gel eluting with $CH_2Cl_2$–MeOH–$H_2O$ (9:1:0, 6:1:0, 20:10:1, 7:4:1), and finally with MeOH to give 3 frs. (I–III). Fr. II was further divided by a Sephadex LH-20 column eluting with MeOH and a silica gel column with $CHCl_3$–MeOH (4:1) into frs IIa–IIc. Fr. IIa was chromatographed on ODS silica gel using MeOH–$H_2O$ (4:1) to give compound **8** with a few impurities, which was acetylated with $Ac_2O$ in pyridine followed by purification by silica gel CC using hexane–$Me_2CO$ (5:2). The pure acetate was hydrolysed with 4% KOH in EtOH to yield **8** (260 mg) as a pure compound. Fr. IIc was chromatographed on silica gel with $CHCl_3$–MeOH–$H_2O$ (35:10:1) to give compound **9** (92 mg).

*Compound* **6**. Amorphous powder, $[\alpha]_D^{25}$ −55.2° (pyridine: *c* 0.25). Negative-ion FAB-MS *m/z*: 1149 $[M-H]^-$, 1167 $[M-xylosyl]^-$; IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400 (OH), 2935 (CH), 1445, 1370, 1240, 1155, 1050, 985, 920, 895, 865, 835, 805, 770 (25*R*-spiroacetal, intensity 920 < 895); $^1H$ NMR (pyridine-$d_5$): δ6.16 (1H, *br s*, H-1″), 5.42 (1H, *d*, *J* = 7.7 Hz, H-1‴), 5.22 (1H, *d*, *J* = 7.8 Hz, H-1⁗), 4.98 (1H, *d*, *J* = 7.9 Hz, H-1‴), 4.84 (1H, *d*, *J* = 7.7 Hz, H-1′), 3.58 (1H, *dd*, *J* = 10.6, 3.0 Hz, H-26a), 3.50 (1H, *dd*, *J* = 10.6, 10.6 Hz, H-26b), 1.71 (3H, *d*, *J* = 6.2 Hz, Me-6″), 1.14 (3H, *d*, *J* = 6.9 Hz, Me-21), 0.86

(3H, *s*, Me-19), 0.81 (3H, *s*, Me-18), 0.70 (3H, *d*, $J = 5.3$ Hz, Me-27); $^{13}$C NMR (pyridine-$d_5$): $\delta$37.3, 30.0, 77.8, 34.5, 44.7, 29.0, 32.5, 35.3, 54.5, 36.0, 21.3, 40.2, 40.8, 56.5, 32.2, 81.2, 63.1, 16.6, 12.4, 42.0, 15.0, 109.2, 31.9, 29.3, 30.6, 66.9, 17.3 (C-1 to C-27), 100.3, 77.1, 76.6[a], 81.4[b], 75.8, 60.5 (C-1′ to C-6′), 102.0, 72.4, 72.7, 74.0, 69.3, 18.4 (C-1″ to C-6″), 105.3, 81.3[b], 87.7, 70.4, 76.7[a], 63.0 (C-1‴ to C-6‴), 105.7, 75.1, 79.0, 70.9, 67.6, (C-1⁗ to C-5⁗), 105.0, 75.1, 78.7, 70.7, 67.3 (C-1⁗′ to C-5⁗′). Assignments marked [a, b] may be reversed.

*Compound* **7**. Amorphous powder, $[\alpha]_D^{25} - 62.9°$ ($CHCl_3$–MeOH, 1:1; *c* 0.25). Anal. Calcd for $C_{45}H_{76}O_{17}.3H_2O$: C, 57.31; H, 8.76. Found: C, 57.59; H, 7.96%. Negative-ion FAB-MS *m/z*: 887 $[M - H]^-$, 743 $[M - \text{rhamnosyl}]^-$, 597 $[M - \text{rhamnosyl} \times 2]^-$; IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3425 (OH), 2900 (CH), 1450, 1380, 1255, 1065, 1035, 975, 905, 830, 805, 695; $^1$H NMR (pyridine-$d_5$): $\delta$5.56 (1H, *br s*, H-1′ or H-1″), 5.48 (1H, *br s*, H-1′ or H-1″), 5.46 (1H, *br d*, $J = 5.4$ Hz H-6), 4.75 (1H, *d*, $J = 7.7$ Hz H-1‴), 1.63 (1H, *d*, $J = 6.0$ Hz Me-6′), 1.60 (3H, *d*, $J = 5.4$ Hz Me-6″), 1.17 (3H, *d*, $J = 7.0$ Hz Me-21), 1.14 (3H, *s*, Me-19), 1.05 (3H, *s*, Me-18), 0.94 (3H, *d*, $J = 6.1$ Hz Me-26 or Me-27), 0.93 (3H, *d*, $J = 6.1$ Hz Me-26 or Me-27).

*Acid hydrolysis of* **7**. A soln of **7** (20 mg) in 1 M HCl (dioxane–$H_2O$, 1:1, 4 ml) was refluxed for 2 hr under an Ar atmosphere. The reaction mixt. was neutralized by passing through Amberlite IRA-93 ZU (Organo), and then transferred to a silica gel column, eluting with $CHCl_3$–MeOH (9:1) followed by MeOH to yield (22*S*)-cholest-5-ene-1$\beta$,3$\beta$,16$\beta$,22-tetrol (**7a**) (5.5 mg) [7] and a mixt. of monosaccharides (9 mg). The mixt. (2 mg) in $H_2O$ (1 ml) was treated with (−)-$\alpha$-methylbenzylamine (5 mg) and Na[$BH_3CN$] (8 mg) in EtOH (1 ml) for 4 hr at 40° 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), initially eluting with $H_2O$–MeCN (4:1, 10 ml), and then with MeCN (10 ml). The MeCN fr. was further passed through a Toyopak IC-SP M (Tosoh) cartridge with EtOH (10 ml) to give a mixt. of the 1-[(*S*)-*N*-acetyl-$\alpha$-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides, which was then analysed by HPLC [18, 19]. Derivatives of D-glucose and L-rhamnose were detected in a ratio of 1:2.

*Partial hydrolysis of* **7**. A soln of **7** (60 mg) in 0.2 M HCl (dioxane–$H_2O$, 1:1, 6ml) was refluxed for 30 min under an Ar atmosphere. After neutralization of the reaction mixt. by passage through an Amberlite IRA-93ZU column, it was subjected to a silica gel column with $CHCl_3$–MeOH (6:1) to yield (22*S*)-cholest-5-ene-1$\beta$,3$\beta$,16$\beta$,22-tetrol 16-*O*-$\beta$-D-glucopyranoside (**7b**) (3 mg) [7].

*Cell culture and assay of $^{32}$P-incorporation into phospholipids of cultured cells.* HeLa cells were cultured as monolayers in Eagle's minimum essential medium supplemented with 10% calf serum in a humidified atmosphere of 5% $CO_2$ in air. HeLa cells were incubated with the test samples (50 $\mu$g ml$^{-1}$ and/or 5 $\mu$g ml$^{-1}$) and, after 1 hr, $^{32}$P (370 kBq culture$^{-1}$) was added with or without TPA (50 nM). Incubation was continued for 4 hr and then the radioactivity incorporated into the phospholipid fr. was measured [20].

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