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A Furostanol Glycoside from Garlic, Bulbs of *Allium sativum* L.

HIROMICHI MATSUURA,* TSUYOSHI USHIROGUCHI, YOICHI ITAKURA, NORIHIRO HAYASHI, and TORU FUWA

Central Research Laboratories, Wakunaga Pharmaceutical Co., Ltd., Shimokotachi 1624, Koda-cho, Takata-gun, Hiroshima 729–64, Japan

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A new furostanol glycoside (**1**) was isolated from garlic, bulbs of *Allium sativum* L. (Liliaceae), and the structure was established to be 26-*O*-β-glucopyranosyl 22-hydroxy-25(*R*)-5α-furostane-3β,6β,26-triol 3-*O*-β-glucopyranosyl(1→2)-[β-glucopyranosyl(1→3)]-β-glucopyranosyl-(1→4)-β-galactopyranoside, *i.e.*, proto-eruboside-B. Eruboside-B (**3**) obtained by enzymatic hydrolysis of **1** inhibited the growth of *Candida albicans in vitro* (25 μg/ml MIC).

Keywords——*Allium sativum*; garlic; Liliaceae; furostanol glycoside; proto-eruboside-B; eruboside-B; antifungal activity

Garlic, bulbs of *Allium sativum* L. (Liliaceae), has been used world-wide as a tonic, a bacteriocide, and a popular remedy for various ailments.[1] There have been many reports on the sulfur-containing constituents.[2] Recently, ajoene obtained from garlic by Block *et al.*[3] was shown to have antithrombotic[3] and antifungal activity.[4] With regard to the saponin constituents of the Allium family, many steroid saponins have been reported by Abubakirov *et al.*[5] However, little is known about the saponins of garlic.[6] The present paper deals with a study on steroidal glycosides of this crude drug, reporting the isolation and structure determination of a new furostanol glycoside, and determination of its antifungal activity.

A crude glycoside fraction from the methanolic extract of garlic was subjected to the combination of silica gel and reversed-phase chromatography on highly porous polymer, followed by heating in aqueous acetone to give compound A (**1**), $C_{57}H_{96}O_{30}\cdot 5H_2O$, in a 0.01% yield. Compound A(**1**) showed a purple coloration with the Ehrlich reagent on thin layer chromatography (TLC).[7] On standing in methanol, **1** was converted to compound B(**2**). The proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra of **1** and **2** indicated the presence of five monosaccharide units (Table II), while the ^{1}H-NMR spectrum of **2** was similar to that of **1** except for a methoxyl signal at 3.26 ppm. On acid hydrolysis, **1** afforded galactose and glucose in the ratio of 1 : 4. Among 27 carbon signals due to the aglycone moiety in the ^{13}C-NMR spectrum of **1** (Table I), the signals assignable to C-22—C-26 appeared at almost the same positions as those of a known furostanol glycoside, proto-aspidistrin, reported by Hirai *et al.*[8] Accordingly, **1** is considered to be a 22-hydroxyfurostanol pentaglycoside.

Enzymatic hydrolysis of **1** with β-glucosidase gave compound C (**3**) and glucose, the former of which was negative to the Ehrlich reagent. On acid hydrolysis, **3** gave galactose and glucose in the ratio of 1 : 3. It can be assumed that **3** is a tetraglycoside of 25(*R*)-spirostanol based on the characteristic 25(*R*)-spiroketal infrared (IR) absorption bands (intensity, 915 cm^{-1} < 900 cm^{-1})[9] and four anomeric carbon signals in the ^{13}C-NMR spectrum. Partial hydrolysis of **3** with aqueous sulfuric acid afforded an aglycone (**4**) and compounds D (**5**) and E (**6**). On inspection of the ^{13}C-NMR spectral data, **4** was proved to be identical with β-chlorogenin, previously isolated from the flower clusters of *Allium erubescens* as a sapo-

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genin.[10] Compound D(**5**) was subjected to acid hydrolysis to give **4** and galactose, while compound E (**6**) gave **4**, galactose and glucose. A comparison of the carbon signals due to the aglycone moieties of **3**, **5**, and **6** with that of **4** indicated that **3**, **5**, and **6** were monodesmosides of **4** with a glycosyl linkage at the 3β-hydroxyl group (Table I). Finally, the structures of **5** and **6** were determined to be β-chlorogenin 3-*O*-β-galactopyranoside and β-chlorogenin 3-*O*-β-glucopyranosyl(1→4)-β-galactopyranoside based on analysis of the ^{1}H- and ^{13}C-NMR spectra. By sequencing analysis (methylation followed by successive hydrolysis, reduction and acetylation), **3** afforded three partially methylated alditol acetates, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (**7**), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (**8**), and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylhexitol (**9**) detected by gas chromatography-mass spectrometry (GC-MS) (alditol acetate analysis).[11] Upon comparison of the ^{13}C-NMR spectrum of **3** with that of the spirostanol glycoside corresponding to PO-d,[12] which was isolated from *Polygonatum odoratum* var. *pluriflorum* and characterized as 26-*O*-β-glucopyranosyl 22-methoxy-(25*R* and *S*)-furost-5-en-3β,14α,26-triol 3-*O*-β-glucopyranosyl(1→2)-[β-glucopyranosyl(1→3)]-β-glucopyranosyl(1→4)-β-galactopyranoside, all the carbon signals due to the sugar moiety of both glycosides appeared at almost the same positions. On the basis of these results, the structure of **3** can be represented as β-chlorogenin 3-*O*-β-glucopyranosyl(1→2)-[β-glucopyranosyl(1→3)]-β-glucopyranosyl(1→4)-β-galactopyranoside, which is identical with eruboside-B isolated from *Allium erubescens*.[10]

Since **1** was a furostanol glycoside corresponding to **3**, it was established to be proto-eruboside-B, 26-*O*-β-glucopyranosyl 22-hydroxy-25(*R*)-5α-furostane-3β,6β,26-triol 3-*O*-β-

TABLE I. ^{13}C-NMR Chemical Shifts: Aglycone Moiety[a]

	4	**5**	**6**	**3**	**1**	**2**
C-1	39.2	38.9	38.9	38.9	38.9	38.8
C-2	32.7	30.2	30.2	30.0	30.0	30.0
C-3	71.0	77.6	77.7	77.9	77.9	77.9
C-4	37.2	32.8	32.7	32.8	32.8	32.8
C-5	48.5	47.9	47.9	47.9	48.0	47.9
C-6	71.4	70.9	70.8	70.8	70.8	70.8
C-7	41.0	40.3	40.2	40.2	40.3	40.5
C-8	30.7	30.7	30.7	30.6	30.6	30.6
C-9	54.8	54.7	54.7	54.6	54.7	54.6
C-10	36.2	36.2	36.2	36.1	36.1	36.1
C-11	21.3	21.3	21.3	21.2	21.3	21.2
C-12	40.3	40.9	40.9	40.8	40.8	40.8
C-13	40.9	40.9	40.9	40.8	41.2	41.2
C-14	56.5	56.4	56.4	56.3	56.3	56.2
C-15	32.3	31.8	31.8	31.8	32.5	32.2
C-16	81.2	81.1	81.1	81.1	81.1	81.3
C-17	63.1	63.1	63.1	63.1	64.0	64.3
C-18	16.7	16.6	16.6	16.6	16.7	16.5
C-19	16.3	16.1	16.0	16.0	16.4	16.3
C-20	42.0	42.0	42.0	42.0	40.6	40.1
C-21	15.1	15.1	15.0	15.0	16.0	16.0
C-22	109.2	109.3	109.3	109.2	110.6	112.6
C-23	31.8	32.3	32.3	32.2	37.2	30.7
C-24	29.2	29.3	29.3	29.2	28.4	28.2
C-25	30.6	30.6	30.6	30.6	34.2	34.2
C-26	66.9	66.9	66.9	66.8	75.3	75.3
C-27	17.3	17.3	17.3	17.3	17.5	17.2
OCH_3						47.3

a) Chemical shifts were measured in pyridine-d_5 at room temperature.

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TABLE II. ^{13}C-NMR Chemical Shifts: Sugar Moiety

		5	6	3	1	2
C-3 sugars						
Galactose	1	102.6	102.4	102.3	102.3	102.3
	2	72.8	73.5	73.2	73.2	73.2
	3	75.5	75.5[a)]	75.6[a)]	75.6[a)]	75.6[a)]
	4	70.5	80.1	80.2	80.3	80.3
	5	77.0	76.0	76.1	76.1	76.1
	6	62.7	61.1	60.6	60.6	60.6
Glucose	1		107.1	105.0[b)]	105.0[b)]	105.0[b)]
(inner)	2		75.3[a)]	81.4	81.5	81.5
	3		78.5[b)]	88.5	88.5	88.4
	4		72.3	70.9[c)]	70.8[c)]	70.8[c)]
	5		78.7[b)]	77.9[d)]	78.0[d)]	78.0[d)]
	6		63.1	63.1[e)]	63.0[e)]	63.0[e)]
Glucose	1			104.9[b)]	104.9[b)]	105.0[b)]
	2			75.3[a)]	75.3[a)]	75.3[a)]
	3			78.6	78.6[f)]	78.6[f)]
	4			70.8[c)]	71.7[c)]	71.7[c)]
	5			77.5[d)]	77.5[d)]	77.5[d)]
	6			62.3[e)]	62.3[e)]	62.3[e)]
Glucose	1			104.5[b)]	104.5[b)]	104.5[b)]
	2			75.3[a)]	75.2[a)]	75.2[a)]
	3			78.6	78.6[f)]	78.6[f)]
	4			71.6[c)]	71.6[c)]	71.6[c)]
	5			78.6	78.6[f)]	78.6[f)]
	6			62.3[e)]	62.3[e)]	62.3[e)]
C-26 sugar						
Glucose	1				104.9[b)]	105.1[b)]
	2				75.2[a)]	75.2[a)]
	3				78.4[f)]	78.5[f)]
	4				71.7[c)]	71.6[c)]
	5				78.4[f)]	78.5[f)]
	6				62.8[e)]	62.9[e)]

a—*f*) Values in any column may be reversed, though those given here are preferred.

TABLE III. Antifungal Activity of Steroidal Glycosides

Sample	MIC (μg/ml)	
	C. albicans	*A. niger*
1	>800	>800
3	25	400
10	0.25	2

MIC: minimum inhibitory concentration.

glucopyranosyl(1→2)-[β-glucopyranosyl(1→3)]-β-glucopyranosyl(1→4)-β-galactopyranoside.

On acid hydrolysis of the crude glycoside fraction of garlic, trace amounts of tigogenin were also isolated, but no corresponding glycosides could be obtained.

Antifungal activity of garlic has been attributed to allicin[13] (produced from alliin by alliinase) and ajoene, a decomposition product of allicin. Imai *et al.* reported that various steroid saponins related to dioscin, diosgenin, and tigogenin inhibited the growth of several

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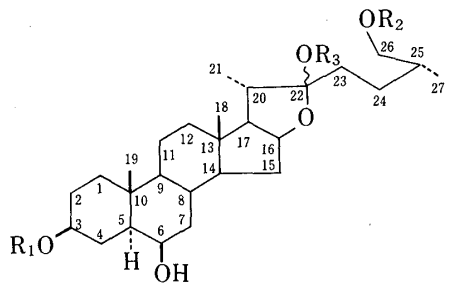

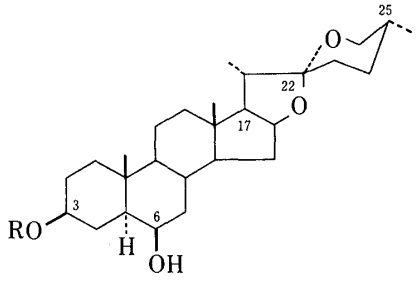

	R_1	R_2	R_3
1:	$-\beta$-Gal^4—β-Glc$^{3}_{2}$ β-Glc, β-Glc	$-\beta$-Glc	$-H$
2:	$-\beta$-Gal^4—β-Glc$^{3}_{2}$ β-Glc, β-Glc	$-\beta$-Glc	$-CH_3$

	R
3:	$-\beta$-Gal^4—β-Glc$^{3}_{2}$ β-Glc, β-Glc
4:	$-H$
5:	$-\beta$-Gal
6:	$-\beta$-Gal^4—β-Glc

Chart 1

fungi.[14] We therefore examined the antifungal activity of two glycosides (**1** and **3**) obtained in this work. The results are shown in Table III. Compound **3** exhibited the growth of only *Candida albicans*, and its activity was less than that of amphotericin B(**10**), which was used as the control drug. It is noteworthy that a compound having antifungal activity has been newly found in garlic.

Experimental

General Procedure——Melting points were measured on a Yanagimoto micromelting point apparatus and are uncorrected. The nuclear magnetic resonance (NMR) spectra were taken on a JEOL JNM GX-270 spectrometer using tetramethylsilane as an internal standard. The MS were recorded on a JEOL JMS DX-300 mass spectrometer. The IR spectra were obtained with a Hitachi model 215 spectrophotometer (KBr disk method), and gas liquid chromatography (GLC) was run on a Shimadzu GC-9AM gas chromatograph. For column chromatography, Silica gel 60 (70—230 mesh or 230—400 mesh, Merck) and MCI gel CHP20P (75—150 μm, Mitsubishi Chem. Ind. Co., Ltd.) were used. TLC was performed on TLC plate Silica gel $100F_{254}$ (Merck) and high performance thin layer chromatography (HPTLC) RP-$8F_{254s}$ (Merck), and spots were visualized by spraying of anisaldehyde–H_2SO_4 followed by heating or by the use of Ehrlich reagent.

Extraction and Isolation of 1——Frozen, freshly skinned garlic cloves, 4 kg (collected in Aomori prefecture, Japan), were crushed in MeOH and twice extracted with hot MeOH (6 l). After removal of the solvent by evaporation, a suspension of the resulting extract in H_2O was applied to a column of MCI gel CHP20P (stepwise elution of H_2O, 20% aqueous MeOH, and MeOH). The fraction (8.6 g) eluted with MeOH was separated by repeated chromatography on silica gel (solvent: $CHCl_3$–MeOH–H_2O (6:4:1, homogeneous)) to give a glycoside fraction (0.60 g) and this was further subjected to reversed-phase chromatography on MCI gel CHP20P (solvent: 80% aqueous MeOH) to afford a mixture of compounds A (**1**) and B (**2**). The mixture was heated with 30% aqueous acetone at 100 °C for 4 h, then the reaction mixture was concentrated to dryness *in vacuo* to give **1** (yield, 0.01%).

Compound A (**1**): White powder (from aqueous acetone), $[\alpha]_D^{27}$ −36.5° (c=0.7, pyridine). *Anal.* Calcd for $C_{57}H_{96}O_{30}\cdot 5H_2O$: C, 50.66; H, 7.92. Found: C, 50.63; H, 7.76. ^{1}H-NMR (pyridine-d_5) δ: 4.82 (1H, d, J=7.9 Hz), 4.95 (1H, d, J=7.3 Hz), 5.16 (1H, d, J=7.6 Hz), 5.31 (1H, d, J=7.6 Hz), 5.58 (1H, d, J=7.0 Hz).

Formation of 2——A methanol solution (2 ml) of **1** (40 mg) was allowed to stand for 2 h at room temperature, and then concentrated to dryness to give **2**. Compounds A (**1**) and B (**2**) showed *Rf* values of 0.35 and 0.50 on silica gel TLC with $CHCl_3$–MeOH–H_2O (6:4:1), respectively. **2**: ^{1}H-NMR (pyridine-d_5) δ: 3.26 (3H, s), 4.86 (1H, d, J=7.9 Hz), 4.95 (1H, d, J=7.6 Hz), 5.16 (1H, d, J=7.9 Hz), 5.31 (1H, d, J=7.6 Hz), 5.58 (1H, d, J=7.3 Hz).

Enzymatic Hydrolysis of 1——An acetate buffer solution (pH 4.3, 50 ml) of **1** (0.25 g) and β-glucosidase from almond (P. L. Biochemicals, 0.25 g) was incubated at 37 °C for 2 h. The reaction mixture was diluted with H_2O and applied to a column of MCI gel CHP20P. The column was washed with H_2O and then eluted with MeOH. After evaporation of MeOH, the residue was reprecipitated from MeOH–AcOEt to afford **3** (0.17 g), while glucose was identified by TLC (*vide post*) in the fraction eluted with H_2O.

Compound C (**3**): White powder, $[\alpha]_D^{27}$ −62.6° (c=0.4, $CHCl_3$–MeOH (10:1)). ^{1}H-NMR (pyridine-d_5) δ: 4.95 (1H, d, J=7.6 Hz), 5.16 (1H, d, J=7.9 Hz), 5.31 (1H, d, J=7.6 Hz), 5.59 (1H, d, J=7.3 Hz). IR ν_{max}^{KBr} cm^{-1}: 3400, 915, 900.

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Partial Hydrolysis of 3——Compound C (**3**, 0.30 g) was heated with 5% aqueous sulfuric acid (25 ml) at 100 °C for 3 h. After cooling, the reaction mixture was diluted with H_2O and applied to a column of MCI gel CHP20P (solvent: H_2O and then MeOH). The fraction (0.19 g) eluted with MeOH was chromatographed on silica gel (solvent: $CHCl_3$–MeOH–H_2O 7:2:0.2, homogeneous) to afford **4** (61 mg), **5** (12 mg), and **6** (35 mg).

The Aglycone (**4**): Colorless needles (from benzene), mp 240—241 °C (lit,[10] 231—233 °C), $[\alpha]_D^{27}$ −73.7° (c=0.5, $CHCl_3$). FD-MS m/z: 432 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 0.79 (3H, d, J=6.1 Hz, 27-CH_3), 0.80 (3H, s, 18-CH_3), 0.96 (3H, d, J=6.9 Hz, 21-CH_3), 1.05 (3H, s, 19-CH_3), 3.65 (1H, tt, J=11.0, 5.5 Hz, 3-H), 3.81 (1H, br q, J=2.7 Hz, 6-H).

Compound D (**5**): White powder (from acetone), $[\alpha]_D^{27}$ −53.9° (c=0.2, $CHCl_3$–MeOH (10:1)). ^{1}H-NMR (pyridine-d_5) δ: 0.69 (3H, d, J=5.5 Hz, 27-CH_3), 0.88 (3H, s, 18-CH_3), 1.15 (3H, d, J=7.0 Hz, 21-CH_3), 1.27 (3H, s, 19-CH_3), 3.95 (1H, br s, 6-H), 5.05 (1H, d, J=7.6 Hz, anomeric-H).

Compound E (**6**): White powder (from EtOH), $[\alpha]_D^{27}$ −68.2° (c=0.2, $CHCl_3$–MeOH (10:1)). ^{1}H-NMR (pyridine-d_5) δ: 4.96 (1H, d, J=7.6 Hz), 5.31 (1H, d, J=7.6 Hz).

Acid Hydrolysis of Glycosides——A glycoside (a few mg) was heated with 2 N HCl–dioxane (1:1, 1 ml) in a sealed tube at 100 °C for 4 h. The reaction mixture was concentrated to dryness by blowing N_2 gas over it at room temperature. TLC was done on HPLC Silica gel 50000F_{254s} (Merck); solvent, 1-BuOH–2-PrOH–H_2O (10:5:4, homogeneous); detection, anisaldehyde–H_2SO_4; Rf values, glucose (0.24) and galactose (0.17). For GLC analysis, the residue was trimethylsilylated with TMS-HT (Tokyo Kasei Kogyo Co., Ltd.) at room temperature. GLC: 1% OV-17 on Chromosorb-W, glass column, 3 mm × 2 m; detection, FID; column temperature, 150 °C; carrier gas, N_2 (40 ml/min); t_R (min), galactose (10.9, 13.5), glucose (13.3, 21.3).

Permethylation Followed by Alditol Acetate Analysis of 3——According to Hakomori's method,[15] **3** (2 mg) was methylated with NaH and dimethylsulfoxide, and CH_3I. The reaction product was purified by column chromatography on silica gel (solvent: $CHCl_3$–MeOH (70:1)) to afford the corresponding permethyl ether of **3**. The resulting permethylated ether was converted to alditol acetates according to the previous paper.[16] GC-MS conditions: 1.5% OV-210 on Chromosorb-W, glass column, 2 mm × 2 m; column temperature, 195 °C; carrier gas, He (50 ml/min); t_R (min): **7** (3.8), **8** (7.2), **9** (11.1).

Antifungal Activity——Antifungal tests were carried out as described in a previous publication,[4] except for the use of MIC defined as the minimum concentration causing complete absence of growth of fungi.

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