Withanolide Derivatives from the Roots of *Withania somnifera* and Their Neurite Outgrowth Activities

Jing ZHAO,[a] Norio NAKAMURA,[a] Masao HATTORI,*[,a] Tomoharu KUBOYAMA,[b] Chihiro TOHDA,[b] and Katsuko KOMATSU[b]

[a] *Department of Metabolic Engineering, Toyama Medical and Pharmaceutical University; and* [b] *Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930–0194, Japan.* Received December 18, 2001; accepted March 7, 2002

Five new withanolide derivatives (1, 9—12) were isolated from the roots of *Withania somnifera* together with fourteen known compounds (2—8, 13—19). On the basis of spectroscopic and physiochemical evidence, compounds 1 and 9—12 were determined to be (20*S*,22*R*)-3α,6α-epoxy-4β,5β,27-trihydroxy-1-oxowitha-24-enolide (1), 27-*O*-β-D-glucopyranosylpubesenolide 3-*O*-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside (withanoside VIII, 9), 27-*O*-β-D-glucopyranosyl (1→6)-β-D-glucopyranosylpubesenolide 3-*O*-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside (withanoside IX, 10), 27-*O*-β-D-glucopyranosylpubesenolide 3-*O*-β-D-glucopyranoside (withanoside X, 11), and (20*R*,22*R*)-1α,3β,20,27-tetrahydroxywitha-5,24-dienolide 3-*O*-β-D-glucopyranoside (withanoside XI, 12). Of the isolated compounds, 1, withanolide A (2), (20*S*,22*R*)-4β,5β,6α,27-tetrahydroxy-1-oxowitha-2,24-dienolide (6), withanoside IV (14), withanoside VI (15) and coagulin Q (16) showed significant neurite outgrowth activity at a concentration of 1 μM on a human neuroblastoma SH-SY5Y cell line.

Key words *Withania somnifera*; Solanaceae; withanolide; withanoside; neurite outgrowth activity

Neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, have attracted more and more public attention due to their damaging impact upon patients and families as well as society generally. Although their pathogeneses are yet to be fully understood, the disease processes are generally believed to be triggered by a number of genetic, environmental, and metabolic factors. Among them, dysfunction of neuronal networks is one of the causes of irreversible cognition impairment among patients. Therefore, the reconstruction of neuronal networks indexed by neurite outgrowth, provides us new insights for drug development to prevent, treat, and cure these diseases.[1)]

Withania somnifera DUN. (family Solanaceae), highly reputed as "Indian ginseng" in Ayurvedic medicine, is noted for its beneficial effects on the nervous system.[2)] To understand this benefit, a MeOH extract of the roots of *W. somnifera* was investigated as reported previously[3)] and showed appreciable activity in the bioassay using a human neuroblastoma SK-N-SH cell line. To search for the active principles, chemical constituents of the crude extract were investigated. In the present paper, we report five new withanolide derivatives (**1**, **9**—**12**) obtained from the MeOH extract, and their neurite outgrowth activities (Chart 1).

The methanol extract of the roots of *W. somnifera* was separated as described in the experimental section to afford compounds **1**—**19**. By comparison with the reported data, fourteen known compounds were identified as withanolide A (**2**),[4)] (20*S*,22*R*)-5α,27-dihydroxy-6α,7α-epoxy-1-oxowitha-2,24-dienolide (**3**),[5)] lycium substance B (**4**),[6)] withacoagin (**5**),[7)] (20*S*,22*R*)-4β,5β,6α,27-tetrahydroxy-1-oxowitha-2,24-dienolide (**6**),[8)] withanolide D (**7**),[9)] and withaferin A (**8**),[10)] withanosides V (**13**),[8,11)] IV (**14**),[11)] VI (**15**),[11)] III (**18**),[11)] and II (**19**),[11)] coagulin Q (**16**),[12)] and physagulin D (**17**).[13)]

Compound **1** was obtained as a white powder. The high resolution (HR)-FAB-MS, *m*/*z* $[M+H]^+$ 489.2843, revealed the molecular formula of $C_{28}H_{40}O_7$, suggesting nine units of unsaturation. The IR spectrum exhibited characteristic absorption bands at 3442 (OH), 1701, 1685 (α,β-unsaturated δ-lactone), and 1720 (ketone) cm^{-1}. The ^{1}H- and ^{13}C-NMR data indicated the presence of three tertiary methyls and one secondary methyl, eight methylenes (one oxygen-bearing sp^3), nine methines (four oxygen-bearing sp^3), and seven quarternary carbons (one oxygen-bearing sp^3, two olefin, and two carbonyl). Their ^{1}H- and ^{13}C-chemical shifts were assigned based on a combination of two-dimensional (2D) NMR [^{1}H–^{1}H shift correlation spectroscopy (COSY) and ^{1}H-detected multiple quantum coherence (HMQC)] techniques. On a basic withanolide skeleton, 1-unconjugated ketone and 20-unsubstituted, 27-hydroxy α,β-unsaturated δ-lactone side chain were assigned. Differing from other withanolides, however, four adjacent oxygenated carbons (δ 74.2, 77.9, 76.9, 77.6) were assigned as C-3—C-6. As a 1-oxowithanolide skeleton could account for only eight units of unsaturation, the remainder unit implied the presence of an intramolecular ether linkage between two of the four oxygen-bearing carbons. The positions of the ether linkage were realised by spectroscopic inspection of its acetate. Treatment of **1** with acetic anhydride in pyridine yielded an acetylated derivative **1a**. The molecular formula of $C_{32}H_{45}O_9$ was deduced from HR-FAB-MS, which implied the addition of two molecules of acetyl groups. The absorption band at 3433 cm^{-1} in its IR spectrum suggested the presence of a tertiary hydroxy group in the structure (5-OH). Apart from one acetyl group at C-27, the heteronuclear multiple bond coherence (HMBC) correlation between signals of the acetoxy carbon (δ 170.9) and an H-4 proton (δ 5.24) confirmed the attachment of the other acetyl group at C-4. Thus the ether linkage sites were exclusively established at C-3 and C-6 as well as free hydroxy groups at C-4 and C-5 in **1**. The stereochemistry of **1** was elucidated as follows. First, the presence of a *trans* B/C/D ring system and a (20*S*,22*R*)-α,β-unsaturated δ-lactone moiety was confirmed by spectroscopic similarity with common withanolides. Next, the stereochemistry of the C-3—C-6 cluster was determined by the circular dichroic (CD) spectrum, where the negative Cotton effect at 289.4 nm suggested 1-unconjugated ketone, *cis*-fused A/B ring junction.[6)] On this

* To whom correspondence should be addressed. e-mail: saibo421@ms.toyama-mpu.ac.jp

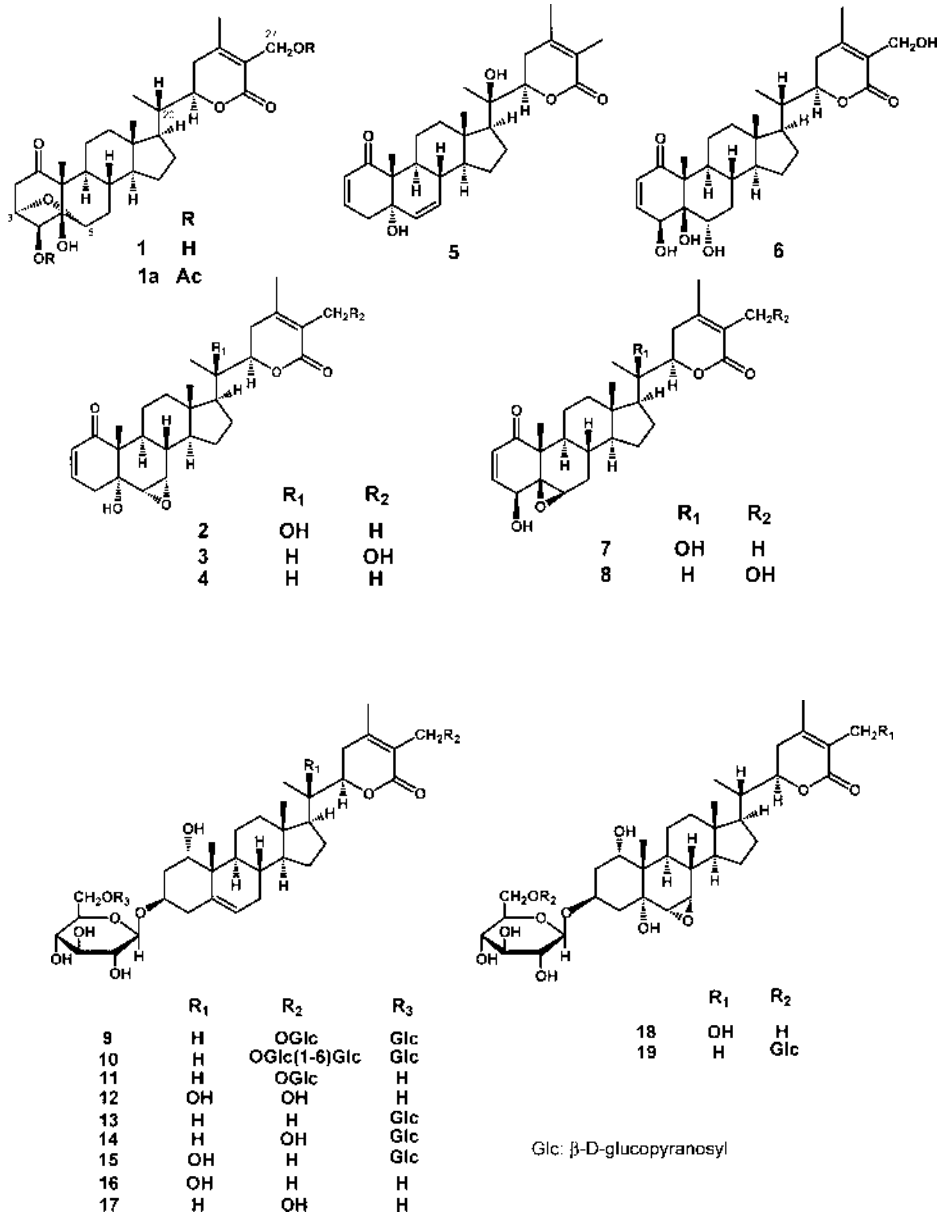

Chart 1. Structures of Compounds **1**—**19**

premise, the orientation of the ether bridge between C-3 and C-6 was rationally assigned to be α relative to the rings A and B in consideration of the ring strain and acceptable distance of an ether linkage between C-3 and C-6. Furthermore, the stereochemistry of C-3—C-6 was deciphered on the basis of the nuclear Overhauser effect spectroscopy (NOESY) spectrum (Fig. 1) and *J* values. First, discrimination of α and β methylene protons at C-7 was crucial for the assignment of chirality. The multiplet at δ 2.20 was confirmed to be β-oriented by the NOE correlations of H-7β/H-8 (δ 1.90) and H-8/H_3-18 (δ 0.52), while H-7α (δ 1.40) showed a cross peak with H-9. Second, the observation of the NOE interaction between H-6 (δ 4.53) and H-7β implied their spatial proximity, namely, they were on the same face of the B-ring, confirming an α-orientation of C-6-oxygen. Third, the NOE cross peak between H-4 (δ 4.86) and H-6 implied that they were axial on the same face of the tetrahydrofuran ring. Correspondingly, 4-OH was determined to be β-positioned. In considera-

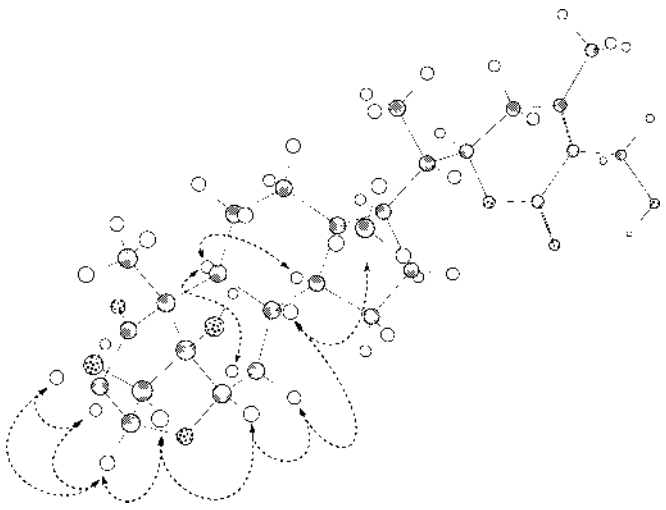

Fig. 1. Selected NOESY Correlations of Compound **1**

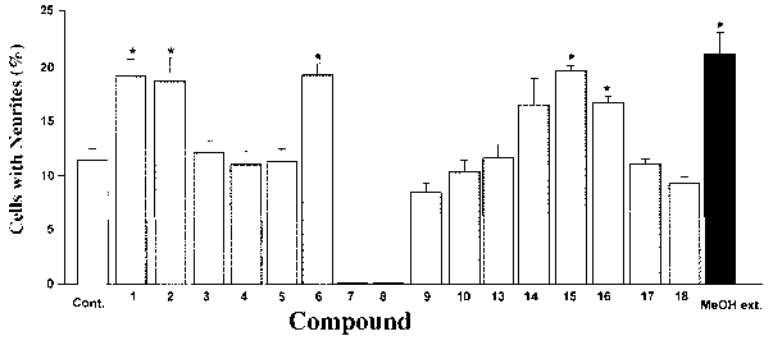

Fig. 2. Effects of Compounds Isolated from the Roots of *Withania somnifera* on Neurite Outgrowth of Human Neuroblastoma SH-SY5Y Cells

The percentages of cells with neuritis were measured 6 d after treatment of compounds and an MeOH extract at doses of 1 μM and 5 μg/ml, respectively. $*p<0.05$ *vs.* control ($n=4$).

tion of C-3, the small coupling constant between H-3 (δ 4.55) and H-4 ($J_{3H,4H}=6.5$ Hz), and the NOE correlation between them revealed a *syn* relationship, thus establishing 3α-oxygen. Consequently, compound **1** was designated as (20*S*,22*R*)-3α,6α-epoxy-4β,5β,27-trihydroxy-1-oxowitha-24-enolide. Although there have been several reports on isolation of 14,20-epoxy withanolides from *Withania* spp.,[12,14] to the best of our knowledge, compound **1** was the first example of a withanolide with an ether bridge between C-3 and C-6.

Withanoside VIII (**9**) was isolated as a white amorphous powder. The IR spectrum indicated the presence of hydroxyl (3406 cm^{-1}) and α,β-unsaturated δ-lactone (1721, 1685 cm^{-1}) groups. In the HR-FAB-MS spectrum, the molecular formula of $C_{46}H_{72}O_{20}$ was assigned by the presence of a quasimolecular ion peak at *m/z* 967.4473 $[M+Na]^+$. In the FAB-MS spectrum, fragment ion peaks at *m/z* 783 $[M+H-C_6H_{10}O_5]^+$, 621 $[M+H-C_{12}H_{22}O_{11}]^+$ and 459 $[M+H-C_{18}H_{34}O_{17}]^+$ implied the stepwise loss of three hexose units, whereas acid hydrolysis of **9** yielded glucose only. Detailed analysis of the 1D and 2D NMR spectra (^{1}H–^{1}H COSY, NOESY, HMQC and HMBC) and comparison with the reported data established the aglycon as pubesenolide [(20*S*,22*R*)-1α,3β,27-trihydroxywitha-5,24-dienolide],[12] the same framework as in compounds **14** and **17**.[11,13] However, deshielding of C-27 at δ 63.4 and upfield shift of C-25 at δ 123.9 were noticed. The presence of three glucose residues was unambiguously confirmed by the observation of three anomeric proton and carbon signals [^{1}H-NMR: δ 4.90 (H-1′), 5.03 (H-1‴), 5.13 (H-1″); ^{13}C-NMR: δ 103.3 (C-1′), 105.5 (C-1″), 104.9 (C-1‴)]. The β-anomeric configurations of all of them were assigned from the large coupling constants, $^3J_{1H,2H}=8.0$—8.5 Hz. D-Glucose was identified by gas chromatography-mass spectroscopic (GC-MS) analysis of the sugar derivative.[15] The connectivities of three glucoses with the aglycon were accomplished by the HMBC experiment. The ^{1}H–^{13}C long-range correlations of the following pairs, H-1′/C-3 (δ 74.3), H-3α (δ 4.77)/C-1′, H-1″/C-6′ (δ 69.7) and H_2-6′ (δ 4.36 and 4.68)/C-1″, confirmed a partial structure of 3-*O*-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside, identical with those of **13**—**15** and **19**. The third glucose was confirmed to attach to C-27 on the basis of HMBC correlations of H_2-27/C-1‴ and H-1‴/C-27, which was also supported by the above glycosylation shifts in the ^{13}C-NMR spectrum. Thus, its structure was determined to be 27-*O*-β-D-glucopyranosylpubesenolide 3-*O*-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside.

Compound **10** was obtained as an amorphous powder. The molecular formula of $C_{52}H_{82}O_{25}$ was assigned on the basis of a quasimolecular ion peak at *m/z* 1129.5001 $[M+Na]^+$ in the HR-FAB-MS. The ^{1}H- and ^{13}C-NMR data due to the aglycon moiety showed high analogy with those of **9**, indicating the identical skeleton bearing two glycosylation sites, C-3 and C-27. With the aid of total correlation spectroscopic (TOCSY) and ^{1}H–^{1}H COSY experiments, four glucosidic proton–proton spin systems were disclosed. In addition to three anomeric protons at δ 4.90 (H-1′), 5.12 (H-1″), and 4.94 (H-1‴), which were in line with those observed in **9**, a fourth one resonated at δ 5.13 (H-1⁗). In the ^{13}C-NMR spectrum, the respective anomeric carbons were observed at δ 103.2 (C-1′), 105.5 (C-1″), 104.4 (C-1‴), and 105.6 (C-1⁗), as inferred by the HMQC experiment. The presence of ^{1}H–^{13}C long-range correlations of H-3 (δ 4.77)/C-1′, H_2-6′ (δ 4.33 and 4.68)/C-1″, together with H_2-27 (δ 4.83 and 5.09)/C-1‴ was also in agreement with those of **9**, implying the same connectivities. Further observation of HMBC cross-peaks between H_2-6‴ (δ 4.32, 4.85) and C-1⁗ accounted for the attachment of the fourth glucose at C-6‴. In conclusion, the structure of **10** was established to be 27-*O*-β-D-glucopyranosyl (1→6)-β-D-glucopyranosylpubesenolide 3-*O*-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside, and was called withanoside IX.

Compound **11** was assigned a molecular formula of $C_{44}H_{63}O_{12}$ by HR-FAB-MS. The ^{1}H- and ^{13}C-NMR data due to the aglycon part exhibited a close resemblance to those of **9** and **10**, revealing the same aglycon structure. In addition, the presence of two monosaccharide residues was manifested by β-anomeric proton signals at δ 5.04 (d, $J=8.0$ Hz, H-1′) and 5.05 (d, $J=7.5$ Hz, H-1‴), as well as anomeric carbon resonances at δ 102.8 (C-1′) and 104.9 (C-1‴). The two monosaccharide residues were determined to be glucose by comparison of the ^{1}H- and ^{13}C-NMR chemical shifts with those of **9** and **10**. Next, HMBC correlations of H-3 (δ 4.87)/C-1′, H-1′/C-3, H_2-27 (δ 4.83 and 5.04)/C-1‴, and H-1‴/C-27 (δ 63.5) suggested their linkage sites at C-3 and C-27, respectively. Based on the above evidence, **11** was determined to be 27-*O*-β-D-glucopyranosylpubesenolide 3-*O*-β-D-glucopyranoside, and called withanoside X. Bisdesmosidic glucosides, **9**—**11**, were isolated for the first time from the roots of *W. somnifera*.

Table 1. ^{1}H- and ^{13}C-NMR Data for **1** in C_5D_5N[a)]

No.	H	C	HMBC (C no.)
1		210.1 (s)	
2a	2.88, dd, J=18.0, 3.5 Hz	42.5 (t)	1, 3, 4
b	2.93, dd, J=18.0, 1.5 Hz		1, 3, 4
3	4.55, m	74.2 (d)	4, 10
4	4.86, d, J=6.5 Hz	77.9 (d)	2, 5, 10
5		76.9 (s)	
6	4.53, m	77.6 (d)	5, 8, 10
7α	1.40, dd, J=14.3, 11.0 Hz	32.9 (t)	5, 6, 8, 14
β	2.20, m		6, 8, 9, 11
8	1.90, m	31.3 (d)	7, 9, 14
9	1.81, m	41.1 (d)	8
10		55.3 (s)	
11a	1.62, td, J=13.3, 3.5 Hz	21.5 (t)	
b	1.74, m		
12a	0.94, m	39.6 (t)	11, 17, 18
b	1.84, m		9, 10
13		42.7 (s)	
14	0.89, m	58.0 (d)	7, 13, 16, 18
15a	0.96, m	24.3 (t)	
b	1.52, m		
16a	1.03, m	27.3 (t)	17, 20
b	1.01, m		13
17	1.01, m	51.7 (d)	
18	0.52, s	11.6 (q)	12, 13, 14, 17
19	1.83, s	16.0 (q)	1, 5, 9, 10
20	1.85, m	39.0 (d)	17
21	0.91, d, J=7.0 Hz	13.4 (q)	17, 20, 22
22	4.33, dt, J=13.5, 3.5 Hz	78.4 (d)	21, 23
23α	2.01, dd, J=17.5, 3.0 Hz	29.9 (t)	24, 25
β	2.32, dd, J=17.5, 13.0 Hz		20, 22, 24, 25
24		154.1 (s)	
25		127.2 (s)	
26		166.4 (s)	
27a	4.71, d, J=11.5 Hz	56.1 (t)	24, 25, 26
b	4.83, d, J=11.5 Hz		24, 25, 26
28	2.11, s	20.0 (q)	24, 25, 26

a) ^{1}H- and ^{13}C-NMR signals were assigned by ^{1}H–^{1}H COSY and HMQC experiments and comparison with the ^{1}H- and ^{13}C-NMR data of **6**.[7)]

Table 2. ^{13}C-NMR Data of Compounds **9**—**12** in C_5D_5N

	9[a)]	**10**[a)]	**11**[b)]	**12**[a)]		**9**[a)]	**10**[a)]	**11**[b)]	**12**[a)]
1	72.3	72.3	72.3	72.3	3-*O*-β-D-glucopyranosyl				
2	37.9	37.9	37.9	37.8	1′	103.3	103.2	102.8	102.8
3	74.4	74.3	73.9	73.8	2′	75.1	75.1	75.4[c)]	75.4
4	39.0	39.1	39.1	39.1	3′	78.4	78.4	78.6	78.6
5	139.3	139.3	139.3	139.1	4′	71.3	71.3	71.5	71.4
6	123.9	123.7	124.0	124.1	5′	76.9	76.9	78.3	78.3
7	32.2	32.2	32.3	32.2	6′	69.7	69.7	62.6	62.5
8	32.1	32.1	32.2	32.0	6′-*O*-β-D-glucopyranosyl				
9	41.4	41.1	41.6	41.5	1′	105.5	105.5		
10	42.0	42.0	42.2	42.2	2″	75.2	75.2		
11	20.5	20.4	20.6	20.5	3″	78.2	78.2		
12	39.1	39.6	39.7	40.1	4″	71.6	71.5		
13	42.8	42.8	42.9	43.1	5″	78.5	78.5		
14	56.3	56.3	56.4	56.1	6″	62.6	62.5		
15	27.2	27.2	27.2	24.4	27-*O*-β-D-glucopyranosyl				
16	24.5	24.5	24.6	22.5	1‴	104.9	104.4	104.9	
17	52.0	52.0	52.1	55.2	2‴	75.2	75.0	75.2[c)]	
18	11.7	11.7	11.8	14.1	3‴	78.5	78.5	78.6	
19	19.5	19.5	19.6	19.6	4‴	71.6	71.5	71.7	
20	39.0	39.0	39.1	74.8	5‴	78.6	77.3	78.7	
21	13.5	13.5	13.5	21.2	6‴	62.7	70.1	62.8	
22	78.2	78.2	78.3	81.9	6-*O*-β-D-glucopyranosyl				
23	29.8	29.8	29.9	31.6	1⁗		105.6		
24	157.0	157.2	156.9	154.1	2⁗		75.2		
25	123.9	123.7	123.8	127.2	3⁗		78.4		
26	166.0	166.0	166.0	166.2	4⁗		71.5		
27	63.4	63.2	63.5	57.0	5⁗		78.5		
28	20.5	20.6	20.5	20.1	6⁗		62.6		

a) 125 MHz for ^{13}C-NMR. *b*) 100 MHz for ^{13}C-NMR. *c*) Interchangeable within the same column.

Compound **12**, an amorphous powder, had the molecular formula of $C_{34}H_{52}O_{11}$, as disclosed by HR-FAB-MS (*m*/*z* 637.3611 $[M+H]^+$). Acid hydrolysis of **12** gave glucose. In contrast with compounds **9**—**11**, appearance of H_3-21 as a singlet in the ^{1}H-NMR spectrum and observation of a quaternary carbon (C-20) at δ 74.8 implied hydroxylation at C-20. Careful inspection of the 2D NMR (^{1}H–^{1}H COSY, NOESY, HMQC and HMBC) spectra allowed interpretation of the aglycon as (20*R*,22*R*)-1α,3β,20,27-tetrahydroxywitha-5,24-dienolide. In addition, the monoglucosidic nature was evident from anomeric signals [^{1}H-NMR: δ 5.04 (d, J=8.0 Hz); ^{13}C-NMR: δ 102.8]. The attachment of glucose at C-3 was corroborated by the HMBC correlation of H-1′/C-3 (δ 73.8). Therefore, compound **12** was determined to be (20*R*,22*R*)-1α,3β,20,27-tetrahydroxywitha-5,24-dienolide 3-*O*-β-D-glucopyranoside, and designated as withanoside XI.

Compounds **1**—**10** and **13**—**18** were evaluated for their effects on neurite outgrowth. Of these compounds, **1**, **2**, **6**, and **14**—**16** showed significant neurite outgrowth activity at 1 μM using a human neuroblastoma SH-SY5Y cell line. Further investigation of the mechanisms of action is now in progress.

Experimental

General Optical rotations were measured with a JASCO DIP-360 automatic polarimeter. IR spectra were measured using a Jasco FT/IR-230 Fourier Transform Infrared Spectrometer. NMR and 2D NMR spectra were recorded on Varian UNITY 500 and JNM-LA 400 WB Lambda (Jeol) NMR spectrometers. HR-FAB-MS spectra were performed with a Jeol JMS-700 mass spectrometer with a resolution of 5000, and glycerol as a matrix. Reversed-phase HPLC separations were carried out on a TSK-GEL ODS-80T$_S$ column (21.5×300 mm; eluent, CH_3OH/H_2O–0.1% trifluoroacetic acid (TFA); flow rate, 5.0 ml/min; UV detection, 210 nm). A human neuroblastoma cell line, SH-SY5Y, (Riken, Tsukuba, Japan) was used for neurite outgrowth bioassay. Minimum essential medium was purchased from GIBCO BRL, Rockville, U.S.A. A twenty-four-well culture dish was purchased from FALCON, Franklin Lakes, U.S.A.

Collection and Extraction Cultivated root material of *W. somnifera* was purchased in Jaipur, India in June 1999 and the botanical source was identified by Dr. K. Komatsu. A voucher specimen is deposited at the Museum of Toyama Medical and Pharmaceutical University (TMPW No. 19975). The crushed roots (1.8 kg) were refluxed with MeOH (1.5 l×3) to give an extract (206.9 g). A 186.0 g portion of the extract was dissolved in water (1.5 l) and extracted with $CHCl_3$ and *n*-BuOH (1.5 l×5) successively to give $CHCl_3$-soluble (20.7 g) and *n*-BuOH-soluble (34.6 g) fractions.

Isolation and Purification The chloroform-soluble fraction (15.0 g) was suspended in EtOH and centrifuged. The supernatant solution was subjected to Sephadex LH-20 column chromatography eluting with MeOH–H_2O (1 : 1), MeOH–H_2O (2 : 1), MeOH and EtOH (2 l), respectively. Similar eluting fractions and the precipitate were combined after TLC examination to provide five subfractions—I (1.21 g), II (6.52 g), III (4.30 g) IV (1.19 g) and V (0.55 g). Subfraction II was chromatographed on silica gel, Sephardex LH-20 and ODS to afford **2** (170 mg), **3** (1.5 mg), **4** (2.0 mg), **5** (4.0 mg), **7** (90 mg), **8** (9 mg). Finally, compound **1** (4.3 mg) was purified by preparative HPLC eluting with MeOH/0.1%TFA–H_2O (7 : 3). The *n*-BuOH-soluble fraction was chromatographed on a Diaion HP-20 column eluting with H_2O, MeOH–H_2O (3 : 7 and 3 : 2) and MeOH to furnish subfractions VI—IX (27.20 g, 2.82 g, 4.45 g and 0.14 g, respectively). Subfractions VII and VIII were further subjected to repeated chromatography on silica gel, Sephardex LH-20 and ODS. Compounds **9** (5.3 mg), **10** (6.5 mg), **11** (0.9 mg) and **12** (2.3 mg) were obtained by preparative HPLC from subfraction VII. RP-18 HPLC separation of subfraction VIII yielded compounds **6** (5.1 mg), **13** (9.8 mg), **14** (33.1 mg), **15** (41.6 mg), **16** (2.0 mg), **17** (7.6 mg), **18** (3.1 mg)

and **19** (1.1 mg).

Acetylatin of (20*S*,22*R*)-3α,6α-Epoxy-4β,5β,27-trihydroxy-1-oxowitha-24-enolide (1) Acetic anhydride (8 mg) was added to 1 ml of pyridine solution of **1** (2 mg) and the reaction mixture was stirred at room temperature for 24 h. The mixture was poured into cold water (2 ml) and extracted with $CHCl_3$ (5 ml×4). Finally, the product was purified by Sephardex LH-20 column chromatography (MeOH–H_2O, 3 : 1; 1.95 mg).

Acid Hydrolysis of Withanosides A solution of withanosides (**9**—**11**, **13**—**15**, **17**, **18**, 1 mg each) in 5% aq. H_2SO_4–dioxane (1 ml, 1 : 1, v/v) was refluxed for 3 h. After cooling, the reaction mixture was neutralized with 1 N NaOH and washed with $CHCl_3$. The remaining water layer was concentrated and subjected to TLC with authentic D-glucose.

Determination of D-Configuration of Glucose Compound **9** (1 mg) was refluxed with 5% aq. H_2SO_4–dioxane (1 : 1, 1 ml) for 3 h, neutralized with 1 N NaOH and extracted with $CHCl_3$. The residual water layer was desalted with Amberlite MB-3 and dried *in vacuo*. The residue was dissolved in pyridine (0.1 ml), then a pyridine solution (0.2 ml) of L-cysteine methyl ester hydrochloride (0.1 M) was added to the sugar solution. The mixture was kept at 60 °C for 1.5 h, dried *in vacuo*, and trimethylsilylated with hexamethyldisilazane–trimethylchlorosilane (HMDS-TMCS) (0.1 ml) at 60 °C for 1.0 h. After partition between hexane (0.3 ml) and H_2O (0.3 ml), the hexane extract was analyzed by GC-MS (column, DB-1, J & W Scientific, 0.25 mm i.d.×30 m; temperature, 50—230 °C, 15 °C/min then 230 °C, 18 min; carrier gas, He). The sugar derivatives showed the retention time of 21.55 min, identical with that of D-glucose. Under the same conditions, L-glucose derivative exhibited the retention time of 22.21 min.

Neurite Outgrowth Assay A human neuroblastoma cell line, SH-SY5Y, was incubated at a density of 5.5×10^4 cells/ml in a 24 well culture dish in minimum essential medium with 5% fetal bovine serum at 37 °C in a humidified atmosphere of 90% air/10% CO_2. The MeOH extract (5 μg/ml), individual compounds (1 μM), and the vehicle solution (0.1% DMSO) were added to the culture medium at the start of culture. Six days after treatment, cells (100—300 cells) were counted in four areas of 650×430 μm, and the percentage of cells with neurites longer than 50 μm was calculated. Statistical comparisons were made by Student's *t*-test with $p<0.05$ being considered as significant.

(20*S*,22*R*)-3α,6α-Epoxy-4β,5β,27-trihydroxy-1-oxowitha-24-enolide (**1**): An amorphous powder, $[\alpha]_D^{23}$ −17.4° (*c*=0.109, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3442, 2939, 1720, 1701, 1685, 1413, 1038; CD (MeOH) $[\theta]_{250}$ +12111 and $[\theta]_{290}$ −12683; HR-FAB-MS: *m/z* $[M+H]^+$ 489.2843 (Calcd for $C_{28}H_{41}O_7$, 489.2852), FAB-MS: *m/z* $[M+H]^+$ 489.3; ^{1}H- and ^{13}C-NMR data: see Table 1.

(20*S*,22*R*)-4β,27-Diacetoxy-3α,6α-epoxy-5β-hydroxy-1-oxowitha-24-enolide (**1a**): A white solid, $[\alpha]_D^{23}$ +27.4° (*c*=0.106, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3433, 2948, 1740, 1720, 1701, 1685, 1400, 1030; CD (MeOH) $[\theta]_{250}$ +7697 and $[\theta]_{290}$ −9942; HR-FAB-MS: *m/z* $[M+H]^+$ 573.3090 (Calcd for $C_{32}H_{45}O_9$, 573.3064), FAB-MS: *m/z* $[M+H]^+$ 573.3; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.70 (3H, s, H_3-18), 1.14 (3H, s, H_3-19), 1.20 (1H, m, H_a-7), 2.06 (3H, s, 4-OAc), 2.08 (3H, s, H_3-28), 2.09 (3H, s, 27-OAc), 2.18 (1H, m, H_b-7), 2.42 (1H, dd, *J*=18.4, 3.5 Hz, H_a-2), 2.75 (1H, dd, *J*=18.4, 1.7 Hz, H_b-2), 4.23 (1H, d-like, *J*=6.8 Hz, H-6), 4.42 (1H, dt, *J*=13.4, 3.4 Hz, H-22), 4.47 (1H, m, H-3), 4.86, 4.90 (2H, ABq, *J*=12.0 Hz, H_2-27), 5.24 (1H, d, *J*=6.6 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 208.7 (C-1), 42.0 (C-2), 71.1 (C-3), 80.4 (C-4), 75.9 (C-5), 77.2 (C-6), 31.8 (C-7), 30.6 (C-8), 41.0 (C-9), 55.5 (C-10), 20.7 (C-11), 39.2 (C-12), 42.8 (C-13), 57.8 (C-14), 24.1 (C-15), 27.4 (C-16), 51.7 (C-17), 11.7 (C-18), 14.6 (C-19), 38.9 (C-20), 13.3 (C-21), 78.2 (C-22), 30.1 (C-23), 157.0 (C-24), 121.9 (C-25), 165.3 (C-26), 58.0 (C-27), 20.7 (C-28), 170.9 (4-O–CO–CH_3), 20.9 (4-O–CO–CH_3), 172.4 (27-O–CO–CH_3), 20.6 (27-O–CO–CH_3).

Withanoside VIII (**9**): An amorphous powder, $[\alpha]_D^{23}$ +10.4° (*c*=0.264, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3406, 2924, 1721, 1685, 1469, 1413, 1075, 900, 800; HR-FAB-MS: *m/z* $[M+Na]^+$ 967.4473 (Calcd for $C_{46}H_{72}O_{20}Na$, 967.4515), FAB-MS *m/z* $[M+Na]^+$ 967.5, $[M+H]^+$ 945.6; ^{1}H-NMR (500 MHz, C_5D_5N) δ: 0.57 (3H, s, H_3-18), 0.88 (1H, m, H-14α), 0.91 (1H, m, H-17α), 0.94 (3H, d, *J*=6.5 Hz, H_3-21), 0.98 (3H, s, H_3-19), 1.01 (1H, m, H_a-12), 1.06 (1H, m, H_a-15), 1.34 (1H, t, *J*=13.5 Hz, H_a-11), 1.38 (1H, m, H-8β), 1.40 (1H, m, H_b-15), 1.44 (1H, m, H_a-16), 1.63 (1H, m, H_b-11), 1.65 (1H, m, H_a-7), 1.66 (1H, m, H_b-16), 1.82 (1H, d, *J*=12.0 Hz, H_b-12), 1.86 (1H, m, H-20β), 1.87 (1H, m, H_b-7), 1.98 (1H, dd, *J*=18.0, 3.0 Hz, H_a-23), 2.09 (3H, s, H_3-28), 2.12 (1H, m, H_a-2), 2.17 (1H, td, *J*=11.8, 4.5 Hz, H-9), 2.25 (1H, t, *J*=15.5 Hz, H_b-23), 2.62 (1H, t, *J*=12.0 Hz, H_a-4), 2.78 (1H, d, *J*=17.5 Hz, H_b-2), 2.81 (1H, d, *J*=13.5 Hz, H_b-4), 3.90 (1H, m, H-5′), 3.92 (1H, m, H-5″), 3.97 (1H, t, *J*=9.0 Hz, H-2′), 3.98 (1H, m, H-5‴), 4.03 (1H, m, H-2″), 4.04 (1H, m, H-1), 4.05 (1H, m, H-2‴), 4.16 (1H, t, *J*=9.0 Hz, H-3′), 4.21 (1H, m, H-4″), 4.22 (1H, m, H-3″), 4.23 (1H, m, H-4′), 4.25 (1H, m, H-4‴), 4.26 (1H, m, H-3‴), 4.32 (1H, m, H-22α), 4.34 (1H, m, H_a-6′), 4.37 (1H, m, H_a-6″), 4.41 (1H, m, H_a-6‴), 4.50 (1H, d, *J*=12.0 Hz, H_b-6″), 4.56 (1H, d, *J*=11.5 Hz, H_b-6‴), 4.68 (1H, d, *J*=11.5 Hz, H_b-6′), 4.77 (1H, m, H-3α), 4.82 (1H, d, *J*=11.0 Hz, H_a-27), 4.90 (1H, d, *J*=8.0 Hz, H-1′), 5.03 (1H, d, *J*=8.5 Hz, H-1‴), 5.04 (1H, d, *J*=11.0 Hz, H_b-27), 5.13 (1H, d, *J*=8.5 Hz, H-1″), 5.56 (1H, br s, H-6); ^{13}C-NMR: see Table 2.

Withanoside IX (**10**): An amorphous powder, $[\alpha]_D^{23}$ +16.7° (*c*=0.096, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3349, 2938, 1721, 1679, 1413, 1050, 911, 801, 606; HR-FAB-MS: *m/z* $[M+Na]^+$ 1129.5001 (Calcd for $C_{52}H_{82}O_{25}Na$, 1129.5043), FAB-MS: *m/z* $[M+Na]^+$ 1129.5, $[M+H]^+$ 1107.5; ^{1}H-NMR (500 MHz, C_5D_5N) δ: 0.58 (3H, s, H_3-18), 0.90 (1H, m, H-14), 0.92 (1H, m, H-17), 0.94 (3H, d, *J*=6.5 Hz, H_3-21), 0.97 (3H, s, H_3-19), 1.02 (1H, m, H_a-12), 1.06 (1H, m, H_a-15), 1.34 (1H, m, H_a-11), 1.37 (1H, m, H-8), 1.40 (1H, m, H_b-15), 1.45 (1H, m, H_a-16), 1.63 (1H, m, H_b-11), 1.65 (1H, m, H_a-7), 1.66 (1H, m, H-16), 1.82 (1H, m, H_b-12), 1.87 (1H, m, H-20), 1.89 (1H, m, H_b-7), 1.96 (1H, dd, *J*=2.5, 18.0 Hz, H_a-23), 2.17 (1H, m, H-9), 2.10 (1H, m, H_a-2), 2.12 (3H, s, H_3-28), 2.21 (1H, m, H_b-23), 2.78 (1H, d, *J*=16.5 Hz, H_b-2), 2.61 (1H, t, *J*=12.3 Hz, H_a-4), 2.81 (1H, d, *J*=14.5 Hz, H_b-4), 3.91 (1H, m, H-5′), 3.92 (1H, m, H-5″), 3.94 (1H, m, H-5‴), 3.96 (1H, t, *J*=8.5 Hz, H-2′), 4.00 (1H, t, *J*=7.5 Hz, H-2‴), 4.03 (1H, t, *J*=7.5 Hz, H-2″), 4.04 (1H, m, H-1), 4.06 (1H, m, H-2⁗), 4.07 (1H, m, H-5⁗), 4.13 (1H, t, *J*=8.5 Hz, H-4‴), 4.15 (1H, t, *J*=9.0 Hz, H-3′), 4.18 (1H, t, *J*=8.5 Hz, H-3‴), 4.21 (1H, t, *J*=9.5 Hz, H-4′), 4.22 (1H, m, H-4″), 4.23 (1H, m, H-3″), 4.24 (1H, m, H-4⁗), 4.25 (1H, m, H-3⁗), 4.28 (1H, dt, *J*=13.5, 3.0 Hz, H-22α), 4.32 (1H, dd, *J*=10.0, 6.0 Hz, H_a-6‴), 4.33 (1H, dd, *J*=12.0, 6.0 Hz, H_a-6′), 4.35 (1H, dd, *J*=12.0, 5.5 Hz, H_a-6″), 4.38 (1H, dd, *J*=11.5, 5.5 Hz, H_a-6⁗), 4.49 (1H, dd, *J*=13.3, 2.5 Hz, H_b-6″), 4.52 (1H, dd, *J*=11.5, 2.5 Hz, H_b-6⁗), 4.68 (1H, d, *J*=10.5 Hz, H_b-6′), 4.77 (1H, m, H-3α), 4.83 (1H, d, *J*=10.5 Hz, H_a-27), 4.84 (1H, d, *J*=10.0 Hz, H_b-6‴), 4.90 (1H, d, *J*=8.0 Hz, H-1′), 4.94 (1H, d, *J*=8.0 Hz, H-1‴), 5.09 (1H, d, *J*=10.5 Hz, H_b-27), 5.12 (1H, d, *J*=7.5 Hz, H-1″), 5.14 (1H, d, *J*=8.0 Hz, H-1⁗), 5.55 (1H, br s, H-6); ^{13}C-NMR: see Table 2.

Withanoside X (**11**): An amorphous powder, $[\alpha]_D^{23}$ +21.1° (*c*=0.11, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3421, 2935, 1700, 1685, 1076, 420; HR-FAB-MS *m/z* $[M+H]^+$ 783.4172 (Calcd for $C_{40}H_{63}O_{15}$, 783.4167), FAB-MS: *m/z* $[M+H]^+$ 783.5; ^{1}H-NMR (500 MHz, C_5D_5N) δ: 0.59 (3H, s, H_3-18), 0.90 (1H, m, H-14), 0.92 (1H, m, H-17), 0.94 (3H, d, *J*=6.0 Hz, H_3-21), 0.99 (3H, s, H_3-19), 1.02 (1H, m, H_a-12), 1.07 (1H, m, H_a-15), 1.39 (1H, m, H_a-11), 1.41 (1H, m, H-8), 1.42 (1H, m, H_b-15), 1.44 (1H, m, H_a-16), 1.63 (1H, m, H_b-11), 1.66 (1H, m, H_a-7), 1.67 (1H, m, H_b-16), 1.85 (1H, m, H_b-12), 1.87 (1H, m, H-20β), 1.90 (1H, m, H_b-7), 1.97 (1H, dd, *J*=18.0, 3.0 Hz, H_a-23), 2.09 (3H, s, H_3-28), 2.10 (1H, m, H_a-2), 2.18 (1H, td, *J*=4.0, 12.0 Hz, H-9), 2.23 (1H, m, H_b-23), 2.63 (1H, m, H_a-4), 2.66 (1H, m, H_b-2), 2.90 (1H, dd, *J*=13.5, 3.5 Hz, H_b-4), 3.84 (1H, dt, *J*=9.5, 4.0 Hz, H-5′), 3.98 (1H, m, H-5‴), 4.03 (1H, m, H-1), 4.07 (2H, m, H-2′ and H-2″), 4.26 (1H, m, H-3′), 4.27 (1H, m, H-4‴), 4.28 (1H, m, H-3‴), 4.32 (1H, dt, *J*=12.5, 3.5 Hz, H-22α), 4.34 (1H, t, *J*=9.0 Hz, H-4′), 4.34—4.41 (2H, m, H_2-6‴), 4.38 (1H, m, H_b-6′), 4.58 (1H, dd, *J*=11.8, 2.0 Hz, H_b-6′), 4.83 (1H, d, *J*=10.5 Hz, H_a-27), 4.87 (1H, m, H-3), 5.04 (1H, d, *J*=8.0 Hz, H-1′), 5.04 (1H, d, *J*=10.5 Hz, H_b-27), 5.05 (1H, d, *J*=7.5 Hz, H-1‴), 5.56 (1H, d, *J*=4.5 Hz, H-6); ^{13}C-NMR: see Table 2.

Withanoside XI (**12**): An amorphous powder, $[\alpha]_D^{23}$ +18.8° (*c*=0.101, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3411, 2938, 1696, 1685, 1388, 1076, 800; HR-FAB-MS: *m/z* $[M+H]^+$ 637.3611 (Calcd for $C_{34}H_{53}O_{11}$, 637.3588), FAB-MS: *m/z* $[M+H]^+$ 637.4; ^{1}H-NMR (500 MHz, C_5D_5N) δ: 0.98 (3H, s, H_3-19), 1.01 (1H, m, H-14), 1.04 (3H, s, H_3-18), 1.16 (1H, m, H_a-15), 1.30 (1H, td, *J*=13.0, 3.5 Hz, H_a-12), 1.39 (3H, s, H_3-21), 1.48 (1H, m, H_a-11), 1.57 (1H, m, H_b-15), 1.64 (1H, m, H-8), 1.65 (1H, m, H_a-16), 1.67 (2H, m, H_b-11 and H_a-7), 1.75 (1H, t, *J*=10.0 Hz, H-17), 1.89 (1H, m, H_b-7), 2.03 (1H, m, H_b-12), 2.09 (1H, m, H_a-2), 2.10 (3H, s, H_3-28), 2.18 (2H, m, H_b-16 and H-9), 2.34 (1H, dd, *J*=17.5, 3.5 Hz, H_a-23), 2.57 (1H, dd, *J*=17.5, 13.5 Hz, H_b-23), 2.63 (1H, m, H_a-4), 2.65 (1H, m, H_b-2), 2.88 (1H, dd, *J*=13.8, 3.5 Hz, H_b-4), 3.85 (1H, dt, *J*=9.5, 3.5 Hz, H-5′), 4.02 (1H, br s, H-1β), 4.05 (1H, t, *J*=8.5 Hz, H-2′), 4.26 (1H, t, *J*=9.0 Hz, H-3′), 4.34 (1H, t, *J*=9.5 Hz, H-4′), 4.41 (2H, br s, H_2-6′), 4.43 (1H, dd, *J*=9.5, 3.5 Hz, H-22α), 4.72 (1H, d, *J*=12.0 Hz, H_a-27), 4.85 (1H, d, *J*=12.0 Hz, H_b-27), 4.86 (1H, m, H-3), 5.04 (1H, d, *J*=8.0 Hz, H-1′), 5.55 (1H, d, *J*=5.5 Hz, H-6); ^{13}C-NMR: see Table 2.

References

1) Tohda C., Nakamura N., Komatsu K., Hattori M., *Biol. Pharm. Bull.*, **22**, 679—682 (1999).
2) Schliebs R., Liebmann A., Bhattacharya S. K., Kumar A., Ghosal S.,

Bigl V., *Neurochem. Int.*, **30**, 181—190 (1997).
3) Tohda C., Kuboyama T., Komatsu K., *Neuro Report*, **11**, 1981—1985 (2000).
4) Subramanian S. S., Sethi P. D., Glotter E., Kirson I., Lavie D., *Phytochemistry*, **10**, 685—688 (1971).
5) Kirson I., Glotter E., Lavie D., *J. Chem. Soc. (C)*, **1971**, 2032—2044 (1971).
6) Hansel R., Huang J. T., Rosenberg D., *Arch. Pharm.*, **308**, 653—654 (1975).
7) Neogi P., Kawai M., Butsugan Y., Mori Y., Suzuki M., *Bull. Chem. Soc. Jpn.*, **61**, 4479—4481 (1988).
8) Kuroyanagi M., Shibata K., Umehara K., *Chem. Pharm. Bull.*, **47**, 1646—1649 (1999).
9) Lavie D., Kirson I., Glotter E., *Israel J. Chem.*, **6**, 671—678 (1968).
10) Lavie D., Glotter E., Shvo Y., *J. Org. Chem.*, **30**, 1774—1778 (1965).
11) Matsuda H., Murakami T., Kishi A., Yoshikawa M., *Bioorg. Med. Chem.*, **9**, 1499—1507 (2001).
12) Atta-ur-Rahman, Shabbir M., Yousaf M., Qureshi S., e-Shahwar D., Naz A., Choudhary M. I., *Phytochemistry*, **52**, 1361—1364 (1999).
13) Shingu K., Yahara S., Nohara T., Okabe H., *Chem. Pharm. Bull.*, **40**, 2088—2091 (1992).
14) Atta-ur-Rahman, Abbas S., e-Shahwar D., Jamal S. A., Choudhary M. I., *J. Nat. Prod.*, **56**, 1000—1006 (1993).
15) Ma C. M., Nakamura N., Hattori M., *Chem. Pharm. Bull.*, **46**, 982—987 (1998).