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Structures of Withanosides I, II, III, IV, V, VI, and VII, New Withanolide Glycosides, from the Roots of Indian *Withania somnifera* DUNAL. and Inhibitory Activity for Tachyphylaxis to Clonidine in Isolated Guinea-Pig Ileum

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Abstract—Seven new withanolide glycosides called withanosides I, II, III, IV, V, VI, and VII were isolated from an Indian natural medicine, Ashwagandha, the roots of Indian *Withania somnifera* DUNAL. (Solanaceae), together with four known compounds, withaferin A, 5α,20α$_F$(*R*)-dihydroxy-6α,7α-epoxy-1-oxowitha-2,24-dienolide, physagulin D, and coagulin Q. The structures of withanosides I, II, III, IV, V, VI, and VII were determined based on chemical and physicochemical evidence. Principal constituents, withanoside VI (10 and 30 μM) and withaferin A (10 μM), attenuated the tachyphylaxis to clonidine on electrically stimulated guinea-pig ileum in vitro.

The roots and leaves of *Withania somnifera* DUNAL. (Solanaceae) have been used for nervous sedative, hypnotic, tonic, astringent, and aphrodisiac purposes in the Ayurvedic system of traditional Indian medicine.[1] The chemical constituents of this plant have been the targets of many investigations and the structures of many withanolides have been characterized.[2] Recently, it was reported that the extract from the roots of *W. somnifera* (Sanskrit name: Ashwagandha) did not show analgesic effect in mice and acute treatment with the extract did not affect the morphine-induced analgesia as well, while after chronic treatment of morphine for 10 days, it attenuated the development of tolerance and dependence on morphine in mice.[3] However, the active constituents of the root extract were not clarified.

As part of our continuing studies on bioactive constituents of natural medicines,[4] we isolated seven new withanolide glycosides termed withanosides I (**1**), II (**2**), III (**3**), IV (**4**), V (**5**), VI (**6**), and VII (**7**) from the roots of Indian *W. somnifera*. This paper describes the structure elucidation of these withanosides (**1**–**7**) based on chemical and physicochemical evidence.[5] In addition, two principal constituents, withanoside VI (**6**) and withaferin A (**8**), were found to attenuate tachyphylaxis to clonidine on electrically stimulated guinea-pig ileum in vitro.[6]

The methanolic extract from the roots of Indian *W. somnifera* was subjected to Diaion HP-20 column chromatography to give the glycosidic fraction (1.26%), which was subjected to normal-phase silica gel column chromatography to furnish four fractions (fr. 1–4). Fractions 1–4 were purified by reversed-phase silica gel column chromatography followed by HPLC to give withanosides I (**1**, 0.0020%), II (**2**, 0.012%), III (**3**, 0.0024%), IV (**4**, 0.048%), V (**5**, 0.017%), VI (**6**, 0.024%), and VII (**7**, 0.0011%) together with withaferin A (**8**,[7] 0.028%), 5α,20α$_F$(*R*)-dihydroxy-6α,7α-epoxy-1-oxowitha-2,24-dienolide (**9**,[2e] 0.0052%), physagulin D (**10**,[8] 0.0083%), and coagulin Q (**11**,[9] 0.0054%) (Charts 1 and 2).

Structures of Withanosides I (1), II (2), III (3), IV (4), V (5), VI (6), and VII (7)

In the positive-ion FAB–MS of withanolide I (**1**), a quasimolecular ion peak was observed at *m/z* 659 $(M+Na)^+$, and high-resolution MS analysis revealed the molecular formula of **1** to be $C_{34}H_{52}O_{11}$. The IR spectrum of **1** showed absorption bands at 1705 and 1655 cm^{-1} assignable to α,β-unsaturated δ-lactone and

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carbonyl functions and broad bands at 3432, 2942, and 1034 cm^{-1} suggestive of an oligoglycosidic structure. The UV spectrum of **1** showed absorption maxima at 227 nm (log ε 3.7), which suggested the presence of a α,β-unsaturated δ-lactone. The enzymatic hydrolysis of **1** with β-glucosidase furnished 1α,3β,5α-trihydroxy-6α,7α-epoxy-22*R*-with-24-enolide (**12**).[10] Acid hydrolysis of **1** with 5% aqueous sulfuric acid–dioxane (1:1, v/v) gave D-glucose, which was identified by GLC analysis of the trimethylsilyl thiazolidine derivatives.[11]

The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[12] of **1** showed signals assignable to a 1α,3β,5α-trihydroxy-6α,7α-epoxy-22*R*-with-24-enolide part [δ 0.65, 0.76, 1.84, 1.94 (all s, 18, 19, 28, 27-H_3), 0.98 (d, J = 6.6 Hz, 21-H_3), 2.93 (d, J = 3.6 Hz, 6-H), 3.17 (dd-like, 7-H), 3.79 (dd-like, 1-H), 4.19 (m, 22-H), 5.11 (m, 3-H)] together with a β-D-glucopyranosyl part [δ 5.06 (d, J = 7.6 Hz, 1′-H)]. The aglycone structure of **1** was also comfirmed by HMBC and NOESY experiments. Namely, long-range correlations were observed between

withanoside I (**1**)

withanoside II (**2**)

withanoside III (**3**)

withanoside IV (**4**)

withanoside V (**5**)

withanoside VI (**6**)

withanoside VII (**7**)

Chart 1.

the 18-protons and the 12-, 13-, 14-, and 17-carbons, between the 19-protons and the 1-, 5-, 9-, and 10-carbons, between the 27-protons and the 24-, 25-, and 26-carbons, and between the 28-protons and the 23-, 24-, and 25-carbons. NOE correlations were observed between the 6-proton and the 4β- and 8-protons and between the 8-proton and the 7-, 18-, and 19-protons. The position of D-glucose in **1** was clarified by HMBC experiment, which showed a long-range correlation between the 1′-proton and 3-carbon, and the assignment of anomeric proton was confirmed by H-H and H-C HOHAHA.[12] Moreover, the circular dichroism (CD) spectrum of **1** showed a positive Cotton effect at 248 nm, which indicated the absolute stereostructure of the 22-position to be *R* configuration.[13] Finally, by comparison of the NMR data for related withanolide oligoglycosides,[9] the structure of withanoside I was determined to be 1α,3β,5α-trihydroxy-6α,7α-epoxy-22*R*-with-24-enolide 3-*O*-β-D-glucopyranoside (**1**).

Withanoside II (**2**) liberated D-glucose by acid hydrolysis,[11] while **12**[10] was obtained by enzymatic hydrolysis of **2**. The IR and UV spectra of **2** was found to be similar to that of **1**. The positive-ion FAB–MS of **2** showed a quasimolecular ion peak at *m*/*z* 821 $(M+Na)^+$ and high-resolution MS analysis revealed the molecular formula of **2** to be $C_{40}H_{62}O_{16}$. The ^1H NMR (pyridine-d_5) spectrum of **2** showed signals due to the aglycone part [δ 0.64, 0.76, 1.84, 1.95 (all s, 18, 19, 28, 27-H_3), 0.98 (d, $J=6.7$ Hz, 21-H_3), 2.93 (d, $J=3.7$ Hz, 6-H), 3.16 (dd-like, 7-H), 3.81 (dd-like, 1-H), 3.90 (m, 22-H), 5.11 (m, 3-H)] and two β-D-glucopyranosyl moieties [δ 4.96 (d, $J=7.6$ Hz, 1′-H), 5.09 (d, $J=7.9$ Hz, 1″-H)]. Comparison of the ^{13}C NMR data (Table 1) for **2** with those for **1** revealed a glycosilation shift[14] around the 6′-position of the β-D-glucopyranosyl part in **1**. Furthermore, long-range correlations were observed between the 1′-proton and the 3-carbon and between the 1″-proton and the 6′-carbon in HMBC

withaferin A (**8**)

5α,20α$_F$(*R*)-dihydroxy-6α,7α-epoxy-1-oxowitha-2,24-dienolide (**9**)

physagulin D (**10**)

coagulin Q (**11**)

1α,3β,5α-trihydroxy-6α,7α-epoxy-22*R*-with-24-enolide (**12**) : R = H

withagenin A (**13**) : R = OH

	R^1	R^2	R^3
sominone (**14**) :	H	H	OH
20*S*,22*R*-1α,3β-dihydroxy-witha-5,24-dienolide (**15**) :	H	H	H
1α,3β,20α$_F$-trihydroxy-20*R*,22*R*-witha-5,24-dienolide (**16**) :	H	OH	H
withagenin B (**17**) :	OH	H	H

Chart 2.

experiment. This evidence led us to elucidate the structure of withanoside II (**2**) as shown.

The molecular formula $C_{34}H_{52}O_{12}$ of withanoside III (**3**) was determined from the negative- and positive-ion FAB–MS [*m*/*z* 651 $(M-H)^-$, *m*/*z* 675 $(M+Na)^+$] and by high-resolution MS measurement. The enzymatic hydrolysis of **3** with β-glucosidase provided a new withanolide, withagenin A (**13**). The ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra[12] of **13**, showed signals due to four methyls [δ 0.77, 0.84, 2.06 (all s, 18, 19, 28-H_3)], a secondary methyl [δ 1.02 (d, $J=6.7$ Hz, 21-H_3)], a hydroxymethylene [δ 4.40 (m, 27-H_2)], and five methines bearing a hydroxyl group [δ 2.98 (d, $J=3.7$ Hz, 6-H], 3.30 (br s, 7-H), 3.67 (br s, 1-H), 4.31 (m, 3-H)). The proton and carbon signals in the ^1H and ^{13}C NMR spectra of **13** were found to be very similar to those of **12**, except for the 27-hydroxymethylene protons in the δ-lactone moiety, while the proton and carbon signals due to the α,β-unsaturated δ-lactone moiety (C-20–C-28) of **13** was superimposable on those of **8** and **10**. In the HMBC experiment of **3**, long-range correlations were observed between the 27-protons and the 24-, 25-, and 26-carbons and between the 28-protons and the 23-, 24-, and 25-carbons (Fig. 1). In the NOESY experiment of **3**, NOE correlations were observed between the 6-proton and the 4β- and 7-protons and between the 8-proton and the 7-, 18-, and 19-protons (Fig. 2). Furthermore, the CD spectrum of **13** showed a positive Cotton effect at 250 nm, which indicated the 22*R* configuration. On the basis of the HMBC and NOESY data of **3** (Figs 1 and 2), the stereostructure of withagenin A was determined as 1α,3β,5α,27-tetrahydroxy-6α,7α-epoxy-22*R*-with-24-enolide (**13**). The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[12] of **3** showed signals due to a β-D-glucopyranosyl moiety [δ 5.07 (d, $J=7.6$ Hz, 1′-H)] together with the withagenin A part. In the HMBC experiment of **3**, a long-range correlation was observed between the 1′-proton and 3-carbon. Consequently, withanoside III was elucidated as withagenin A 3-*O*-β-D-glucopyranoside (**3**).

Table 1. ^{13}C NMR data for withanosides I (**1**), II (**2**), III (**3**), IV (**4**), V (**5**), VI (**6**), and VII (**7**) and withagenins A (**13**) and B (**17**)

	1[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[a]	**7**[a]	**13**[b]	**17**[b]
C-1	73.3	73.4	73.3	72.4	72.4	72.4	72.5	72.9	72.8
C-2	38.1	38.0	38.1	37.8	37.8	37.8	37.8	38.7	38.1
C-3	71.7	72.2	71.7	74.5	74.5	74.4	73.7	63.8	64.9
C-4	39.4	39.4	39.4	39.2	39.1	39.1	39.5	41.2	41.4
C-5	74.3	74.3	74.3	139.3	139.2	139.2	143.0	73.9	143.0
C-6	57.8	57.8	57.8	124.0	124.0	124.1	127.9	58.0	127.2
C-7	56.2	56.2	56.2	32.2	32.2	32.1	64.8	57.8	64.8
C-8	36.4	36.3	36.3	32.2	32.2	31.7	38.2	35.8	37.4
C-9	35.4	35.3	35.4	41.5	41.5	41.4	34.1	34.9	41.3
C-10	40.6	40.5	40.6	42.0	42.0	42.1	42.7	39.4	42.6
C-11	20.7	20.6	20.7	20.6	20.5	20.5	20.4	20.2	20.1
C-12	40.0	39.9	39.9	39.8	39.7	40.2	38.9	39.9	38.8
C-13	44.0	44.0	44.0	42.9	42.9	43.2	42.9	44.0	42.9
C-14	51.5	51.5	51.5	56.4	56.4	57.0	49.8	51.2	49.3
C-15	27.5	27.5	27.4	27.3	27.3	24.4	27.6	27.5	27.3
C-16	23.6	23.6	23.6	24.6	24.6	22.5	24.7	23.4	24.2
C-17	52.0	52.0	51.9	52.2	52.2	55.2	52.4	51.7	52.0
C-18	12.1	12.1	12.1	11.8	11.8	14.0	11.8	12.0	11.5
C-19	16.3	16.3	16.3	19.6	19.6	19.6	18.3	16.1	13.6
C-20	39.3	39.3	39.2	39.2	39.2	79.7	39.4	38.9	39.0
C-21	13.4	13.4	13.4	13.5	13.5	21.3	13.6	13.3	20.6
C-22	78.6	78.4	78.6	78.5	78.5	81.9	78.6	78.7	78.4
C-23	29.6	29.7	30.0	30.0	29.6	31.8	29.6	29.8	33.6
C-24	149.7	149.8	153.8	154.2	149.8	149.6	149.7	152.8	149.0
C-25	121.9	121.9	127.4	127.2	121.8	121.7	121.9	125.7	122.0
C-26	166.6	166.9	166.3	166.5	166.9	166.5	166.6	167.0	166.2
C-27	12.7	12.7	56.3	56.1	12.7	12.6	12.7	57.5	12.5
C-28	20.1	20.2	20.1	20.1	20.2	20.1	20.2	20.0	20.7
Glc-1′	102.8	103.2	102.9	103.2	103.2	103.1	102.9		
2′	75.3	75.1	75.4	75.0	74.9	75.0	75.2		
3′	78.4	78.3	78.4	78.2	78.1	78.2	78.3		
4′	71.7	71.5	71.7	71.4	71.3	71.4	71.7		
5′	78.3	77.2	78.4	76.9	76.8	76.8	76.8		
6′	62.7	70.0	62.7	69.8	69.7	69.8	70.2		
Glc-1″		105.3		105.2	105.1	105.2	105.3		
2″		75.1		75.0	75.0	75.0	75.2		
3″		78.2		78.1	78.0	78.2	78.1		
4″		71.7		71.6	71.5	71.6	71.8		
5″		78.4		78.2	78.2	78.2	78.4		
6″		62.7		62.6	62.6	62.6	62.7		

68 or 125 MHz.
[a]pyridine-d_5.
[b]$CDCl_3$.

Withanoside IV (**4**) liberated sominone (**14**)[15] by enzymatic hydrolysis with β-glucosidase, while D-glucose was detected by acid hydrolysis.[11] The IR spectrum of **4** showed absorption bands at 3422, 1692, and 1655 cm^{-1} due to hydroxyl and α,β-unsaturated δ-lactone carbonyl functions. In the negative- and positive-ion FAB–MS of **4**, quasimolecular ion peaks were observed at *m*/*z* 781 $(M-H)^-$, 783 $(M+H)^+$, and 805 $(M+Na)^+$ and high-resolution MS analysis revealed the molecular formula of **4** to be $C_{40}H_{62}O_{15}$. The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[12] of **4** showed signals due to a sominone pant [δ 0.63, 1.01, 2.15 (all s, 18, 19, 28-H_3), 0.99 (d, $J=6.9$ Hz, 21-H_3), 4.05 (dd-like, 1-H), 4.38 (m,

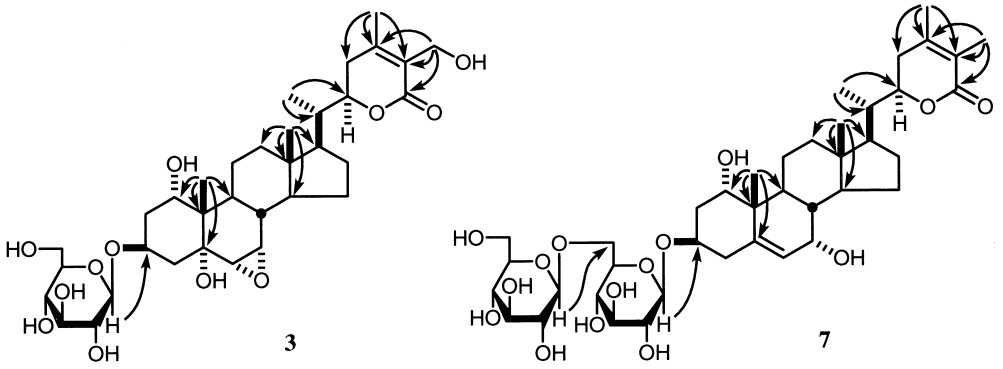

Figure 1. HMBC correlations of withanosides III (**3**) and VII (**7**).

22-H), 4.74 (m, 3-H), 4.73, 4.89 (both d, $J=11.6$ Hz, 27-H_2), 5.59 (dd-like, 6-H)] and a β-D-glucopyranosyl moiety [δ 4.89 (d, $J=7.6$ Hz, 1′-H), 5.08 (d, $J=7.6$ Hz, 1″-H)]. The HMBC experiment of **4** showed long-range correlations between the following protons and carbons: the 1′-proton and the 3-carbon; the 1″-proton and the 6′-carbon. Consequently, withanoside IV was elucidated as sominone 3-*O*-[β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside (**4**).

Withanoside V (**5**) showed absorption bands ascribable to hydroxyl and carbonyl functions at 3432, 2938, and 1698 cm^{-1}. The positive- and negative-ion FAB–MS of **5** showed quasimolecular ion peaks at *m/z* 765 $(M-H)^-$, 767 $(M+H)^+$, and 789 $(M+Na)^+$, and high-resolution MS analysis revealed the molecular formula of **5** to be $C_{40}H_{62}O_{14}$. Enzymatic hydrolysis of **5** liberated 20*S*,22*R*-1α,3β-dihydroxy-witha-5,24-dienolide (**15**),[16] while D-glucose was detected by acid hydrolysis.[11] The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[12] of **5** showed signals assignable to a 20*S*,22*R*-1α,3β-dihydroxy-witha-5,24-dienolide part [δ 0.64, 1.00, 1.87, 1.94 (all s, 18, 19, 28, 27-H_3), 1.00 (d-like, 21-H_3), 4.06 (m, 1-H), 4.12 (m, 22-H), 4.68 (m, 3-H), 5.59 (br s, 6-H)] and two β-D-glucopyranosyl parts (δ 4.88 (d, $J=7.6$ Hz, 1′-H), 5.04 (d, $J=7.6$ Hz, 1″-H)). The HMBC experiment on **5** showed long-range correlations between the 1′-proton and the 3-carbon and between the 1″-proton and the 6′-carbon. Consequently, withanoside VI was elucidated as 20*S*,22*R*-1α,3β-dihydroxy-witha-5,24-dienolide 3-*O*-[β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside (**5**).

Withanoside VI (**6**) liberated 1α,3β,20α_F-trihydroxy-20*R*,22*R*-witha-5,24-dienolide (**16**)[17] and D-glucose[11] by enzymatic hydrolysis or acid hydrolysis. The molecular formula $C_{40}H_{62}O_{15}$ of **6** was clarified from a quasimolecular ion peaks observed in negative- and positive-ion FAB–MS [*m/z* 781 $(M-H)^-$, *m/z* 805 $(M+Na)^+$] and by high-resolution MS analysis. The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[12] of **6** showed the presence of two β-D-glucopyranosyl moieties [δ 4.88 (d, $J=7.6$ Hz, 1′-H), 5.06 (d, $J=7.6$ Hz, 1″-H)] together with an aglycone moiety [δ 1.00, 1.07, 1.43, 1.85, 1.92 (all s, 19, 18, 21, 28, 27-H_3), 4.03 (m, 1-H), 4.37 (m, 22-H), 4.72 (m, 3-H), 5.58 (dd-like, 6-H)]. The glycosidic structure of **6** was characterized by the HMBC experiment, in which long-range correlations were observed between the 1′-proton and the 3-carbon and between the 1″-proton and the 6′-carbon. The carbon signals in the ^{13}C NMR spectrum of **6** closely resembled those of **10**,[8] except for the signals due to the 6′-*O*-β-D-glucopyranosyl moiety of **6**. On the basis of this evidence, withanoside VI was elucidated as 1α,3β,20α_F-trihydroxy-20*R*,22*R*-witha-5,24-dienolide 3-*O*-[β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside (**6**).

Withanolide VII (**7**) liberated D-glucose by acid hydrolysis.[11] The IR spectrum of **7** showed absorption bands at 3425, 1701, and 1655 cm^{-1} due to hydroxyl and α,β-unsaturated δ-lactone functions. In the negative- and positive-ion FAB–MS of **7**, quasimolecular ion peaks were observed at *m/z* 781 $(M-H)^-$ and 805 $(M+Na)^+$, respectively, and high-resolution MS analysis revealed the molecular formula of **7** to be $C_{40}H_{62}O_{15}$. The enzymatic hydrolysis of **7** with β-glucosidase furnished withagenin B (**17**). The ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra[12] of **17**, showed signals due to four tertiary methyls [δ 0.72, 1.01, 1.88, 1.94 (all s, 18, 19, 27, 28-H_3)], a secondary methyl [δ 1.02 (d, $J=7.0$ Hz, 21-H_3)], and four methines bearing a hydroxyl group [δ 3.65 (br s, 7-H), 3.85 (dt-like, 1-H), 4.04 (dd-like, 3-H), 4.38 (dd-like, 22-H)]. The proton and carbon signals in the ^1H and ^{13}C NMR spectra of **17** were found to be very similar to those of 20*S*,22*R*-1α,3β-dihydroxy-witha-5,24-dienolide (**15**), except for the 6-position in **17.** In the HMBC experiment of **7**, long-range correlations were observed between the 18-protons and the 12-, 13-, 14-, and 17-carbons, between the 19-protons and the 1-, 5-, 9-, and 10-carbons, between the 21-protons and the 17-, 20-, and 22-carbons, between the 27-protons and the 24-, 25-, and 26-carbons, and between the 28-protons and the 23-, 24-, 25-carbons (Fig. 1). Furthermore, in the NOESY experiment of **7**, NOE correlations were observed between the 19-protons and the 1- and 4β-protons and between the 8-protons and the 7-, 18-, and 19-protons. The CD spectrum of **17** showed a positive Cotton effect at 245 nm to confirm a 22*R* configuration. On the basis of above mentioned evidence, the stereostructure of withagenin B was determined as 1α,3β,7α-trihydroxy-20*S*,22*R*-witha-5,24-dienolide (**17**). The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[12] of **7** showed signals due to two β-D-glucopyranosyl moieties [δ 4.90 (d, $J=7.7$ Hz, 1′-H), 5.13 (d, $J=7.6$ Hz, 1″-H)] together with the withagenin B part. The HMBC experiment of **7** showed long-range

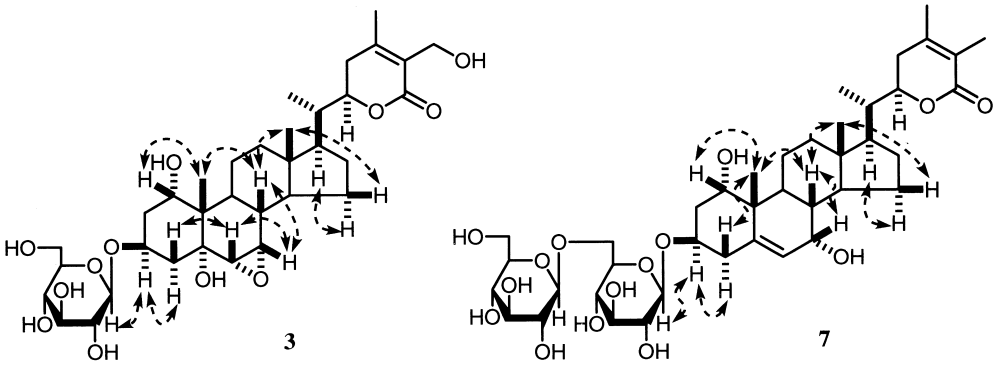

Figure 2. NOE correlations of withanosides III (**3**) and VII (**7**).

correlations between the 1′-proton and the 3-carbon and between the 1″-proton and the 6′-carbon. Consequently, withanoside VII was elucidated as withagenin B 3-*O*-[β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside (**7**).

Effects of Withanoside VI (6) and Withaferin A (8) on the Tachyphylaxis to Clonidine

Clonidine (0.3 to 100 nM) produced a concentration-dependent inhibition of twitch responses on electrically stimulated guinea-pig ileum. The EC_{50} value of clonidine was 4.2 nM. After 90-min incubation with clonidine (10 nM) that gave an initial reduction in twitch height of 69 ± 11% ($n = 5$), their effective concentrations in the first treatment were less effective to the twitch responses. Namely, EC_{50} value shifted to 15 nM (Table 2).

Withanoside VI (**6**, 10 and 30 μM) and withaferin A (**8**, 10 μM), when applied 10 min before the second dose–response curve of clonidine in preparations without the 90-min incubation with clonidine, did not show any significant effect on the action of clonidine. While, when **6** and **8** were incubated with clonidine for 90 min, they shifted the dose–responses curve of clonidine left nearly to the normal level (Table 2).

It has been noted that the sensitivity of isolated smooth muscle could serve as a reliable index to assess the tolerance and development of morphine.[18] Clonidine, an α_2-adrenoceptor agonist, is well-known to be a hypotensive drug. Apart from this, clonidine also showed a specific action on the myenteric plexus. Acute treatment with clonidine in vitro caused a concentration-dependent inhibition of the twitch responses on electrically stimulated guinea-pig ileum by means of inhibiting the release of ACh via acting on α_2-adrenoceptors in these tissues.[19] Chronic treatment with clonidine in vitro induced dependence (withdrawal) on the preparations.[20] In agreement with these previous studies, the present experiments demonstrated that treatment of clonidine for 90 min in isolated guinea-pig ileum induces desensitization to the inhibitory effect of clonidine.

Since opioid receptor and α_2-adrenoceptor share a common second messenger signal, such as c-AMP resulting from adenylate cyclase activity, they act via the same intracellular effector process. Therefore, an alteration in the intracellular effector linked to the opioid receptor could result in cross-tolerance to α_2-adrenoceptor agonist, and vice vasa. It was reported that mice pretreated with clonidine showed a great cross-tolerance to morphine, and morphine pretreatment also caused desensitization to morphine itself along with cross-tolerance to clonidine.[21]

In the present study, withanoside VI (**6**, 10 and 30 μM) and withaferin A (**8**, 10 μM) did not show any significant effect on acute clonidine treatment, while they attenuated the desensitization to clonidine after treatment of clonidine for 90 min. These results suggest that **6** and **8** can inhibit the development of tolerance to clonidine and could explain the effect of *W. somnifera* on morphine-tolerance and dependence.

Table 2. Effects of withanoside VI (**6**) and withaferin A (**8**) on twitch responses induced by electrical stimulation in 90-min clonidine-treated ileum

Treatment	Concn (μM)	Clonidine treatment EC_{50} (nM)[a]	Clonidine untreatment EC_{50} (nM)[b]
Control (Vehicle)	—	15.0 ± 4.7	4.2 ± 0.2
Withanoside VI (**6**)	10	4.2 ± 1.7**	5.0 ± 1.7
	30	3.0 ± 1.3**	4.6 ± 0.3
Withaferin A (**8**)	10	8.3 ± 1.1**	4.3 ± 1.2
	30	3.1 ± 0.7**	3.0 ± 0.3**

Each value represents the mean ± SEM (**$p < 0.01$, $n = 4$–6).
[a]EC_{50} of clonidine after treatment with 10 nM clonidine for 90 min and test sample.
[b]Test sample was added to the organ bath 10 min before second dose-response curve of clonidine in preparations without 90-min incubation of clonidine.

Experimental

The following instruments were used to obtain physical data:specific rotations, Horiba SEPA-300 digital polarimeter ($l = 5$ cm); UV spectra, Shimadzu UV-1200 spectrometer; CD spectra; J-720WI spectropolarimeter; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H NMR spectra, JEOL EX-270 (270 MHz) and JNM-LA500 (500 MHz) spectrometers; ^{13}C NMR spectra, JEOL EX-270 (68 MHz) and JNM-LA500 (125 MHz) spectrometers with tetramethylsilane as an internal standard; MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer and JMS-GCMATE; HPLC, Shimadzu LC-10AS chromatograph.

The following experimental conditions were used for chromatography: normal-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh):TLC, pre-coated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm) (normal-phase) and Silica gel RP-18 $60F_{254}$ (Merck, 0.25 mm) (reversed-phase); HPTLC, pre-coated TLC plates with Silica gel RP-18 $60WF_{254s}$ (Merck, 0.25 mm) (reversed-phase). Detection was done by spraying with 1% aqueous $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

Isolation of withanosides I (1), II (2), III (3), IV (4), V (5), VI (6), and VII (7) and known compounds (8–11) from the roots of *W. somnifera* DUNAL. The roots of *W. somnifera* DUNAL. (2.1 kg, purchased in Bonbay, India) was finely cut and extracted three times with MeOH under reflux. Evaporation of the solvent under reduced pressure provided the MeOH extract (210 g, 10.0%), and the MeOH extract (209 g) was subjected to Diaion HP-20 column chromatography [2.8 kg (Nippon Rensou Co.), $H_2O \rightarrow MeOH \rightarrow CHCl_3$] to give the H_2O eluate, MeOH eluate (26.3 g, 1.26%), and $CHCl_3$ eluate (9.7 g, 0.46%). Normal-phase silica gel column chromatography [BW-200 (Fuji Silysia Chemical Ltd., 750 g), $CHCl_3$–MeOH–H_2O (30:3:1, lower layer→15:3:1, lower

layer→10:3:1, lower layer →7:3:1, lower layer)→MeOH] of the MeOH eluate provided four fractions [Fr. 1 (4.5 g), Fr. 2 (3.2 g), Fr. 3 (7.5 g), Fr. 4 (9.2 g)]. Fraction 1 (4.1 g) was purified by reversed-phase silica gel column chromatography [Chromatorex DM1020T (Fuji Silysia Chemical Ltd., 123 g), MeOH–H_2O (50:50→70:30→80:20, v/v)→MeOH] and HPLC [YMC-Pack ODS-A (250×20 mm i.d.), MeOH–H_2O (65:35, v/v)] to give withaferin A (**8**, 163.2 mg, 0.028%) and 5α,20α$_F$(*R*)-dihydroxy-6α,7α-epoxy-1-oxowitha-2,24-dienolide (**9**, 30.0 mg, 0.0052%). Fraction 2 (3.0 g) was separated by reversed-phase silica gel column chromatography [90 g, MeOH–H_2O (50:50→60:40→70:30, v/v)→MeOH] and HPLC [(1) MeOH–H_2O (65:35, v/v), (2) MeOH–H_2O (60:40, v/v), (3) CH_3CN–H_2O (35:65, v/v)] to furnish withanosides I (**1**, 37.6 mg, 0.0020%) and III (**3**, 23.3 mg, 0.0024%), **8** (16.1 mg, 0.00086%), physagulin D (**10**, 155.5 mg, 0.0083%), and coagulin Q (**11**, 101.3 mg, 0.0054%). Fraction 3 (7.0 g) was subjected to reversed-phase silica gel column chromatography [210 g, MeOH–H_2O (50:50→60:40→70:30, v/v)→MeOH] and HPLC [(1) MeOH–H_2O (65:35, v/v), (2) CH_3CN–H_2O (35:65, v/v), (3) CH_3CN–H_2O (40:60, v/v)] to give withanosides II (**2**, 40.0 mg, 0.012%), IV (**4**, 160.9 mg, 0.048%), V (**5**, 60.3 mg, 0.017%), VI (**6**, 326.3 mg, 0.024%), VII (**7**, 19.3 mg, 0.0011%). The known (**8**–**11**) were identified by comparison of their physical data ($[\alpha]_D$, IR, ^{1}H NMR, ^{13}C NMR) with reported values.[2e,7–9]

Withanoside I (**1**): A white powder, $[\alpha]_D^{28}$ +48.6° (*c* = 0.1, MeOH). High-resolution positive-ion FAB–MS: calcd for $C_{34}H_{52}O_{11}Na$ $(M+Na)^+$: 659.3407. Found: 659.3419. CD (*c* = 0.0041, MeOH) Δε (nm): +5.8 (248) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 227 (3.7). IR (KBr): 3432, 2942, 1705, 1655, 1306, 1034 cm^{-1}. ^{1}H NMR (270 MHz, pyridine-d_5) δ: 0.65, 0.76, 1.84, 1.94 (3H each, all s, 18, 19, 28, 27-H_3), 0.98 (3H, d, *J* = 6.6 Hz, 21-H_3), 2.93 (1H, d, *J* = 3.6 Hz, 6-H), 3.17 (1H, dd-like, 7-H), 3.79 (1H, dd-like, 1-H), 4.19 (1H, m, 22-H), 5.06 (1H, d, *J* = 7.6 Hz, 1′-H), 5.11 (1H, m, 3-H). ^{13}C NMR (68 MHz, pyridine-d_5) δc: given in Table 1. Negative-ion FAB–MS: *m*/*z* 635 $(M-H)^-$, 473 $(M-C_6H_{11}O_5)^-$. Positive-ion FAB–MS: *m*/*z* 659 $(M+Na)^+$.

Withanoside II (**2**): A white powder, $[\alpha]_D^{28}$ −9.6° (*c* = 0.6, MeOH). High-resolution positive-ion FAB–MS: calcd for $C_{40}H_{62}O_{16}Na$ $(M+Na)^+$: 821.3936. Found: 821.3948. CD (*c* = 0.0056, MeOH) Δε (nm): +3.7 (247) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 227 (3.8). IR (KBr): 3451, 2938, 1701, 1655, 1385, 1044 cm^{-1}. ^{1}H NMR (500 MHz, pyridine-d_5) δ: 0.64, 0.76, 1.84, 1.95 (3H each, all s, 18, 19, 28, 27-H_3), 0.98 (3H, d, *J* = 6.7 Hz, 21-H_3), 2.93 (1H, d, *J* = 3.7 Hz, 6-H), 3.16 (1H, dd-like, 7-H), 3.81 (1H, dd-like, 1-H), 4.16 (1H, m, 22-H), 4.96 (1H, d, *J* = 7.6 Hz, 1′-H), 5.09 (1H, d, *J* = 7.9 Hz, 1′-H), 5.11 (1H, m, 3-H). ^{13}C NMR (125 MHz, pyridine-d_5) δc: given in Table 1. Negative-ion FAB–MS: *m*/*z* 797 $(M-H)^-$, 635 $(M-C_6H_{11}O_5)^-$, 473 $(M-C_{12}H_{21}O_{10})^-$. Positive-ion FAB–MS: *m*/*z* 821 $(M+Na)^+$.

Withanoside III (**3**): A white powder, $[\alpha]_D^{28}$ −24.0° (*c* = 0.1, MeOH). High-resolution positive-ion FAB–MS: calcd for $C_{34}H_{52}O_{12}Na$ $(M+Na)^+$: 675.3356. Found: 675.3367. CD (*c* = 0.0031, MeOH) Δε (nm): +1.9 (252) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 228 (3.7). IR (KBr): 3430, 2936, 1695, 1655, 1365, 1043 cm^{-1}. ^{1}H NMR (270 MHz, pyridine-d_5) δ: 0.64, 0.76, 2.12 (3H each, all s, 18, 19, 28-H_3), 0.96 (3H, d, *J* = 6.9 Hz, 21-H_3), 2.93 (1H, d, *J* = 3.7 Hz, 6-H), 3.17 (1H, dd-like, 7-H), 3.79 (1H, dd-like, 1-H), 4.22 (1H, m, 22-H), 4.74, 4.84 (1H each, both d, *J* = 11.5 Hz, 27-H_2), 5.07 (1H, d, *J* = 7.6 Hz, 1′-H), 5.17 (1H, m, 3-H). ^{13}C NMR (68 MHz, pyridine-d_5) δc: given in Table 1. Negative-ion FAB–MS: *m*/*z* 651 $(M-H)^-$. Positive-ion FAB–MS: *m*/*z* 675 $(M+Na)^+$.

Withanoside IV (**4**): A white powder, $[\alpha]_D^{28}$ +5.2° (*c* = 0.2, MeOH). High-resolution positive-ion FAB–MS: calcd for $C_{40}H_{62}O_{15}Na$ $(M+Na)^+$: 805.3986. Found: 805.3977. CD (*c* = 0.0053, MeOH) [Δε] (nm): +5.2 (255) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 228 (3.7). IR (KBr): 3422, 2930, 1692, 1655, 1420, 1042 cm^{-1}. ^{1}H NMR (270 MHz, pyridine-d_5) δ: 0.63, 1.01, 2.15 (3H each, all s, 18, 19, 28-H_3), 0.99 (3H, d, *J* = 6.9 Hz, 21-H_3), 4.05 (1H, dd-like, 1-H), 4.38 (1H, m, 22-H), 4.74 (1H, m, 3-H), 4.73, 4.84 (1H each, both d, *J* = 11.6 Hz, 27-H_2), 4.89 (1H, d, *J* = 7.6 Hz, 1′-H), 5.08 (1H, d, *J* = 7.6 Hz, 1″-H), 5.59 (1H, dd-like 6-H). ^{13}C NMR (68 MHz, pyridine-d_5) δc: given in Table 1. Negative-ion FAB–MS: *m*/*z* 781 $(M-H)^-$. Positive-ion FAB–MS: *m*/*z* 783 $(M+H)^+$, 805 $(M+Na)^+$.

Withanoside V (**5**): A white powder, $[\alpha]_D^{28}$ +7.8° (*c* = 0.3, MeOH). High-resolution positive-ion FAB–MS: calcd for $C_{40}H_{62}O_{14}Na$ $(M+Na)^+$: 789.4037. Found: 789.4053. CD (*c* = 0.0052, MeOH) Δε (nm): +5.0 (252) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 227 (3.7). IR (KBr): 3432, 2938, 1698, 1655, 1385, 1044 cm^{-1}. ^{1}H NMR (270 MHz, pyridine-d_5) δ: 0.64, 1.00, 1.87, 1.94 (3H each, all s, 18, 19, 28, 27-H_3), 1.00 (3H, d-like, 21-H_3), 4.06 (1H, m, 1-H), 4.12 (1H, m, 22-H), 4.68 (1H, m, 3-H), 4.88 (1H, d, *J* = 7.6 Hz, 1′-H), 5.04 (1H, d, *J* = 7.6 Hz, 1″-H), 5.59 (1H, dd-like, 6-H). ^{13}C NMR (68 MHz, pyridine-d_5) δc: given in Table 1. Negative-ion FAB–MS: *m*/*z* 765 $(M-H)^-$. Positive-ion FAB–MS: *m*/*z* 767 $(M+H)^+$, 789 $(M+Na)^+$.

Withanoside VI (**6**): A white powder, $[\alpha]_D^{27}$ −11.6° (*c* = 0.5, MeOH). High-resolution positive-ion FAB–MS: calcd for $C_{40}H_{62}O_{15}Na$ $(M+Na)^+$: 805.3986. Found: 805.4005. CD (*c* = 0.0065, MeOH) Δε (nm): +4.2 (242) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 228 (3.8). IR (KBr): 3425, 2936, 1690, 1655, 1385, 1043 cm^{-1}. ^{1}H NMR (270 MHz, pyridine-d_5) δ: 1.00, 1.07, 1.43, 1.85, 1.92 (3H each, all s, 19, 18, 21, 28, 27-H_3), 4.03 (1H, m, 1-H), 4.37 (1H, m, 22-H), 4.72 (1H, m, 3-H), 4.88 (1H, d, *J* = 7.6 Hz, 1′-H), 5.06 (1H, d, *J* = 7.6 Hz, 1″-H), 5.58 (1H, dd-like, 6-H). ^{13}C NMR (68 MHz, pyridine-d_5) δc: given in Table 1. Negative-ion FAB–MS: *m*/*z* 781 $(M-H)^-$. Positive-ion FAB–MS: *m*/*z* 805 $(M+Na)^+$.

Withanoside VII (**7**): A white powder, $[\alpha]_D^{29}$ +5.0° (*c* = 0.1, MeOH). High-resolution positive-ion FAB–MS: calcd for $C_{40}H_{62}O_{15}Na$ $(M+Na)^+$: 805.3986. Found:

805.3973. CD ($c=0.0085$, MeOH) $\Delta\varepsilon$ (nm): +4.9 (248) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 228 (3.7). IR (KBr): 3425, 2928, 1701, 1655, 1405, 1038 cm^{-1}. ^1H NMR (500 MHz, pyridine-d_5) δ: 0.69, 1.00, 1.84, 1.94 (3H each, all s, 18, 19, 28, 27-H_3), 1.01 (3H, d, $J=7.0$ Hz, 21-H_3), 4.02 (1H, m, 7-H), 4.09 (1H, dd-like, 1-H), 4.17 (1H, m, 22-H), 4.79 (1H, m, 3-H), 4.90 (1H, d, $J=7.7$ Hz, 1′-H), 5.13 (1H, d, $J=7.6$ Hz, 1″-H), 5.97 (1H, d, $J=4.0$ Hz, 6-H). ^{13}C NMR (125 MHz, pyridine-d_5) δc: given in Table 1. Negative-ion FAB–MS: m/z 781 $(M-H)^-$. Positive-ion FAB–MS: m/z 805 $(M+Na)^+$.

Acid hydrolysis of withanosides I (1), II (2), III (3), IV (4), V (5), VI (6), and VII (7). A solution of withanosides (2 mg of **1**, **2**, **3**, **4**, **5**, **6**, or **7**) in 5% aq H_2SO_4–dioxane (2 mL, 1:1, v/v) was heated under reflux for 3 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and the resin was filtered. After removal of the solvent in vacuo from the filtrate, the residue was passed through a Sep-Pak C18 cartridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (4 mg) in pyridine (0.02 mL) at 60 °C for 1 h. After reaction, the solution was treated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.01 mL) at 60 °C for 1 h. The supernatant was then subjected to GLC analysis to identify the derivative of D-glucose from **1**, **2**, **3**, **4**, **5**, **6** and **7**. GLC conditions: column, Supeluco STB™-1 (30 m×0.25 mm i.d.); column temperature, 230 °C; t_R, 24.1 min.

Enzymatic hydrolysis of withanosides I (1), II (2), IV (4), V (5), and VI (6). A solution of withanosides (10 mg of **1**, **2**, **4**, **5**, or **6**) in 0.2 M acetate buffer (pH 4.4, 4 mL) was treated with β-glucosidase (Oriental yeast Co., Ltd., 15 mg) and the whole mixture was stirred at 38 °C for 2 days. After treatment of the reaction mixture with EtOH, the whole mixture was evaporated to dryness under reduced pressure and the residue was purified by normal-phase silica gel column chromatography [1 g, $CHCl_3$–MeOH–H_2O (15:3:1, lower layer)] to give withanolides [1α,3β,5α-trihydroxy-6α,7α-epoxy-22*R*-with-24-enolide (**12**, 5.3 mg from **1**; 5.3 mg from **2**), sominone (**14**, 5.2 mg from **4**), 20*S*,22*R*-1α,3β-dihydroxy-witha-5,24-dienolide (**15**, 4.3 mg from **5**), and 1α,3β,20α_F-trihydroxy-20*R*,22*R*-witha-5,24-dienolide (**16**, 5.9 mg from **6**)], which were identified by comparison of the $[\alpha]_D$, IR, and ^1H NMR data with reported values.[10,15–17]

Enzymatic hydrolysis of withanosides III (3) and VII (7). A solution of withanosides (10 mg of **3** or **7**) in 0.2 M acetate buffer (pH 4.4, 4 mL) was treated with β-glucosidase (Oriental yeast Co., Ltd., 15 mg) and the whole mixture was stirred at 38 °C for 2 days. Work-up of the reaction mixture as described above furnished a product, which was purified by normal-phase silica gel column chromatography [1 g, $CHCl_3$–MeOH–H_2O (15:3:1, lower layer)] to give new withanolides [withagenins A (**13**, 5.8 mg, 81% from **3**) and B (**17**, 4.8 mg, 83% from **7**)].

Withagenin A (**13**): A white powder, $[\alpha]_D^{28}$ −28.0° ($c=0.1$, $CHCl_3$). High-resolution positive-ion FAB–MS: calcd for $C_{28}H_{42}O_7Na$ $(M+Na)^+$: 513.2828. Found: 513.2812. CD ($c=0.0039$, MeOH) $\Delta\varepsilon$ (nm): +4.5 (250) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 228 (3.6). IR (KBr): 3418, 2928, 1695, 1655, 1360, 1043 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.77, 0.84, 2.06 (3H each, all s, 18, 19, 28-H_3), 1.02 (3H, d, $J=6.7$ Hz, 21-H_3), 2.98 (1H, d, $J=3.7$ Hz, 6-H), 3.30 (1H, br s, 7-H), 3.67 (1H, br s, 1-H), 4.31 (1H, m, 3-H), 4.40 (2H, m, 27-H_2). ^{13}C NMR (125 MHz, pyridine-d_5) δ_c: given in Table 1. Positive-ion FAB–MS: m/z 513 $(M+Na)^+$.

Withagenin B (**17**): A white powder, $[\alpha]_D^{28}$ +8.3° ($c=0.1$, $CHCl_3$). High-resolution positive-ion FAB–MS: calcd for $C_{28}H_{42}O_5Na$ $(M+Na)^+$: 481.2930. Found: 481.2918. CD ($c=0.0039$, MeOH) $\Delta\varepsilon$ (nm): +3.9 (245) (positive max.). UV λ_{max}^{MeOH} nm (log ε): 227 (3.8). IR (KBr): 3418, 2928, 1705, 1655, 1410, 1045 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.72, 1.01, 1.88, 1.94 (3H each, all s, 18, 19, 27, 28-H_3), 1.02 (3H, d, $J=7.0$ Hz, 21-H_3), 3.65 (1H, br s, 7-H), 3.85 (1H, dt-like, 1-H), 4.04 (1H, m, 3-H), 4.38 (2H, dd-like, 22-H), 5.82 (1H, dd, $J=1.6$, 5.3 Hz, 6-H). ^{13}C NMR (125 MHz, pyridine-d_5) δ_c: given in Table 1. Positive-ion FAB–MS: m/z 481 $(M+Na)^+$.

Bioassay methods

Tissue preparation. Under ether anesthesia, female guinea-pigs weighing 250–350 g were bled to death by severing both carotid arteries, and the distal ileum was removed. Isolated segments (1.5–2.0 cm long) of the ileum were suspended under ca. 1 g tension in an organ bath filled with a physiological salt solution with a 95%O_2/5%CO_2 gas mixture and kept at 37 °C. Physiological salt solution contained the following composition (mM); NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.0; $NaHCO_3$, 25; KH_2PO_4, 1.2; D-glucose, 11.1. To prevent effects via β-adrenoceptors, propranolol (1 μM) was routinely included in the medium. Transmural electrical stimulation of the ileum was accomplished with two platinum electrodes, and the intraluminal electrode was the cathode. Rectangular pulses used were of 0.5 msec duration at the frequency of 0.1 Hz and were of a strength sufficient to give a maximal response. Tension was recorded by an isometric force–displacement transducer (Type 45196A, NEC San-ei Instruments, Tokyo, Japan) on a polygraph. Electrical stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan) was used for electrical stimulation of the ileum preparation. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to the organ bath (final concentration of DMSO was 0.1%).

Tachyphylaxis to clonidine on electrically stimulated guinea-pig ileum. After equilibration, the ileum preparations were electrically stimulated. Dose-response curves (0.3–100 nM) were obtained from the effect of clonidine on the contraction of the electrically stimulated ileum. After the first dose–response curves were established, tissues were washed and then clonidine at 10 nM, which induced approximately 70% inhibition of the twitch responses, was added to the bath. A contact period of 90 min was chosen because it was found that

tachyphylaxis to clonidine could thus be most reliably reproduced. After 90 min, the tissues were washed several times until the contraction return to the pre-incubation level, then dose–response curves of clonidine were re-established. Second responses to clonidine after 90-min incubation with 10 nM clonidine were compared to the maximal inhibition in first responses, and the inhibitions (%) were obtained. EC_{50} values (concentrations that produce a 50% inhibition of twitch response) of clonidine were graphically estimated.

To examine the direct relaxing effects of test samples on electrically induced twitch responses, test sample solution was applied 10 min before the second dose–response curve of clonidine in clonidine-untreated preparations. In addition, withanoside VI (**6**) and withaferin A (**8**) at 100 μM always caused the preparations unrecovered-relaxing effects (data not shown). Therefore, 30 μM was chosen as the maximal concentration for **6** and **8**.

Statistics

Values were expressed as means ± SEM One-way analysis of variance following Dunnett's test was used for statistical analysis.

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