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Withanolides with Antibacterial Activity from *Nicandra john-tyleriana*

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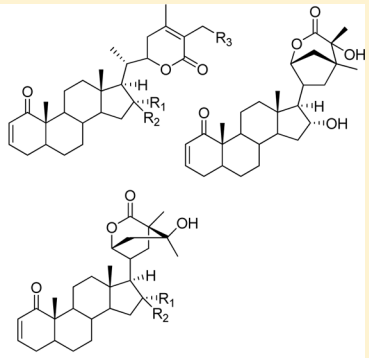
Supporting Information



ABSTRACT: Eleven new withanolides (**1**−**11**) were isolated and characterized from the aerial parts of *Nicandra john-tyleriana*. Five of these withanolides have an unmodified skeleton (**1**−**5**), two are acnistins (**6**, **7**), and four are withajardins (**8**−**11**). These new isolates were fully characterized using a combination of spectroscopic techniques (including multidimensional NMR) and mass spectrometry. All compounds were evaluated for their antibacterial activity against *Bacillus*, *Enterococcus*, *Escherichia*, *Listeria*, *Pseudomonas*, and *Staphylococcus* strains.

Withanolides comprise a group of naturally occurring C_{28} steroids based on an ergostane skeleton, in which C-26 and C-22, or C-26 and C-23, are oxidized in order to form a δ- or γ-lactone. Biogenetic transformations of withanolides can produce highly modified compounds in both the steroid nucleus and side chain, including the formation of additional rings. Their chemistry and occurrence have been the subject of several reviews.[1−4]

Nicandra Adans. (Solanaceae) is a small genus comprising three species. Two of these are *Nicandra john-tyleriana* S. Leiva & Pereyra, which grows in the northern Andean region of the Department San Martín (Prov. Otuzco, ca. 3000 m), and *Nicandra yacheriana* S. Leiva, from the "lomas" of the Department Arequipa (Prov. Caravelí, ca. 600 m), both endemic in Peru.[5] The third species in this group is the well-known and most widespread species of the genus, *Nicandra physalodes* (L.) Gaertn., which occurs in a region from Peru to northern Argentina, as well as being found as a ruderal species in tropical and subtropical areas worldwide.[6] Species in this genus are vigorous annual herbs with showy pale violet bell-shaped corollas with a white throat and sagittate calyx. The three above-mentioned species are distinguished by their floral and fruit characters.[5b]

A family of aromatic D-ring withanolides and withanolides with an unmodified skeleton has been isolated from *N. physalodes*,[7] with some of these compounds having exhibited interesting biological activities such as insecticidal[7a] or potential anticancer properties.[8]

In the present investigation into the withanolides of the genus *Nicandra*, reported is the isolation of 11 new withanolides from *N. john-tyleriana* (**1**−**11**). Antibacterial activity has been previously reported for the ethanolic extract of *N. john-tyleriana* against *Escherichia*, *Pseudomonas*, *Proteus*, and *Staphylococcus* bacteria.[9] In order to determine the antimicrobial activity, all compounds obtained in the present study were evaluated against different strains of *Bacillus*, *Enterococcus*, *Escherichia*, *Listeria*, *Pseudomonas*, and *Staphylococcus* by utilizing a disk diffusion method and bioautography. Finally, the most active compounds were tested by direct contact against the most sensitive bacteria cells.[10]

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Chart 1

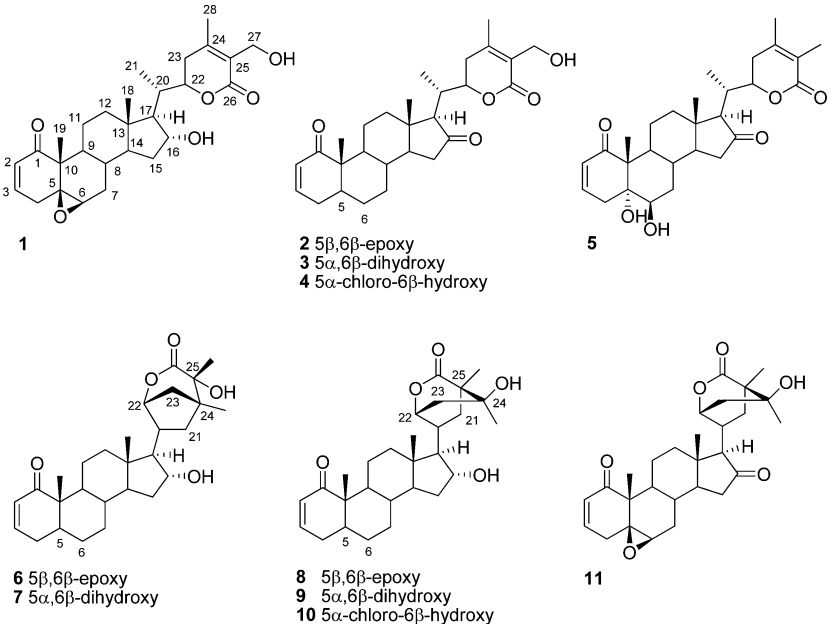

Table 1. ^{1}H NMR Data of Compounds 1−5 in $CDCl_3$[a]

position	1	2	3	4	5
2	6.03 dd (10.0, 2.8)	6.04 dd (10.0, 2.8)	5.90 dd (10.1, 2.5)	5.95 dd (10.2, 2.8)	5.90 dd (10.2, 2.5)
3	6.85 ddd (10.0, 6.2, 2.3)	6.87 ddd (10.0, 6.1, 2.3)	6.63 ddd (10.1, 5.0, 2.2)	6.68 ddd (10.2, 5.2, 2.2)	6.63 ddd (10.2, 5.1, 2.2)
4α	1.91 dd (18.9, 6.2)	1.94 m	2.10 dd (19.8, 5.0)	2.53 dd (19.9, 5.2)	2.12 m
4β	2.99 dt (18.9, 2.8)	3.01 dt (19.0, 2.8)	3.33 dt (19.8, 2.5)	3.53 dt (19.9, 2.8)	3.32 dt (19.7, 2.5)
6	3.13 d (2.5)	3.17 d (2.6)	3.70 t (2.5)	4.06 t (2.7)	3.71 t (2.6)
7α	1.33 m	1.37 m	1.49 m	1.55 m	1.50 m
7β	2.03 m	2.01 m	1.80 brt (13.3)	2.08 m	1.81 m
8	1.57 dd (11.4, 3.9)	1.76 cd (11.0, 3.7)	1.96 m	1.98 m	1.96 m
9	1.19 td (11.4, 4.1)	1.34 m	1.97 m	2.12 m	1.97 m
11α	2.05 m	2.20 m	2.41 m	2.47 m	2.35 m
11β	1.42 m	1.56 m	1.38 m	2.15 m	1.39 m
12α	1.93 m	2.02 m	2.03 m	2.06 m	2.03 m
12β	1.29 m	1.55 m	1.70 m	1.73 m	1.72 m
14	1.36 m	1.43 m	1.68 m	1.74 m	1.68 m
15α	1.72 td (13.3, 8.3)	2.23 m	2.23 dd (17.8, 7.1)	2.25 dd (18.1, 7.4)	2.24 dd (17.8, 7.3)
15β	1.50 m	1.87 m	1.89 t (17.8)	1.90 m	1.89 m
16	4.11 t (7.0)				
17	1.12 dd (17.8, 6.3)	1.96 m	2.01 m	1.99 m	2.07 m
18	0.73 s	0.89 s	0.93 s	0.93 s	0.92 s
19	1.25 s	1.28 s	1.35 s	1.41 s	1.34 s
20	2.13 m	2.40 m	2.39 m	2.38 m	2.39 m
21	1.02 d (6.6)	1.03 d (7.0)	1.04 d (7.0)	1.05 d (7.1)	1.02 d (6.9)
22	4.72 ddd (11.6, 4.6, 3.6)	5.10 ddd (12.9, 5.8, 3.4)	5.13 ddd (13.3, 8.9, 3.5)	5.19 ddd (13.0, 5.3, 3.5)	5.00 ddd (13.0, 6.1, 3.1)
23α	2.44 m	2.44 m	2.46 dd (17.8, 13.3)	2.47 m	2.39 m
23β	2.37 m	2.14 dd (17.6, 3.3)	2.17 m	2.15 m	2.10 m
27a	4.40 d (12.5)	4.38 d (13.6)	4.39 d (12.7)	4.39 d (13.9)	1.88 s
27b	4.34 d (12.5)	4.35 d (13.6)	4.35 d (12.7)	4.36 d (13.9)	
28	2.02 s	2.04 s	2.05 s	2.05 s	1.93 s

[a]Chemical shifts (δ) downfield from TMS, J couplings (in parentheses) in Hz, run at 400.13 MHz.

RESULTS AND DISCUSSION

Withanolides with an Unmodified Skeleton. The dichloromethane extract of the aerial parts of *N. john-tyleriana* was subjected to chromatographic purification and gave 11 new withanolides (**1**−**11**). Of these, HRESIMS of 16α-hydroxyjaborosalactone A (**1**) showed a quasimolecular ion $[M + 1]^+$ at m/z 471.2735, corresponding to an elemental formula of $C_{28}H_{39}O_6$. The ^{1}H NMR spectrum of **1** (Table 1) revealed the characteristic signals of H-2, H-3, and H-6 for a 1-oxo-2-ene-5β,6β-epoxywithanolide at δ 6.03 dd (J = 10.0, 2.8 Hz), 6.85 ddd (J = 10.0, 6.2, 2.3 Hz), and 3.13 d (J = 2.5 Hz), respectively. The substitution pattern of rings A/B was

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Table 2. ^{13}C NMR Data of Compounds 1−11[a]

position	1[b]	2[b]	3[b]	4[b]	5[b]	6[b]	7[b]	8[b]	8[c]	9[d]	10[b]	11[b]
1	203.4	203.2	204.1	201.0	204.1	203.5	204.2	203.6	203.6	205.1	201.4	203.3
2	129.4	129.2	128.8	128.6	128.8	129.3	128.8	129.2	129.7	129.8	128.6	129.1
3	144.4	144.5	141.2	141.3	141.2	144.4	141.1	144.5	145.5	142.9	141.4	144.6
4	33.0	32.9	35.8	37.2	35.8	33.0	35.8	33.0	33.6	37.3	37.2	33.0
5	62.1	62.0	77.2	79.9	77.2	62.0	77.5	62.1	62.5	78.5	80.1	62.0
6	63.2	63.0	74.2	74.3	74.3	63.2	74.4	63.2	63.5	75.7	74.5	63.1
7	31.2	31.2	33.7	33.6	33.7	31.1	33.7	31.1	31.9	35.1	33.6	31.2
8	29.4	28.9	29.0	29.1	28.9	29.4	29.5	29.4	30.3	31.3	29.7	29.2
9	44.6	44.4	41.3	42.0	41.3	44.6	41.6	44.6	45.5	43.2	42.2	44.4
10	48.4	48.3	52.0	52.5	52.0	48.4	52.1	48.4	49.1	53.2	52.6	48.3
11	23.3	23.3	22.9	22.8	22.8	23.2	22.8	23.2	24.0	24.3	22.7	23.5
12	39.7	39.1	39.4	39.3	39.4	39.6	40.1	39.4	40.4	41.9	39.7	39.2
13	44.1	43.0	43.5	43.4	43.5	43.8	44.3	43.9	44.3	45.7	44.4	42.8
14	53.3	50.7	50.5	50.4	50.5	52.9	52.6	52.9	53.7	54.6	52.5	50.2
15	37.6	38.8	38.9	38.8	38.9	37.4	37.4	37.2	37.9	38.7	37.2	38.9
16	76.5	217.2	217.8	217.5	217.9	76.9	76.8	75.9	75.5	76.7	76.0	217.0
17	61.2	63.2	63.5	63.5	63.4	64.5	64.9	61.6	62.8	63.6	62.0	64.0
18	13.2	14.0	14.4	14.3	14.6	14.7	15.2	14.4	14.9	16.0	14.9	14.4
19	15.0	15.2	15.6	16.3	15.6	15.1	15.7	15.1	15.7	16.8	16.3	15.3
20	37.6	35.0	35.1	34.9	35.3	47.3	47.5	38.5	40.0	40.7	38.5	34.7
21	13.6	13.4	13.5	13.4	13.5	38.6	38.6	30.7	32.1	32.5	30.8	30.6
22	79.3	77.1	77.2	77.2	76.8	85.9	86.0	77.7	78.9	79.1	77.8	75.3
23	30.0	31.6	31.6	31.2	31.6	39.0	39.1	38.2	40.3	40.5	38.3	38.3
24	154.0	152.6	152.9	152.8	148.9	46.9	46.9	71.7	71.5	72.3	71.7	71.7
25	125.3	125.7	125.7	125.7	122.1	76.7	76.8	47.2	48.5	48.8	47.2	46.9
26	167.4	166.7	166.9	166.9	166.9	178.7	178.8	177.7	178.7	178.4	177.6	177.1
27	57.5	57.5	57.4	57.5	12.4	25.1	25.1	14.1	15.8	15.7	14.2	14.2
28	20.0	20.0	20.0	20.0	20.5	20.1	20.1	28.9	29.6	29.8	29.0	29.0

[a]Chemical shifts (δ) downfield from TMS, run at 100.03 MHz. [b]$CDCl_3$. [c]C_5D_5N. [d]$(CD_3)_2CO$.

confirmed by the signals at δ 203.4 (C-1), 129.4 (C-2), 144.4 (C-3), 62.1 (C-5), and 63.2 (C-6) in the ^{13}C NMR spectrum (Table 3). A β-orientation of the 5,6-epoxy function was supported by the chemical shifts of C-5 and C-6 as well as the small value of the coupling constant between H-6α and H-7β (2.5 Hz).[11] Regarding the side chain, an α,β-unsaturated δ lactone was established, although its NMR data [^{13}C: δ 79.3 (C-22), 30.0 (C-23), 154.0 (C-24), 125.3 (C-25), 167.4 (C-26); ^{1}H: δ 4.72 ddd (J = 11.6, 4.6, 3.6 Hz, H-22), 2.02 s (H_3-28)], the absence of a singlet signal corresponding to H_3-27 at the high-field region of the ^{1}H NMR spectrum, and the appearance of two doublets at δ 4.40 d (J = 12.5 Hz) and 4.34 d (J = 12.5 Hz) suggested the presence of an isolated C-27 hydroxymethylene group. The ^{13}C NMR spectrum showed only four methyl groups, at δ 13.2, 15.0, 13.6, and 20.0, corresponding to C-18, C-19, C-21, and C-28, respectively, and the methylene signal at δ 57.5 confirmed the presence of a hydroxy group at C-27. Compound **1** exhibited ^{1}H and ^{13}C NMR data closely resembling those of jaborosalactone A isolated initially from *Jaborosa integrifolia*[12] but differing only in the substitution pattern of ring D. The signal at δ 4.11 t (J = 7.0 Hz) in the ^{1}H NMR spectrum in conjunction with the ^{13}C NMR data of ring D suggested an α-hydroxy substitution at C-16, and the location of this group was supported by HMBC correlations between the signals of H-16 and C-14, C-15, and C-17 at δ 53.3, 37.6, and 61.2, respectively. The α-orientation of the hydroxy group at C-16 was confirmed by the NOE observed between H-16 and the resonance corresponding to H_3-18 (δ 0.73), while the β-orientation of the side chain at C-17 was established by the ^{1}H NMR chemical shift of the angular methyl group H_3-18[1] and by the cross-correlation peak observed between H-16 and the signal corresponding to H-22 in the NOESY experiment. The structure of **1** was elucidated as (17*R*,20*S*,22*R*)-5β,6β-epoxy-16α,27-dihydroxy-1-oxowitha-2,24-dien-26,22-olide.

16-Oxojaborosalactone A (**2**) revealed a molecular formula of $C_{28}H_{36}O_6$ by HRESIMS, with the ^{1}H and ^{13}C NMR data (Tables 1 and 2) being closely comparable to those of 16α-hydroxyjaborosalactone A (**1**). The only difference between **2** and **1** was the absence of signals corresponding to the oxygenated methine (C-16, δ_H 4.11 and δ_C 76.5) in **1** and the appearance of the keto carbonyl signal at δ_C 217.2 in **2**, thus suggesting the presence of a keto group at C-16, with this assumption being confirmed by the cross-correlation peaks between H_2-15 (δ 2.23 m and 1.87 m), H-17 (δ 1.96 m), and H-20 (δ 2.40 m) and C-16 in the HMBC experiment. The structure of **2** was elucidated as (17*R*,20*S*,22*R*)-5α,6α-epoxy-27-hydroxy-1,16-dioxowitha-2,24-dien-26,22-olide.

The ^{1}H and ^{13}C NMR spectra of 16-oxojaborosalactone D (**3**) and 16-oxojaborosalactone E (**4**) were closely related to those of **2** (Tables 1 and 2). The almost identical ^{13}C NMR data for rings C and D and the side chain of compounds **2**−**4** indicated that structural differences were restricted to substituents in rings A and B. Furthermore, the presence of a 1-oxo-2-ene functionality in ring A was evident for the three compounds. The ^{1}H and ^{13}C NMR data of **3** were consistent with a 5α,6β-diol typical of many withanolides,[11] and the small couplings in the H-6 resonance at δ 3.70 t (J = 2.5 Hz) confirmed the axial orientation (β) of the 6-hydroxy group. Moreover, the ^{13}C NMR spectrum revealed the expected

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Table 3. ^{1}H NMR Data of Compounds 6−11[a]

position	6[b]	7[b]	8[b]	9[c]	10[b]	11[b]
2	6.02 dd (10.0, 2.9)	5.90 dd (10.3, 2.7)	6.02 dd (10.0, 2.8)	5.71 dd (10.1, 2.6)	5.93 dd (10.1, 2.8)	6.03 dd (10.0, 2.9)
3	6.85 ddd (10.0, 6.1, 2.3)	6.61 ddd (10.3, 5.1, 2.3)	6.86 ddd (10.0, 6.2, 2.3)	6.59 ddd (10.1, 5.0, 2.3)	6.66 ddd (10.1, 5.1, 2.2)	6.87 ddd (10.0, 6.1, 2.2)
4α	1.91 dd (19.0, 6.1)	2.06 ddd (19.8, 5.1, 0.7)	1.92 dd (19.1, 6.2)	2.08 m	2.51 dd (20.1, 5.2)	1.95 m
4β	2.98 dt (19.0, 2.9)	3.30 dt (19.8, 2.7)	2.99 dt (19.1, 2.8)	3.32 dt (19.7, 2.3)	3.53 dt (20.1, 2.8)	3.02 dt (19.2, 2.7)
6	3.13 d (2.5)	3.66 brs	3.14 d (2.5)	3.63 brs	4.03 t (2.7)	3.17 d (2.7)
7α	1.34 m	1.51 m	1.33 m	1.52 m	1.56 m	1.40 m
7β	2.04 m	1.70 m	2.03 m	1.76 m	2.05 m	2.01 m
8	1.57 m	1.73 m	1.58 m	1.79 m	1.74 m	1.77 m
9	1.21 m	1.81 m	1.23 m	1.93 m	2.02 m	1.38 m
11α	2.05 m	2.24 m	2.07 m	2.27 m	2.36 m	2.28 m
11β	1.27 m	1.29 m	1.43 m	1.29 m	1.30 m	1.58 m
12α	1.78 m	1.80 m	1.81 m	1.84 m	1.83 dt (12.6, 3.4)	1.97 m
12β	1.29 m	1.47 m	1.43 m	1.49 m	1.63 dd (12.6, 3.8)	1.61 m
14	1.37 m	1.61 m	1.42 m	1.60 m	1.75 m	1.48 m
15α	1.72 m	1.73 m	1.73 m	1.70 m	1.76 m	2.22 m
15β	1.52 m	1.52 m	1.55 m	1.57 m	1.56 m	1.85 m
16	4.14 brt (6.9)	4.13 brt (6.8)	4.06 brt (7.2)	4.02 brt (7.7)	4.07 brt (7.0)	
17	1.09 dd (11.6, 6.1)	1.16 dd (11.6, 6.1)	1.40 m	1.49 m	1.48 m	2.03 m
18	0.69 s	0.73 s	0.69 s	0.78 s	0.74 s	0.84 s
19	1.24 s	1.31 s	1.24 s	1.30 s	1.38 s	1.28 s
20	2.32 m	2.32 m	2.13 m	2.07 m	2.16 m	2.28 m
21a	2.63 ddd (13.9, 8.8, 1.9)	2.64 ddd (13.9, 8.6, 1.6)	2.15 m	2.28 m	2.21 m	2.15 m
21b	1.21 m	1.24 m	1.51 m	1.50 m	1.54 m	1.52 m
22	5.05 brs	5.08 brs	4.69 brt (3.2)	4.66 brt (3.5)	4.71 brt (3.4)	5.56 brt (3.5)
23a	2.15 brd (14.0)	2.16 brd (13.2)	2.21 brd (15.0)	2.31 m	2.24 d (14.6)	2.05 m
23b	1.69 m	1.73 m	2.05 m	1.92 m	2.07 m	1.94 m
27	1.47 s	1.47 s	1.13 s	1.09 s	1.15 s	1.13 s
28	1.18 s	1.20 s	1.25 s	1.17 s	1.27 s	1.27 s
OH-C24				3.93 s		
OH-C16				3.70 d (5.8)		

[a]Chemical shifts (δ) downfield from TMS, J couplings (in parentheses) in Hz, run at 400.13 MHz. [b]$CDCl_3$. [c]$(CD_3)_2CO$.

chemical shifts for signals of carbons C-5 and C-6 at δ 77.2 and 74.2, respectively. The ^{1}H and ^{13}C NMR data of **4** were consistent with a 5α-chloro-6β-hydroxy arrangement. Thus, the signal at δ 4.06 t (J = 2.7 Hz) was assigned to the equatorial H-6, with the unusually high chemical shift observed for H-4β at δ 3.53 (dt, J = 19.9 and 2.8 Hz) being indicative of a chlorine atom at C-5 with an α-orientation.[11] The substitution pattern in ring B was confirmed by the signals at δ 79.9 and 74.3 in the ^{13}C NMR spectrum, which were assigned to C-5 and C-6, respectively.

The full and unambiguous proton and carbon NMR assignments for compounds **3** and **4** (Tables 1 and 2) were confirmed using a combination of COSY, HSQC, and HMBC experiments. The high-resolution mass measurements were in agreement with the proposed molecular formulas. Thus, the structures of compounds **3** and **4** were established as (17*R*,20*S*,22*R*)-5α,6β,27-trihydroxy-1,16-dioxowitha-2,24-dien-26,22-olide and (17*R*,20*S*,22*R*)-5α-chloro-6β,27-dihydroxy-1,16-dioxowitha-2,24-dien-26,22-olide, respectively.

The molecular formula of 16-oxo-27-deoxyjaborosalactone D (**5**) was determined by HRESIMS as $C_{28}H_{38}O_6$. Comparison of its ^{1}H and ^{13}C NMR spectra with those of compound **3** showed signals almost identical with respect to the carbons and protons of rings A−D, which differed only in the side chain. The presence of an α,β-unsaturated δ lactone was inferred from the NMR data. However, compound **5** did not show the characteristic signals of the hydroxymethylene group, but revealed two methyls bonded to vinylic positions C-24 and C-25 at δ 1.93 and 1.88, respectively. The ^{13}C NMR spectrum (Table 3) showed the expected chemical shifts at δ 76.8, 31.6, 148.9, 122.1, 166.9, 12.4, and 20.5 assigned to the C-22−C-28 positions, respectively. The structure of compound **5** was determined as (17*R*,20*S*,22*R*)-5α,6β-dihydroxy-1,16-dioxowitha-2,24-dien-26,22-olide.

Acnistins. *Nicandra john-tyleriana* afforded acnistins I (**6**) and J (**7**). Acnistins are characterized by a bicyclic side chain involving C-21 and the lactone ring, where C-21 is directly bonded to C-24 via a C−C bond. These withanolide types have been isolated previously from several genera of the tribe Physalideae (*Acnistus*,[13] *Discopodium*,[14] *Dunalia*,[15] and *Tubocapsicum*[16]). Acnistin I (**6**), $C_{28}H_{38}NaO_6$, showed a peak at m/z 493.2557, corresponding to [M + Na] in the HRESIMS, and the ^{1}H and ^{13}C NMR data of rings A−D (Tables 2 and 3) were closely related to those of compound **1**, indicating a 1-oxo-2-ene-5β,6β-epoxy substitution pattern in rings A/B and the presence of an α-hydroxy group at C-16. Regarding the side chain, the NMR spectroscopic data of withanolide **6** closely resembled those of acnistins A−H[13−15] from the following observations: (i) the ^{1}H NMR spectrum revealed the characteristic signal corresponding to the carbinyl hydrogen H-22 at δ 5.05 and two singlets at δ 1.47 and 1.18 assigned to H_3-27 and H_3-28, respectively; (ii) the absence of a signal for

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CH_3-21 and the presence of strong cross-correlation peaks of H-22 with the methylene carbon at δ 38.6 (C-21) and the quaternary carbon at δ 46.9 (C-24) and of H-21a (δ 2.63) with C-22 (δ 85.9), C-23 (δ 39.0), C-24, and C-25 (δ 76.7) in the HMBC experiment suggested a C-21−C-24 bond; (iii) the signals at δ 85.9 (C-22), 46.9 (C-24), 76.7 (C-25), 178.7 (C-26), 25.1 (C-27), and 20.1 (C-28), observed in the ^{13}C NMR spectrum, were in agreement with a side chain acnistin arrangement; (iv) the *R* configuration of C-25 was established from the NOE observed between H_3-27 and H-23a (δ 2.15) (see the Supporting Information). Thus, the structure of **6** was elucidated as (17*R*,20*S*,22*R*,24*R*,25*R*)-5β,6β-epoxy-16α,25α-dihydroxy-21,24-cycloergost-2-en-1-one. Compound **7** revealed a molecular formula of $C_{28}H_{40}O_7$ by HREIMS, with its ^{1}H and ^{13}C NMR spectra being very similar to those of **6** (Tables 2 and 3). The ^{13}C NMR spectrum indicated that the only difference between **6** and **7** was in the substitution pattern at C-5 and C-6. Instead of the signals corresponding to the epoxy group at δ 62.0 (C-5) and 63.2 (C-6) in **6**, the spectrum of **7** showed two signals at δ 77.5 (C-5) and 74.4 (C-6) typical of a 5α,6β-diol. Furthermore, the multiplicity and the chemical shift of H-6 (δ 3.66 brs) were in good agreement with the β-orientation of the hydroxy group at C-6. Spectroscopic NMR assignments were confirmed from the COSY, HSQC, and HMBC spectra. The structure of **7** was determined as (17*R*,20*S*,22*R*,24*R*,25*R*)-5α,6β,16α,25α-tetrahydroxy-21,24-cycloergost-2-en-1-one.

Withajardins. The four new withajardins F−I (**8**−**11**) were isolated from *N. john-tyleriana*. Withajardins have been previously isolated only from the *Deprea*[17] and *Tubocapsicum*[16b,18] genera. They exhibit a bicyclic side chain involving C-21 and the lactone ring, but, in contrast with the acnistins, C-21 is bonded to C-25 instead of C-24.

Withajardin F (**8**) revealed a molecular formula of $C_{28}H_{38}O_6$ by HRESIMS. The ^{1}H and ^{13}C NMR data of rings A−D (Tables 2 and 3) were closely related to those of compounds **1** and **6**, indicating a 1-oxo-2-ene-5β,6β-epoxy substitution pattern in rings A/B, the presence of a hydroxy group at C-16 with an α-orientation, and the side chain at C-17 with a β-orientation. With respect to the side chain, compound **8** exhibited ^{1}H and ^{13}C NMR spectra closely related to those of the withajardins tuboanosides A and B.[18] The characteristic NMR spectroscopic data for this side chain were the signals at δ 4.69 (brt, J = 3.2 Hz), 1.13 s, and 1.25 s, assigned to H-22, H_3-27, and H_3-28, respectively, in the ^{1}H NMR spectrum, and with the key cross-correlation peaks observed between the signal corresponding to H-21a (δ 2.15) and the signals at δ 38.5 (C-20), 47.2 (C-25), 61.6 (C-17), and 177.7 (C-26) in the HMBC experiment, thus confirming the characteristic C-21−C-25 bond of the withajardin skeleton. The ^{13}C NMR spectrum of **8** was in agreement with the structure proposed. Regarding the configuration of C-24, the NOE observed between H_3-28 and H-12β (δ 1.43 m) supported the *S* configuration at this position (see the Supporting Information). The ^{1}H and ^{13}C NMR spectra of compound **8** were run in pyridine-d_5 in order to correlate the chemical shifts with those of tuboanosigenin *p*-bromobenzoate, a compound for which X-ray analysis has been performed.[18] The observed differences between both spectra were in good agreement with compound **8** having the opposite configuration at C-24 (Table 2). Accordingly, the structure of **8** was elucidated as (17*R*,20*S*,22*R*,24*S*,25*R*)-5β,6β-epoxy-16α,24α-dihydroxy-21,25-cycloergost-2-en-1-one.

The ^{1}H and ^{13}C NMR spectra of withajardin G (**9**) and H (**10**) were closely related to those of **8** (Tables 2 and 3), showing patterns typical of the withajardin arrangement at the side chain, for the resonances of carbons 17−28 and their protons. The almost identical ^{13}C NMR data for rings C and D and the side chain of compounds **8**−**10** indicated that structural differences were restricted to substituents in rings A and B. The ^{1}H and ^{13}C NMR spectra of **9** revealed a 1-oxo-2-ene-5α,6β-dihydroxy substitution pattern from the characteristic signals corresponding to C-1−C-6 and the corresponding protons. Regarding withajardin H (**10**), the resonances from rings A and B were almost identical to those in compound **4**, especially with respect to the unusually high chemical shifts observed for H-4β (δ 3.53 dt, J = 20.1, 2.8 Hz) and the signal at δ 4.03 t (J = 2.7 Hz), which was assigned to the H-6α proton, consistent with a 5α-chloro-6β-hydroxy arrangement in ring B. The ^{13}C NMR and HSQC spectra of **10** were found to be in agreement with the proposed structure.

Finally, withajardin I (**11**) revealed closely related ^{1}H and ^{13}C NMR spectra to those of compound **8**. In fact, the only difference between **11** and **8** was the presence of a carbonyl group at C-16 (δ_C 217.0) instead of a hydroxy group (δ_H 4.06, δ_C 75.9).

The full and unambiguous proton and carbon NMR assignments for compounds **9**−**11** were confirmed using a combination of COSY, HSQC, HMBC, and NOESY experiments. High-resolution mass measurements for compounds **9** and **11** were in agreement with the proposed formulas. Thus, the structures of **9**, **10**, and **11** were determined as (17*R*,20*S*,22*R*,24*S*,25*R*)-5α,6β,16α,24α-tetrahydroxy-21,25-cycloergost-2-en-1-one, (17*R*,20*S*,22*R*,24*S*,25*R*)-5α-chloro-6β,16α,24α-trihydroxy-21,25-cycloergost-2-en-1-one, and (17*R*,20*S*,22*R*,24*S*,25*R*)-5β,6β-epoxy-24α-hydroxy-21,25-cycloergost-2-ene-1,16-dione, respectively.

The three withanolide subtypes found in *N. john-tyleriana*, namely, withanolides with an unmodified skeleton, acnistins, and withajardins, are the same as those isolated from *Tubocapsicum anomalum* but distinct from those isolated from *N. physalodes*.[16,18] In the latest phylogenetic classifications for the family Solanaceae,[19,20] none of these genera have been placed in any particular tribe. It would be premature to use the present phytochemical evidence to draw any chemotaxonomic conclusions at the present time. A comprehensive phytochemical and molecular study of both genera is still required.

The potential antibacterial activity of all the compounds described above was evaluated in vitro against strains of *Bacillus*, *Enterococcus*, *Escherichia*, *Listeria*, *Pseudomonas*, and *Staphylococcus* by different bioassay techniques. Compounds **2** and **6** showed significant antibacterial activity against *Bacillus cereus* using a disk-diffusion technique and the bioautographic TLC assay. The antibacterial activity of compound **2** was also quantified by direct contact against *B. cereus* BAC1 cells. This compound exerted bactericidal and bacteriostatic effects at 1000 ppm and close to 750 ppm, respectively (Figure 1).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1010 polarimeter. The UV spectra were obtained using a Shimadzu-260 spectrophotometer, and IR spectra were produced using a Nicolet 5-SXC spectrophotometer. NMR spectra were recorded on a Bruker AVANCE II AV-400 operating at 400.13 MHz for ^{1}H and 100.63 MHz for ^{13}C, while 2D spectra (COSY, HSQC, HMBC, and NOESY) were obtained using standard Bruker software. Chemical shifts are given in ppm (δ) downfield from the TMS internal standard. HRESIQTOFMS were determined on a

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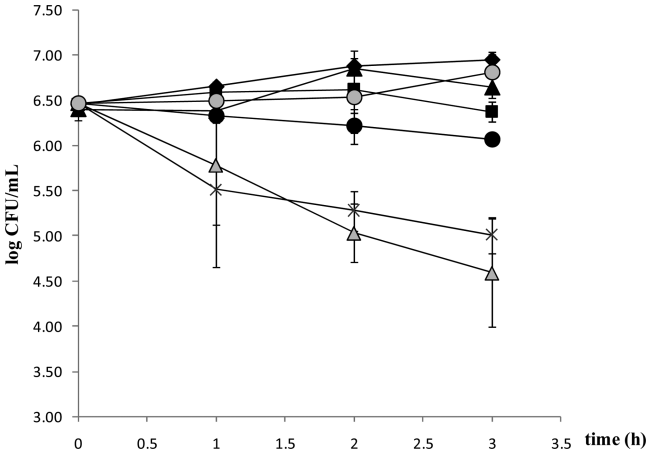

Figure 1. Antibacterial activity of compound **2** against vegetative cells of *Bacillus cereus* BAC1, expressed as log CFU/mL (colony forming units) and determined as means of two experiments (△ 2000 ppm; × 1500 ppm; ● 1000 ppm; ■ 750 ppm, ○ 500 ppm; ▲ sterile culture medium without compound; ◆ solvent).

Micro TOF II Bruker Daltonics. The chomatographic separations were performed by column chromatography on silica gel 60 (0.063−0.200 mm) and Sephadex LH-20, and preparative TLC was carried out on silica gel 60 F_{245} (0.2 nm thick) plates.

Plant Material. *Nicandra john-tyleriana* was collected in Plozapampa-El Tablón, District Salpo, Departament La Libertad, Peru, at 1781 m above sea level in April 2010. A voucher specimen, Leiva 4692, identified by one of the authors (S.L.), is housed at the Atenor Orrego Herbarium (HAO) of the Universidad Privada A. Orrego (Trujillo, Peru).

Extraction and Isolation. The dry and pulverized aerial parts of *N. john-tyleriana* (ca. 267 g) were exhaustively extracted with EtOH, and the solvent was evaporated at reduced pressure. The residue was defatted by partition in *n*-hexane−MeOH−H_2O (10:3:1), with the resultant MeOH−H_2O phase being washed with *n*-hexane (3 × 100 mL) and MeOH evaporated at reduced pressure. The residue was diluted with H_2O and extracted with CH_2Cl_2 (3 × 100 mL). The CH_2Cl_2 extract was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness at reduced pressure. The residue (2.86 g) was chromatographed initially on a silica gel column, using a mixture of CH_2Cl_2−MeOH of increasing polarity as eluent, to afford 255 fractions. Then, fractions with similar TLC profiles were combined to form eight pooled fractions (I−VIII). Fraction I was purified by preparative TLC with *n*-hexane−EtOAc (1:9) to yield (in order of elution) compounds **2** (10.2 mg), **6** (5.4 mg), and **11** (5.0 mg). Fraction II was separated by preparative TLC with CH_2Cl_2−MeOH (8.5:1.5) to obtain compound **4** (8.3 mg). Fraction III was subjected to preparative TLC with EtOAc, yielding compounds **1** (19.2 mg) and **8** (21.4 mg). Fraction IV was processed by TLC with CH_2Cl_2−MeOH (8:2) to afford compound **8** (17.0 mg) and an impure fraction, which was further separated by preparative TLC with *n*-hexane−EtOAc (1:9) as eluent to yield compound **5** (14.8 mg). Compound **10** (12.2 mg) was purified from fraction V by preparative TLC using *n*-hexane−EtOAc (1:9). Preparative TLC of fraction VI with EtOAc gave compound **3** (2.4 mg), and preparative TLC of fraction VII with EtOAc gave compounds **3** (3.6 mg) and **7** (15.5 mg). Finally, compounds **7** (25 mg) and **9** (7.0 mg) were obtained by preparative TLC with CH_2Cl_2−MeOH (8:2) from fraction VIII.

*16α-Hydroxyjaborosalactone A [(17R,20S,22R)-5α,6α-epoxy-16α,27-dihydroxy-1-oxowitha-2,24-dien-26,22-olide] (**1**):* white, amorphous solid; $[\alpha]^{21}_D$ +38.1 (*c* 0.5, acetone); UV (MeOH) λ_{max} (log ε) 221 (3.94) nm; IR (dried film) ν_{max} 3433, 2926, 2854, 1731, 1695, 1655, 1463, 1397, 1209, 1188, 1037, 755 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESITOFMS *m*/*z* $[M + 1]^+$ 471.2750 (calcd for $C_{28}H_{39}O_6$, 471.2741).

*16-Oxojaborosalactone A [(17R,20S,22R)-5α,6α-epoxy-27-hydroxy-1,16-dioxowitha-2,24-dien-26,22-olide] (**2**):* white, amorphous solid; $[\alpha]^{21}_D$ −22.4 (*c* 0.9, acetone); UV (MeOH) λ_{max} (log ε) 223 (3.96) nm; IR (dried film) ν_{max} 3457, 2925, 2848, 1732, 1699, 1674, 1655, 1392, 1189, 1127, 1013, 754 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESITOFMS *m*/*z* $[M + 1]^+$ 469.2576 (calcd for $C_{28}H_{37}O_6$, 469.2585).

*16-Oxojaborosalactone D [(17R,20S,22R)-5α,6β,27-trihydroxy-1,16-dioxowitha-2,24-dien-26,22-olide] (**3**):* white, amorphous solid; $[\alpha]^{21}_D$ −20.4 (*c* 0.6, acetone); UV (MeOH) λ_{max} (log ε) 221 (3.90) nm; IR (dried film) ν_{max} 3395, 2921, 2848, 1724, 1712, 1671, 1634, 1454, 1393, 1013, 751, 453 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESITOFMS *m*/*z* $[M + Na]^+$ 509.2497 (calcd for $C_{28}H_{38}NaO_7$, 509.2510).

*16-Oxojaborosalactone E [(17R,20S,22R)-5α-chloro-6β,27-dihydroxy-1,16-dioxowitha-2,24-dien-26,22-olide] (**4**):* white, amorphous solid; $[\alpha]^{21}_D$ −26.8 (*c* 0.3, acetone); UV (MeOH) λ_{max} (log ε) 222 (3.83) nm; IR (dried film) ν_{max} 3458, 2928, 2855, 1731, 1686, 1655, 1455, 1383, 1234, 1130, 1077, 756 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESITOFMS *m*/*z* $[M + Na]^+$ 527.2168 (calcd for $C_{28}H_{37}ClNaO_6$, 527.2171).

*16-Oxo-27-deoxyjaborosalactone D [(17R,20S,22R)-5α,6β-dihydroxy-1,16-dioxowitha-2,24-dien-26,22-olide] (**5**):* white, amorphous solid; $[\alpha]^{21}_D$ −44.6 (*c* 0.4, acetone); UV (MeOH) λ_{max} (log ε) 225 (4.08) nm; IR (dried film) ν_{max} 3466, 2927, 2852, 1731, 1684, 1655, 1383, 1197, 1132, 758, 670 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESITOFMS *m*/*z* $[M + 1]^+$ 471.2750 (calcd for $C_{28}H_{39}O_6$, 471.2741).

*Acnistin I [(17R,20S,22R,24R,25R)-5β,6β-epoxy-16α,25α-dihydroxy-21,24-cycloergost-2-en-1-one] (**6**):* white, amorphous solid; $[\alpha]^{21}_D$ −19.0 (*c* 0.3, acetone); UV (MeOH) λ_{max} (log ε) 219 (3.69) nm; IR (dried film) ν_{max} 3467, 2922, 2850, 1732, 1673, 1392, 1130, 1037, 756 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRESITOFMS *m*/*z* $[M + Na]^+$ 493.2557 (calcd for $C_{28}H_{38}NaO_6$, 493.2561).

*Acnistin J [(17R,20S,22R,24R,25R)-5α,6β,16α,25α-tetrahydroxy-21,24-cycloergost-2-en-1-one] (**7**):* white, amorphous solid; $[\alpha]^{21}_D$ −16.8 (*c* 1.0, acetone); UV (MeOH) λ_{max} (log ε) 223 (3.76) nm; IR (dried film) ν_{max} 3412, 2925, 2856, 1724, 1671, 1450, 1381, 1127, 1042, 645 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRESITOFMS *m*/*z* $[M + Na]^+$ 511.2657 (calcd for $C_{28}H_{40}NaO_7$, 511.2666).

*Withajardin F [(17R,20S,22R,24S,25R)-5β,6β-epoxy-16α,24α-dihydroxy-21,25-cycloergost-2-en-1-one] (**8**):* white, amorphous solid; $[\alpha]^{21}_D$ −19.1 (*c* 1.4, acetone); UV (MeOH) λ_{max} (log ε) 229 (4.08) nm; IR (dried film) ν_{max} 3459, 2974, 2930, 2868, 1731, 1673, 1454 1383, 1132, 754 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRESITOFMS *m*/*z* $[M + 1]^+$ 471.2735 (calcd for $C_{28}H_{39}O_6$, 471.2741).

*Withajardin G [(17R,20S,22R,24S,25R)-5α,6β,16α,24α-tetrahydroxy-21,25-cycloergost-2-en-1-one] (**9**):* white, amorphous solid; $[\alpha]^{21}_D$ −14.1 (*c* 0.3, acetone); UV (MeOH) λ_{max} (log ε) 221 (3.76) nm; IR (dried film) ν_{max} 3420, 2921, 2852, 1728, 1671, 1650, 1532, 1385, 1037, 878, 760 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRESITOFMS *m*/*z* $[M + Na]^+$ 511.2662 (calcd for $C_{28}H_{40}NaO_7$, 511.2666).

*Withajardin H [(17R,20S,22R,24S,25R)-5α-chloro-6β,16α,24α-trihydroxy-21,25-cycloergost-2-en-1-one] (**10**):* white, amorphous solid; $[\alpha]^{21}_D$ −3.1 (*c* 0.8, acetone); UV (MeOH) λ_{max} (log ε) 224 (3.64) nm; IR (dried film) ν_{max} 3395, 2925, 2847, 1732, 1683, 1450, 1383, 758 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRMS *m*/*z* $[M + Na]^+$ 529.2334 (calcd for $C_{28}H_{39}ClNaO_6$, 529.2327).

*Withajardin I [(17R,20S,22R,24S,25R)-5β,6β-epoxy-24α-hydroxy-21,25-cycloergost-2-en-1,16-dione] (**11**):* white, amorphous solid; $[\alpha]^{21}_D$ −45.9 (*c* 0.4, acetone); UV (MeOH) λ_{max} (log ε) 220 (3.63) nm; IR (dried film) ν_{max} 3452, 2925, 2848, 1728, 1704, 1675, 1446, 1377, 1233, 1140, 523 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRESITOFMS *m*/*z* $[M + 1]^+$ 469.2576 (calcd for $C_{28}H_{37}O_6$, 469.2585).

Biological Activity Assays. *Microorganisms and Media.* The test organisms used in this study were as follows: *Staphylococcus aureus*

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ATCC29213, *Enterococcus faecium* SM21, *Pseudomonas aureginosa*, *Escherichia coli* CS, *Listeria monocytogenes* 01/155, *L. monocytogenes* 00/110, *L. monocytogenes* 00/270, *Bacillus cereus* BC1, *B. cereus* 5, *B. cereus* 1, *B. cereus* BC3, and *B. subtilis*. The strains were obtained from the culture collection of the Applied Bacteriology Laboratory of INIQUI (Salta, Argentina) and were activated on Mueller-Hinton broth (Britania), at 37 °C for 72 h, without any special atmosphere. When a solid medium was needed, 1.5% w/v of agar was used.

Disk Diffusion Method. The compounds were seeded onto paper disks 0.5 mm in diameter to a final concentration of 2000 ppm. Bacteria were grown in Mueller-Hinton broth, and an aliquot of 100 μL was added to a Petri dish with a concentration of 10^8 CFU/mL. The disks were put into contact with bacteria for 16 h at 25 °C to allow diffusion of the compounds. Then, the plates were incubated at 37 °C for 24−48 h and examined to determine the presence or absence of inhibition halos. All experiments were carried out in duplicate. Disks used as negative controls contained methanol or chloroform, and the positive control was chloramphenicol (30 μg).

Bioautography. Using a modification of the assay described by Chomnawang et al.,[21] the compounds at 2000 ppm in TLC plates without development were covered with 5 mL of medium (BHI with 1.5% w/v of agar) containing an aliquot of 200 μL with a concentration of 10^8 CFU/mL of *Bacillus cereus* and *Staphylococcus aureus*. The plates were incubated at 37 °C for 24 h, and the areas of inhibition were compared with the positive control containing chloramphenicol (30 μg).

Microplate Direct Contact. This assay was carried out in order to quantify the biological effect of compound **2** on the indicator strain *B. cereus* BAC1. Cells from overnight cultures grown in MH broth were diluted in peptone water in order to obtain a suspension of ca. 10^5 CFU/mL. The effects of the pure compounds at different concentrations (from 2000 ppm to 500 ppm) on the indicator strain were analyzed in the following manner. Thus, 96-well microplates were used, and the different concentrations of pure compound were put in direct contact with the indicator strain suspensions at a 1:10 ratio at 37 °C for 1, 2, and 3 h. Viable indicator cells were determined by plating in duplicate using MH (1.5%, w/v) agar. The plates were incubated at 37 °C for 24 h.[10]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **1**−**11** and the relevant NOE and HMBC correlations of **6** and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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