Trypanocidal Constituents in Plants 6.[1] Minor Withanolides from the Aerial Parts of *Physalis angulata*

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Further study of the methanol extract of the aerial parts of *Physalis angulata* (Solanaceae) resulted in the isolation of new withanolides, designated physagulins L, M and N, together with known withanolide, physagulin D and flavonol glycoside, quercetin 3-*O*-rhamnosyl-(1→6)-galactoside. The chemical structures of these new withanolides were elucidated by detailed spectroscopic analyses to be (20*R*,22*R*)-15α-acetoxy-5α,6β,14β,17β,27-pentahydroxy-1-oxo-witha-2, 24-dienolide, (20*S*,22*S*)-15α-acetoxy-5α,6β,14α,23β-tetrahydroxy-1-oxo-witha-2,16,24-trienolide and (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-14α-hydoxy-3β-methoxy-1-oxo-witha-16,24-dienolide, respectively. All these compounds showed weak trypanocidal activity against trypomastigotes, an infectious form of *Trypanosoma cruzi*, the etiologic agent for Chagas' disease. Withanolides obtained in the previous paper showed considerable trypanocidal activity, suggesting the structure–activity relationships.

Key words withanolide; *Physalis angulata*; trypanocidal activity; *Trypanosoma cruzi*; Solanaceae

Physalis angulata L. (Solanaceae) is indigenous in tropical America and was naturalized in Japan in the Edo era. It is an annual small herb and has been traditionally utilized for antipyretic purposes in Japan.[2] In the previous paper in this series,[3] we described the investigation of constituents in *P. angulata* based on the plant's trypanocidal activity against epimastigotes of *Trypanosoma cruzi*, the etiologic agent for Chagas' disease (American trypanosomiasis). Ten withanolides (**1**—**10**), including four new ones, were isolated from the active fraction. Trypanocidal activity of these withanolides against trypomastigotes, an infectious form of *T. cruzi*, was also estimated, as well as cytotoxic activity against human uterine carcinoma (HeLa) cells *in vitro* to examine the possibility of chemotherapeutic utility.

Continuous study of the same plants resulted in the isolation of three new withanolides (**11**—**13**), designated physagulin L, M and N, respectively, together with known withanolide, physagulin D[4] (**14**) and flavonol glycoside, quercetin 3-*O*-rhamnosyl-(1→6)-galactoside (**15**).[5] The structures of the known compounds were identified from their spectral data and confirmed by comparison with those published in the literature. In this paper we describe the structural elucidation of the three new withanolides and the trypanocidal activity of the isolates.

Compound **11** has the molecular formula $C_{30}H_{42}O_{10}$, one oxygen atom more than physagulin K (**10**).[3] The ^{1}H- and ^{13}C-NMR spectra of **11** were similar to those of **10**, except for signals in ring E, suggesting the same substitution pattern in rings A—D. In the ^{1}H-NMR spectrum, one olefinic methyl signal at δ 2.04 (br s) and one hydroxymethylene signal at δ 4.74 and 4.88 (d each, J=12 Hz) were observed in **11**, instead of two olefinic methyl signals in **10**. In the ^{13}C-NMR spectrum of **11**, the corresponding signals due to the olefinic methyl and hydroxymethylene groups were observed at δ 20.0 and 56.2 accompanied with downfield shifts of C-24 (+4.4 ppm) and C-25 (+5.5 ppm) in comparison with those of **10**. Therefore, it was suggested that one of the methyl groups at C-24 or C-25 was replaced with a hydroxymethylene group in **11**. The methyl proton signal showed 3-bond connectivity with C-23 as well as C-25, and the hydroxymethylene proton signals with C-26 in the heteronuclear multiple-bond connectivity (HMBC) spectrum. Thus, the structure of **11** was elucidated to be (20*R*,22*R*)-15α-acetoxy-5α,6β,14β,17β,27-pentahydroxy-1-oxo-witha-2,24-dienolide, and it is called physagulin L.

The molecular formula of **12** was determined to be $C_{30}H_{40}O_9$ by HR-FAB-MS, one oxygen atom more than withaminimin (**8**).[6] The ^{1}H- and ^{13}C-NMR spectra showed characteristic signals for a 1-one-2-ene system, 5α,6β-glycol moiety, and a 14α-hydroxy-15α-acetoxy-16-ene system. In fact, signals due to rings A—D were in good agreement with those of **8**. As a part of the side chain, a carbon signal of hy-

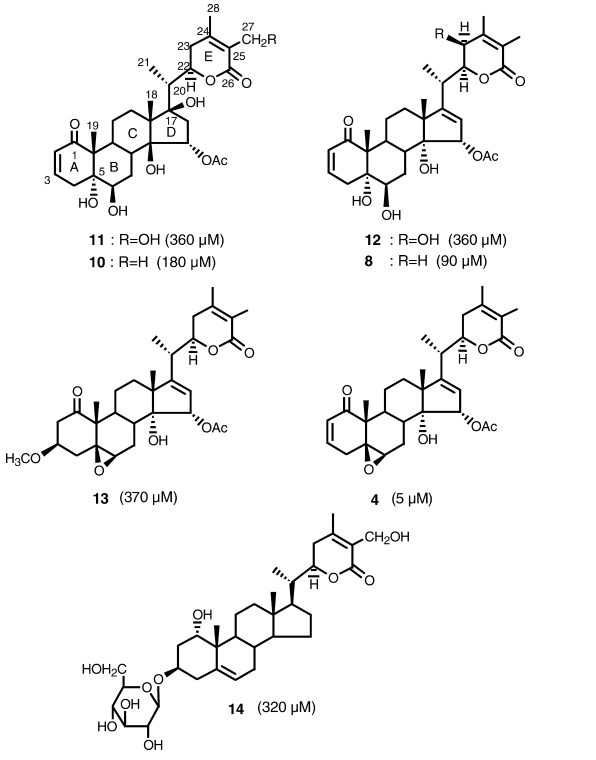

Chart 1. Chemical Structures of Compounds **4**, **8**, and **10**—**14** and Their Trypanocidal Activity (MC_{100})

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droxymethine was observed at δ 66.7 besides C-22. Downfield shifts of C-22 (+6.2 ppm) and C-24 (+4.1 ppm) were also observed in comparison with those of **8**, suggesting the hydroxylation at C-23. Orientation of the hydroxyl group was assigned as β, since H-23 appeared as a *quasi*-equatorial proton at δ 4.52 with small coupling constant (J=3 Hz). Furthermore, the nuclear Overhauser effect (NOE) between H-23 and H-28 supported α-orientation (*quasi*-equatorial) of H-23. NOEs between H-20/H-22 and H-21/H-12 were also observed. Compound **12** was determined to be (20*S*,22*S*)-15α-acetoxy-5α,6β,14α,23β-tetrahydoxy-1-oxo-witha-2,16,24-trienolide and was called physagulin M.

Compound **13** was obtained as a colorless powder. The molecular formula of **13** was determined to be $C_{31}H_{42}O_8$, based on the HR-FAB-MS. Unlike other withanolides obtained from *P. angulata*, **13** has 31 carbons. The extra carbon was considered to be a methoxyl group by the signals δ_H 3.21 (3H, s) and δ_C 55.7. The ^{1}H- and ^{13}C-NMR spectra of **13** were similar to those of physagulin A (**4**), except for signals in ring A, suggesting the same substitution pattern in rings B—E. In ring A, signals of two methylenes (δ_H 2.88 and 2.94, δ_C 42.9; δ_H 1.68 and 2.35, δ_C 42.9) and one methine bearing oxygen (δ_H 3.72, δ_C 73.3) were observed along with an isolated carbonyl carbon signal (δ 210.5). The methoxyl proton signal showed correlation with the carbon signal at δ 73.3. A methoxyl group was located at C-3 with β-orientation, since both methylene signals (H-2 and 4) showed 3-bond connectivity to C-10 and had small coupling constants between H-3 as shown in Table 2. Compound **13** was determined as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-14α-hydoxy-3β-methoxy-1-oxo-witha-16,24-dienolide and was called physagulin N. It is possible that **13** might be an artifact of **4** because it corresponds to MeOH addition product.

Trypanocidal activity of the newly isolated compounds from the aerial parts of *P. angulata* was examined against trypomastigotes of *T. cruzi*. Withanolides **11**—**14** as well as **15** showed weak activity, although the related compounds **4**, **8** and **10** showed activity 5, 94 and 183 μM, respectively, as MC_{100} values. Hydroxylation in ring E (**11**, **12**, **14**) caused loss of activity. It seems that an enone system in ring A is indispensable for showing activity. To clarify structure–activity relationships of withanolides further investigation on genus

Table 1. ^{13}C-NMR Data for Compounds **4, 8**, **10**, **11**, **12** and **13** (δ ppm from TMS in Pyridine-d_5)

No.	**10**	**11**	**8**	**12**	**4**	**13**
1	204.7	204.7	204.9	204.9	203.6	210.5
2	129.1	129.1	129.0	129.1	128.7	42.9
3	141.9	142.0	142.1	142.1	145.6	73.3
4	36.8	36.8	36.9	36.9	33.2	36.5
5	77.3	77.3	77.3	77.3	61.7	61.9
6	74.8	74.8	74.9	74.9	63.8	62.4
7	27.9	27.9	28.5	28.5	25.4	25.2
8	36.2	36.2	37.3	37.2	36.2	35.7
9	35.3	34.9	36.5	36.5	40.1	38.1
10	52.6	52.7	52.6	52.6	48.7	52.1
11	23.2	23.3	24.3	24.4	24.0	22.7
12	31.7	31.6	39.2	39.9	38.0	37.8
13	51.3	51.3	53.2	53.5	52.7	52.8
14	87.7	87.7	82.0	81.9	81.0	81.2
15	79.2	79.2	83.5	83.6	84.5	84.5
16	48.2	48.2	122.2	123.0	122.3	122.2
17	86.2	86.2	162.1	162.2	162.1	162.0
18	15.9	15.9	17.0	17.6	16.3	16.2
19	15.1	15.1	15.4	15.3	15.3	14.5
20	42.4	42.3	35.3	32.7	35.2	35.2
21	10.0	9.9	17.8	20.4	17.8	17.7
22	77.3	77.3	78.9	85.1	78.8	78.7
23	32.3	32.7	32.4	66.7	32.4	32.3
24	150.3	154.7	149.3	153.4	149.2	149.2
25	121.6	127.1	121.7	121.5	121.9	121.7
26	166.6	166.4	166.1	164.7	166.1	166.1
27	12.5	56.2	12.5	13.0	12.5	12.5
28	20.1	20.0	19.7	15.8	19.7	19.7
OAc	21.4	21.4	21.6	21.5	21.1	21.1
	170.3	170.4	170.7	170.7	169.8	169.8
OMe						55.7

Table 2. ^{1}H-NMR Spectral Data for Compounds **11**, **12** and **13** (δ ppm from TMS in Pyridine-d_5) and HMBC Correlations

No.	**11**	HMBC	**12**	HMBC	**13**	HMBC
2	6.13 (dd, 10.1, 2.1)		6.13 (dd, 10.2, 2.2)	C-4, 10	2.88 (ddd, 14.1, 4.4, 1.3)	C-1, 3, 4, 10
					2.94 (dd, 14.1, 5.5)	C-1, 3, 4, 10
3	6.66 (ddd, 10.1, 5.0, 2.1)		6.67 (ddd, 10.2, 5.2, 2.2)	C-1, 5	3.72 (m)	
4	2.37 (dd, 20.1, 5.0)	C-2, 3, 5, 10	2.37 (dd, 19.8, 5.2)	C-2, 3, 5, 10	1.68 (br d, 14.5)	C-2, 3, 10
	3.72 (dt, 20.1, 2.1)		3.74 (dt, 19.8, 2.2)		2.35 (dd, 14.5, 3.8)	
6	4.14 (t, 2.7)	C-8	4.17 (br s)	C-5, 8, 10	3.40 (br s)	C-7, 8
15	5.88 (dd, 8.6, 3.8)	C-13	6.20 (d, 2.8)	C-13, 14, 17, Ac[a)]	5.82 (d, 2.7)	C-13, 14, 17, Ac[a)]
16	2.09 (dd, 15.4, 3.8)		6.28 (d, 2.8)	C-13, 14, 15, 17	6.07 (d, 2.7)	C-13, 14, 15, 17
	2.91 (dd, 15.4, 8.6)	C-13, 17				
18	1.39 (s)	C-12, 13, 14, 17	1.55 (s)	C-12, 13, 14, 17	1.30 (s)	C-12, 13, 14, 17
19	1.68 (s)	C-1, 5, 9, 10	1.67 (s)	C-1, 5, 9, 10	1.35 (s)	C-1, 5, 9, 10
21	1.24 (d, 7.0)	C-17, 20, 22	1.41 (d, 7.3)	C-17, 20, 22	1.21 (d, 7.0)	C-17, 20, 22
22	5.06 (dt, 12.8, 3.1)		4.50 (t, 3.1)	C-17, 20, 21, 23, 24, 26	4.38 (dt, 13.7, 3.9)	
23	2.61 (dd, 18.3, 12.8)		4.52 (d, 3.1)	C-24, 25	2.00 (m)	
	2.77 (dd, 18.3, 3.1)				2.44 (m)	
27	4.74 (d, 11.7)	C-24, 25, 26	1.87 (br s)	C-24, 25, 26	1.84 (br s)	C-24, 25, 26
	4.88 (d, 11.7)	C-24, 25, 26				
28	2.04 (br s)	C-23, 24, 25	1.87 (br s)	C-23, 24, 25	1.53 (br s)	C-23, 24, 25
OAc	2.24 (s)	Ac[a)]	2.16 (s)	Ac[a)]	2.09 (s)	Ac[a)]
OMe					3.21 (s)	C-3

a) Correlated with carbonyl carbon signal of acetyl group.

Physalis is now in progress.

Experimental

General Experimental Procedures The instruments and reagents used in this study are the same as those in described in the previous paper.[3)]

Plant Material *P. angulata* was cultivated in the medicinal plant garden of Fukuoka University and harvested in September 2002. The aerial parts were air-dried and powdered.

Extraction and Isolation *P. angulata* (950 g) was treated as described in the previous paper.[3)] The MeOH extract (125 g) was suspended in 60% MeOH and centrifuged. The precipitates were extracted with MeOH, and then with AcOEt. The 60% MeOH solution was passed through a column of Diaion HP-20, and the column was washed with 60% MeOH (fr. 1, 70.1 g). The MeOH solution was passed through the same column and the column washed again with MeOH (fr. 2, 19.9 g). The AcOEt solution was treated in the same way (fr. 3, 20.9 g). Fraction 2 showed activity against epimastigotes (MC_{100} 50 μg/ml). Fraction 2 was chromatographed successively on silica gel (hexane–AcOEt), Sephadex LH-20 ($CHCl_3$), and YMC gel (MeOH–H_2O) columns to afford physagulin C[7)] (**1**, 35 mg), physagulin H[3)] (**2**, 20 mg), withangulatin A[8)] (**3**, 180 mg), physagulin A[4)] (**4**, 140 mg), physagulin I[3)] (**5**, 15 mg), physagulin F[4)] (**6**, 290 mg), physagulin B[4)] (**7**, 51 mg), withaminimin[6)] (**8**, 140 mg), physagulin J[3)] (**9**, 39 mg), physagulin K[3)] (**10**, 90 mg), **11** (20 mg), **12** (12 mg), **13** (35 mg), physagulin D[4)] (**14**, 35 mg) and quercetin 3-*O*-rhamnosyl-(1→6)-galactoside[5)] (**15**, 21 mg). Compounds **14** and **15** were identified by comparison of the spectral data with those reported.

Compound **11**: A colorless powder, $[\alpha]_D^{27}$ +34.3° (c=0.92, MeOH), HR-FAB-MS (m/z): 585.2687 $[M+Na]^+$ (Calcd for $C_{30}H_{42}NaO_{10}$: 585.2675). ^{13}C- and ^{1}H-NMR data: see Tables 1 and 2.

Compound **12**: A colorless powder, $[\alpha]_D^{27}$ +85.6° (c=0.70, MeOH), HR-FAB-MS (m/z): 567.2576 $[M+Na]^+$ (Calcd for $C_{30}H_{40}NaO_9$: 567.2570). ^{13}C- and ^{1}H-NMR data: see Tables 1 and 2.

Compound **13**: A colorless powder, $[\alpha]_D^{27}$ +100.5° (c=0.20, MeOH), HR-FAB-MS (m/z): 565.2794 $[M+Na]^+$ (Calcd for $C_{31}H_{42}NaO_8$: 565.2778). ^{13}C- and ^{1}H-NMR data: see Tables 1 and 2.

Trypanocidal Assay MC_{100} values of compounds **11**—**15** against trypomastigotes were estimated by the same method as mentioned in the previous paper.[10)] The activity was expressed as the MC_{100} value (μM), which was defined as the minimum concentration at which all the trypomastigotes become immobilized after 24 h incubation. The strain of *T. cruzi* was H6 (international code: MHOM/GT/95/SMI-06).[11)] The trypomastigotes were harvested from the culture supernatant of rhesus monkey kidney (LLC-MK2) cells infected with *T. cruzi*. LLC-MK2 cells were maintained in DMEM containing 10% FBS at 37 °C under 5% CO_2. After inoculation of trypomastigotes into LLC-MK2 cells, the medium was changed to DMEM containing 10% NCS. Samples were first dissolved in DMSO and then diluted with DMEM. Fifty microliters of each sample solution prepared by the two-fold dilution method and cell suspension (*ca.* 2×10^6 trypomastigotes/ml) was placed in 96-well microplates in duplicate and incubated at 37 °C for 24 h under 5% CO_2. The control was free from the test samples. The motion of trypomastigotes was observed under an inverted light microscope. MC_{100} values of **11**—**15** were 360, 360, 370, 320, and 330 μM, respectively.

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References and Notes

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