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Triterpenoid saponins from *Pteleopsis suberosa* stem bark

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Dedicated to the memory of Prof. Ivano Morelli.

Abstract

Thirteen oleanane saponins (**1**–**13**), four of which were new compounds (**1**–**4**), were isolated from *Pteleopsis suberosa* Engl. et Diels stem bark (Combretaceae). Their structures were determined by 1D and 2D NMR spectroscopy and ESI-MS spectrometry. The compounds were identified as 2α,3β,19α,23,24-pentahydroxy-11-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**1**), 2α,3β,19β,23,24-pentahydroxy-11-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**2**), 2α,3β,19α,23-tetrahydroxy-11-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**3**), and 2α,3β,6β,19α,24-pentahydroxy-11-oxo-olean-12- en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**4**). The presence of α,β-unsaturated carbonyl function was not common in the oleanane class and the aglycons of these compounds were not found previously in the literature. Moreover, the isolated compounds were tested against *Helicobacter pylori* standard and *vacA*, and *cagA* clinical virulence genotypes. Results showed that compound **6** has an anti-*H. pylori* activity against three metronidazole-resistant strains (Ci 1 *cagA*, Ci 2 *vacA*, and Ci 3).

Keywords: *Pteleopsis suberosa*; Combretaceae; *Helicobacter pylori*; Triterpenoid saponins

1. Introduction

In the course of our investigations on plants belonging to Malian flora, the chemical composition of *Pteleopsis suberosa* Engl. et Diels bark has been studied. The genus *Pteleopsis* belongs to Combretaceae family which includes 20 genus and about 600 species of plants distributed especially in the Tropical and Sub-Tropical regions, and in West Africa (Hutchinson, 1959). *P. suberosa* is a small tree quite widespread in the savanna and in African regions as Mali, Senegal, Guinea, Ghana, Togo, Benin, and Nigeria. The leaves of *P. suberosa* are popularly known for the treatment of meningitis, convulsive fever, and headache, while the bark is reported able to increase cereal productivity (Kerharo and Adam, 1974) and to treat jaundice, asthenia, and dysentery (Adjanhoun et al., 1986). In the Malian folk medicine the stem bark, commonly named "terenifù", is known as a traditional remedy against cough, asthma, hemorrhoids, virus, and especially against ulcer (Mariko, 1989). In the Malian Pharmacopea, the powdered bark constitutes the Calmogastryl®, a Traditional Improved Drug orally administered as decoction for the treatment of gastric and duodenal ulcers. Extracts obtained from the bark demonstrated fungicidal and fungistatic activity (Baba-Moussa et al., 1999), antitussive properties (Occhiuto et al., 1999), and antiulcer action (De Pasquale et al., 1995). Moreover, the methanolic extract showed an *in vitro* anti-*Helicobacter pylori* activity (Germanò et al., 1998). Previous phytochemical studies on the genus *Pteleopsis* were performed only on

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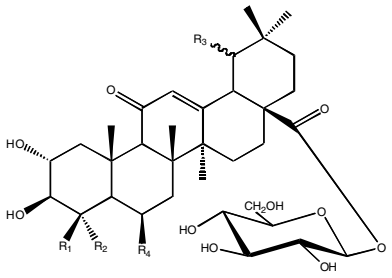
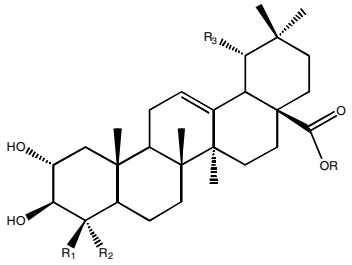
the species *P. hylodendron*, reporting the isolation and elucidation of two oleanane saponins (Ngounou et al., 1999). The aim of our work was to carry out the chemical study of *P. suberosa* bark never reported before; herein we describe the isolation and structural characterization of 13 triterpenoids (**1**–**13**), four of which (**1**–**4**) were new triterpenoid glycosides, together with their anti-*Helicobacter pylori* activities against standard and *vacA* and *cagA* clinical virulence genotypes.



Compound	R_1	R_2	R_3	R_4
1	-CH_2OH	-CH_2OH	αOH	-H
2	-CH_2OH	-CH_2OH	βOH	-H
3	-CH_3	-CH_2OH	αOH	-H
4	-CH_2OH	-CH_3	αOH	-OH



Compound	R	R_1	R_2	R_3
5	Glc	-CH_2OH	-CH_2OH	-OH
6	Glc	-CH_3	-CH_2OH	-OH
7	Glc	-CH_2OH	-CH_3	-OH
8	Glc	-CH_3	-CH_3	-OH
9	Glc	-CH_3	-CH_2OH	-H
10	Glc	-CH_2OH	-CH_2OH	-H
11	-H	-CH_2OH	-CH_3	-OH
12	-H	-CH_3	-CH_2OH	-OH
13	-H	-CH_2OH	-CH_2OH	-OH

Glc = β-D-glucopyranoside

2. Results and discussion

The chemical study of *n*-butanol and chloroform-methanol extracts of *P. suberosa* bark, by different chromatographic techniques, afforded 13 triterpenoid compounds, including 10 saponins and 3 aglycons. Compounds **1**–**4** resulted new natural products and were identified by 1D, 2D NMR spectroscopy, and ESI-MS analyses while the others compounds are known triterpenes identified by comparison of their NMR data with those reported in the literature as trachelosperidoside E-1 (**5**) (Fumiko and Tatsuo, 1987), arjunglucoside I (**6**) (Zhou et al., 1992), sericoside (**7**) (Terreaux et al., 1996), arjunetin (**8**) (Braca et al., 2001), arjunglucoside II (**9**) (Jayasinghe et al., 1993), bellericoside (**10**) (Nandy et al., 1989), sericic acid (**11**) (Bombardelli et al., 1974), arjungenin (**12**) (Jossang et al., 1996), and trachelosperogenin (**13**) (Mahato et al., 1992).

Compound **1** had a molecular formula $C_{36}H_{56}O_{13}$, as determined by ^{13}C, ^{13}C-DEPT NMR, positive ESI-MS spectrum (quasi-molecular ion peak at *m/z* 719 $[M+Na]^+$), and elemental analysis. In the ESI-MS spectrum of **1** was also evident a fragment at *m/z* 557 $[M+Na\text{-}162]^+$, due to the loss of a hexose unit. The ^{13}C NMR data (see Table 1) of compound **1** showed the presence of 36 signals, 30 of which were assigned to a triterpenoid moiety and 6 to the saccharide portion. The UV spectrum of **1** (λ_{max} at 255 nm) suggested the presence of α,β-unsaturated carbonyl function which was confirmed by carbon signals in the ^{13}C NMR spectrum at δ 128.9 (C-12), 174.0 (C-13), and 203.0 (C-11) whose resonances are consistent with references reported in the literature for 11-oxo-oleanolic aglycon type (Ikuta et al., 1995). Also the downfield shift (+4 ppm) of olefinic carbon C-12 (δ 128.9) is consistent with presence of conjugated carbonyl group (Seebacher et al., 2003). The ^1H NMR spectrum (Table 1) exhibited also the presence of signals of five tertiary methyl groups (δ 0.95, 0.98, 0.99, 1.20, and 1.58), while the resonances at δ 3.40 (*d*, $J = 5.0$ Hz), 3.50 (*d*, $J = 10.0$ Hz), and 3.91 (*ddd*, $J = 12.0$, 10.0, 3.0 Hz) were due to three protons linked at carbons bearing one hydroxyl group. The relative configurations of the hydroxylated carbons were assigned as 2α, 3β, and 19α mainly on the basis of ^1H NMR coupling and by comparison with those reported for related compounds (Fumiko and Tatsuo, 1987). We also noted that the presence of the OH group at the 19 position induced a downfield shift of the resonance of the axial proton H-16 (δ 2.47, *ddd*, $J = 13.0$, 13.0, 4.5 Hz), thus supporting the 19α-OH stereochemistry and being compatible only with a *cis* stereochemistry of the ring D/E junction. The observation of two AB doublets (δ 3.56 and 4.05, $J = 9.5$ Hz; δ 3.70 and 4.08, $J = 11.0$ Hz) indicated the presence of two hydroxymethylene. Assignments of all chemical shifts of protons and carbons of aglycon portion were ascertained from a combination of 1D-TOCSY, DQF-COSY, and HSQC spectral analysis. The substitution sites on the triterpene skeleton were confirmed by HMBC experiment showing correlation peaks between H-12 and C-14, C-9, and C-11, demonstrating the 11,12 position of enone group; between Me-30 and C-20, C-19, and C-21, consistent with the presence of a hydroxy group at C-19; finally, between H-23a and C-4, C-24, and C-3, in agreement with all the assignments of A ring. Identification of the saccharide unit was performed by analysis of NMR data: the chemical shifts, the multiplicity of signals, and the values of the coupling constants were in agreement with

Table 1
^{1}H and ^{13}C NMR data for compounds **1** and **2** (CD_3OD, 600 MHz)[a]

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1a	0.92 *dd* (12.0, 10.0)	48.1	0.94 *dd* (12.0, 10.0)	48.0
1b	3.18 *dd* (12.0, 3.0)		3.08 *dd* (12.0, 3.0)	
2	3.91 *ddd* (12.0, 10.0, 3.0)	69.4	3.93 *ddd* (12.0, 10.0, 3.0)	69.1
3	3.50 *d* (10.0)	78.9	3.49 *d* (10.0)	78.7
4		49.0		48.7
5	1.41 *br d* (11.0)	48.1	1.40 *br d* (11.0)	47.8
6a	1.48 *m*	18.7	1.48 *m*	18.7
6b	1.65 *m*		1.66 *m*	
7a	1.38 *m*	33.8	1.39 *m*	33.6
7b	1.78 *m*		1.78 *m*	
8		46.3		45.8
9	2.68 *s*	63.6	2.58 *s*	62.6
10		36.5		38.0
11		203.0		203.0
12	5.68 *s*	128.9	5.70 *s*	131.7
13		174.0		164.0
14		39.3		44.5
15a	1.23 *m*	29.2	1.33 *m*	28.7
15b	1.84 *m*		1.92 *m*	
16a	1.87 *ddd* (13.0, 4.5, 2.0)	27.8	1.89 *ddd* (13.5, 5.0, 2.0)	25.2
16b	2.47 *ddd* (13.0, 13.0, 4.5)		2.19 *ddd* (13.5, 13.5, 5.0)	
17		45.0		49.0
18	3.20 *d* (5.0)	46.3	2.81 *d* (9.0)	50.0
19	3.40 *d* (5.0)	81.1	3.56 *d* (9.0)	75.7
20		36.5		36.1
21a	1.08 *m*	29.2	1.42 *m*	35.1
21b	1.73 *m*		1.67 *m*	
22a	1.79 *m*	32.2	1.67 *m*	31.6
22b	1.85 *m*		1.84 *m*	
23a	4.05 *d* (9.5)	64.4	4.06 *d* (10.0)	64.2
23b	3.56 *d* (9.5)		3.56 *d* (10.0)	
24a	4.08 *d* (11.0)	62.2	4.09 *d* (11.0)	62.0
24b	3.70 *d* (11.0)		3.69 *d* (11.0)	
25	0.95 *s*	19.3	1.25 *s*	18.4
26	1.20 *s*	18.0	1.01 *s*	19.4
27	1.58 *s*	22.6	1.47 *s*	21.7
28		178.3		177.0
29	0.98 *s*	27.9	0.91 *s*	17.5
30	0.99 *s*	24.5	1.05 *s*	30.0
1′	5.43 *d* (8.5)	95.7	5.44 *d* (8.5)	95.7
2′	3.35 *dd* (9.0, 8.5)	73.7	3.34 *dd* (9.0, 8.5)	73.6
3′	3.43 *t* (9.0)	78.1	3.44 *t* (9.0)	78.0
4′	3.37 *t* (9.0)	71.0	3.38 *t* (9.0)	70.9
5′	3.38 *m*	78.7	3.38 *m*	78.6
6′a	3.66 *dd* (12.0, 5.5)	62.2	3.69 *dd* (12.0, 5.5)	62.0
6′b	3.85 *dd* (12.0, 3.0)		3.85 *dd* (12.0, 3.0)	

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

presence of a glucopyranoside portion linked at C-28 of the aglycon as revealed from the resonances of the anomeric proton (δ 5.43, $J = 8.5$ Hz) and carbon (δ 95.7). After hydrolysis of **1** with 1 N HCl and its trimethylsilylation for GC analysis, the retention time of the sugar was the same of an authentic sample of D-glucose. Thus, the structure of compound **1** was established as 2α,3β,19α,23,24-pentahydroxy-11-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

The ESI-MS spectrum of compound **2** (molecular formula $C_{36}H_{56}O_{13}$) showed a quasi-molecular peak at *m*/*z* 719 corresponding to sodium cationized species $[M+Na]^+$ and a fragment at *m*/*z* 557 due to the loss of a hexose unit $[M+Na\text{-}162]^+$, suggesting that **2** was an isomer of compound **1**. Analysis of ^{1}H and ^{13}C NMR spectra of **2** (see Table 1) and comparison with those of **1**, showed differences in the E ring signals of the aglycon. In fact, the doublet at δ 3.20 (H-18, $J = 5.0$ Hz) in **1** underwent highfield shift at δ 2.81 ($J = 9.0$ Hz) in **2**, while the resonance of H-19 (δ 3.40, $J = 5.0$ Hz) shifted downfield at δ 3.56 ($J = 9.0$ Hz) for compound **2**. Analogously, C-19 exhibited a resonance at δ 75.7, with difference of −5 ppm in comparison with

compound **1**, due to the opposite configuration of C-19 chiral carbon. In addition, the chemical shift values of Me-29 and Me-30 were shifted both in the ^{1}H and ^{13}C NMR spectra of **2** in comparison with those of **1**. The β-orientation of the hydroxyl group at C-19 was also derived from the coupling constant values of the H-19 signal at δ 3.56 (*d*, $J = 9.0$ Hz). Assignments of the chemical shifts were obtained by analysis of DQF-COSY, HSQC, and HMBC data. Therefore, **2** was identified as 2α,3β,19β,23,24-pentahydroxy-11-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

The molecular formula of compound **3** ($C_{36}H_{56}O_{12}$) was deduced from full mass spectrum acquired in the ESI-MS analysis, ^{1}H and ^{13}C NMR data, and elemental analysis. A quasi-molecular peak $[M+Na]^{+}$ at *m*/*z* 703 revealed a difference of 16 mass unit in comparison with compound **1**, while the fragment $[M+Na-162]^{+}$ at *m*/*z* 541 indicated the loss of a hexose unit, identified as D-glucose by spectral data and GC analyses. Comparing the ^{1}H NMR spectrum of compounds **3** and **1** (see Table 2), the presence of an additional singlet at δ 0.73 and the absence of one AB doublet was observed for **3**, suggesting one more methyl group

Table 2
^{1}H and ^{13}C NMR data for compounds **3** and **4** (CD_3OD, 600 MHz)[a]

Position	**3**		**4**	
	δ_H	δ_C	δ_H	δ_C
1a	0.95 *dd* (11.5, 10.0)	48.0	0.91 *dd* (12.0, 10.0)	50.1
1b	3.18 *dd* (11.5, 3.5)		3.16 *dd* (12.0, 3.0)	
2	3.79 *ddd* (11.5, 10.0, 3.5)	69.0	3.82 *ddd* (12.0, 10.0, 3.0)	69.4
3	3.43 *d* (10.0)	78.0	3.44 *d* (10.0)	78.1
4		45.1		45.0
5	1.32 *br d* (10.5)	48.0	1.29 *d* (2.0)	48.7
6a	1.38 *m*	18.5	4.42 *m*	67.0
6b	1.60 *m*			
7a	1.35 *m*	33.6	1.60 *m*	40.9
7b	1.73 *m*		1.97 *m*	
8		48.3		47.3
9	2.69 *s*	63.0	2.72 *s*	63.5
10		36.0		38.1
11		203.0		203.0
12	5.68 *s*	128.0	5.70 *s*	129.0
13		174.2		174.0
14		39.6		39.9
15a	1.25 *m*	29.7	1.21 *m*	29.2
15b	1.86 *m*		1.86 *m*	
16a	1.88 *ddd* (13.0, 4.5, 2.0)	27.6	1.87 *ddd* (13.0, 4.0, 2.0)	28.0
16b	2.45 *ddd* (13.0, 13.0, 4.5)		2.47 *ddd* (13.0, 13.0, 4.0)	
17		45.3		45.2
18	3.20 *d* (5.0)	46.0	3.23 *d* (5.0)	46.0
19	3.39 *d* (5.0)	81.2	3.41 *d* (5.0)	81.0
20		36.5		36.4
21a	1.09 *m*	28.8	1.08 *m*	29.2
21b	1.74 *m*		1.80 *m*	
22a	1.78 *m*	32.3	1.79 *m*	32.4
22b	1.85 *m*		1.85 *m*	
23a	3.50 *d* (12.0)	65.4	1.33 *s*	23.7
23b	3.29 *d* (12.0)			
24a	0.73 *s*	13.4	3.64 *d* (11.0)	65.4
24b			3.48 *d* (11.0)	
25	0.96 *s*	19.6	1.01 *s*	15.0
26	1.20 *s*	18.0	1.27 *s*	20.7
27	1.60 *s*	23.4	1.55 *s*	22.0
28		178.0		178.1
29	0.98 *s*	27.9	0.99 *s*	28.1
30	0.99 *s*	24.7	0.98 *s*	24.0
1′	5.43 *d* (8.5)	95.8	5.42 *d* (8.0)	95.8
2′	3.34 *dd* (9.5, 8.5)	73.7	3.34 *dd* (9.0, 8.0)	73.7
3′	3.38 *t* (9.5)	78.6	3.34 *t* (9.0)	77.6
4′	3.37 *t* (9.5)	71.0	3.37 *t* (9.0)	71.0
5′	3.40 *m*	78.0	3.37 *m*	78.7
6′a	3.69 *dd* (11.5, 5.0)	62.0	3.70 *dd* (12.0, 5.0)	62.3
6′b	3.84 *dd* (11.5, 3.0)		3.85 *dd* (12.0, 3.5)	

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

linked to C-4. HMBC correlations confirmed this evidence: the signal at δ 0.73 (Me-24) showed a cross-peaks with resonances at δ 48.0 (C-5), 45.1 (C-4), 69.0 (C-2), and 78.0 (C-3). Therefore, the structure assigned at compound **3** was 2α,3β,19α,23-tetrahydroxy-11-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

Compound **4** showed the same molecular formula ($C_{36}H_{56}O_{13}$) of **1** and **2**, as well as the same fragmentation pattern in the ESI MS spectrum: *m/z* 719 $[M+Na]^+$, *m/z* 557 $[M+Na-162]^+$. On the contrary, ^1H and ^{13}C NMR analyses (see Table 2) revealed some structural differences in the aglycon portion. The C-23 hydroxymethyl group in compound **1** was replaced in **4** by a methyl group (δ 23.7) inducing a highfield shift (−4 ppm) of C-4 resonance. The presence of a signal at δ 67.0 (C-6) in the ^{13}C NMR spectrum indicated that an additional hydroxyl unit was linked to the aglycon, as showed by multiplet at δ 4.42 (H-6) in the ^1H NMR spectrum. The position of this OH group was deduced from downfield shift (+7 ppm) of C-7 and unequivocal HMBC correlations (H-6–C-5, H-6–C-7, H-6–C-4). The β configuration of this OH group was observable from the coupling constant of H-5 ($J = 2.0$ Hz) and the unresolved signal of the equatorial H-6 proton (δ 4.42, 1H, *m*, $W_{1/2} = 6.0$ Hz). This was supported also by the resonances of Me-25 (δ 1.01) and Me-26 (δ 1.27), which were significantly shifted downfield by 1,3-diaxial interactions with respect to compounds **1**, **3**, and 6α-OH triterpenic derivatives spectra (Aquino et al., 1997). Moreover, 1D-ROESY experiments confirmed 6β-OH configuration from the observed correlation peak H-6–H-23. Thus, compound **4** was determined to be 2α,3β,6β,19α,24-pentahydroxy-11-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

The anti-*H. pylori* activities of isolated compounds (**1**–**13**), based on the disk diffusion method, evidenced that compound **6** (arjunglucoside I) has activity against all the *H. pylori* strains tested (inhibition zone diameter between 32 and 40 mm for 200 μg/disk). The remaining compounds exhibited no activities (inhibition zone diameter < 12 mm). The MIC values of **6**, reported in Table 3, showed inhibitory effects at higher concentrations compared to clarithromycin. However, it should be interesting to note that arjunglucoside I was more active respect to metronidazole against three antibiotic resistant strains, two of which were *vacA* and *cagA* clinical virulence genotypes. It is well known that the treatment of *H. pylori* infection is mainly based on the use of combinated therapies such as clarithromycin, amoxicillin, and metronidazole (Ochi et al., 2005; Wang and Huang, 2005a,b). Anyway, there is a great interest in the search for new therapeutic agents towards *H. pylori* since the extent of its antibiotic-resistant strains has been reported (Megraud and Doermann, 1998).

Table 3
MIC values of compound **6** (arjunglucoside I) against *H. pylori*

Strain	MIC (μg/ml)		
	Clarithromycin	Metronidazole	Compound **6**
ATCC 43504	0.06	7.8	3.9
Ci 1 *cag A*	0.12	31.2	7.8
Ci 2 *vac A*	0.48	31.2	7.8
Ci 3	0.12	>250	1.9

3. Experimental

3.1. General procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. Elemental analyses were obtained using a Carlo Erba 1106 elemental analyzer. UV spectra where recorded on a Perkin-Elmer-Lambda 12 spectrophotometer. A Bruker DRX-600 NMR spectrometer provided with an UXNMR software package was used for NMR experiments. ESI-MS spectra were obtained from a LCQ Advantage ThermoFinnigan spectrometer, equipped with a Xcalibur software. HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Waters R401 refractive index detector and Shimadzu injector.

3.2. Plant material

The bark of *P. suberosa* was collected in Kolokani, Mali, on February 2001 and identified by Prof. N'Golo Diarra of the Departement Medicine Traditionelle (DMT), Bamako, Mali, where a voucher specimen (no. D-194) is deposited.

3.3. Extraction and isolation

The dried powdered bark of *P. suberosa* (405.0 g), defatted with *n*-hexane, was successively extracted for 48 h with $CHCl_3$, $CHCl_3$–MeOH (9:1), and MeOH, by exhaustive maceration (3 × 2 l), to give 3.2, 5.0, and 70.4 g of the respective dried residues. A portion of methanol extract (50.0 g) was partitioned between *n*-BuOH and H_2O, in order to obtain a *n*-butanol soluble portion (12.5 g) that was submitted to a Sephadex LH-20 column using MeOH as eluent and collecting fractions of 8 ml each. Three major fractions (A–C) were obtained by TLC results on silica 60 F_{254} gel-coated glass sheets with *n*-BuOH–AcOH–H_2O 60:15:25 and $CHCl_3$–MeOH–H_2O 40:9:1. Fraction B (940.0 mg) was purified by RP-18 flash column chromatography ($\varnothing = 40$ mm, flow 20 ml min^{-1}) eluting with MeOH–H_2O (3:7) followed by increasing concentrations of MeOH in H_2O. The following mixtures of solvents were used to give compound **6** (113.8 mg) and five major groups (1–5): MeOH–H_2O (3:7), MeOH–H_2O (2:3), MeOH–H_2O (1:1), MeOH–H_2O (3:2), MeOH–H_2O (7:3), and finally MeOH–H_2O (4:1). The group 1 (20.9 mg) was successively chromatographed over RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (3:7) to yield compounds **4** (1.2 mg), **1**

(1.8 mg), and **2** (1.0 mg). Group 3 (139.0 mg) was further purified by RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (1:1) to afford compounds **5** (26.1 mg) and **3** (2.5 mg). Isolation of compounds **7** (10.7 mg) and **8** (22.7 mg) occurred submitting group 4 to RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (3:2). The $CHCl_3$–MeOH residue (5.0 g) was chromatographed on Sephadex LH-20 using MeOH as eluent; fractions of 8 ml were collected and grouped into four major fractions (A–D), using the same TLC analyses of the *n*-BuOH extract. Fraction B (2.2 g) was submitted to Si gel flash column chromatography (∅ = 2.5 cm, flow 20 ml min^{-1}) eluting with mixtures of $CHCl_3$–MeOH. The chromatography was carried out at increasing polarity starting from $CHCl_3$ followed by $CHCl_3$–MeOH (9:1), $CHCl_3$–MeOH (8.5:1.5), $CHCl_3$–MeOH (4:1), $CHCl_3$–MeOH (7:3), $CHCl_3$–MeOH (1:1), and ending with MeOH to give compounds **13** (11.1 mg), **8** (22.6 mg), and **7** (10.2 mg). All the others tubes collected were combined to obtain seven major groups (1–7). Group 1 (100.0 mg) was subjected to RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (6.5:3.5) to give compounds **11** (1.6 mg) and **12** (2.2 mg). Group 3 (188.0 mg) was further purified by RP-HPLC using C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (5.5:4.5) yielding compound **9** (2.1 mg), while group 5 (121.3 mg) was separated on the same column eluting with MeOH–H_2O (1:1) to afford compound **10** (12.6 mg).

Compound 1: white solid; mp 185 °C; $[\alpha]_D^{25}$: −20° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 255 (3.83); ^1H and ^{13}C NMR: see Table 1; ESI-MS: *m/z* 719 $[M+Na]^+$, 557 $[M+Na\text{-}162]^+$, 539 $[M+Na\text{-}162\text{-}18]^+$; *anal.* C 62.12%, H 8.11% calcd. for $C_{36}H_{56}O_{13}$, C 62.05%, H 8.10%.

Compound 2: white solid; mp 180 °C *dec*; $[\alpha]_D^{25}$: −27.6° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.92); ^1H and ^{13}C NMR: see Table 1; ESI-MS: *m/z* 719 $[M+Na]^+$, 557 $[M+Na\text{-}162]^+$, 539 $[M+Na\text{-}162\text{-}18]^+$; *anal.*: C 62.08%, H 8.13% calcd. for $C_{36}H_{56}O_{13}$, C 62.05%, H 8.10%.

Compound 3: white solid; mp 130 °C *dec*; $[\alpha]_D^{25}$: −8° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 257 (3.97); ^1H and ^{13}C NMR: see Table 2; ESI-MS: *m/z* 703 $[M+Na]^+$, 541 $[M+Na\text{-}162]^+$, 523 $[M+Na\text{-}162\text{-}18]^+$; *anal.*: C 63.52%, H 8.31% calcd. for $C_{36}H_{56}O_{12}$, C 63.51%, H 8.29%.

Compound 4: white solid; mp 195-200 °C; $[\alpha]_D^{25}$: −32° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 255 (3.65); ^1H and ^{13}C NMR: see Table 2; ESI-MS: *m/z* 719 $[M+Na]^+$, 557 $[M+Na\text{-}162]^+$, 539 $[M+Na\text{-}162\text{-}18]^+$; *anal.*: C 62.11%, H 8.08% calcd. for $C_{36}H_{56}O_{13}$, C 62.05%, H 8.10%.

3.4. Acid hydrolysis of compounds 1–4

A solution of each compound (**1**–**4**, 1.0 mg) in 1 N HCl (1 ml) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. Each residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 ml), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic sample D-glucose after treatment with 1-(trimethylsilyl)imidazole in pyridine.

3.5. Bacterial strains and cultures

H. pylori ATCC 43504, and three clinical isolates (Ci 1 *cagA*, Ci 2 *vacA*, Ci 3) were obtained by biopsy from individual patients with gastritis or duodenal ulcers and they were identified using standard diagnostic procedure. The *cagA* and *vacA*, virulence genotypes, were determined by multiplex PCR (van Doorn et al., 1998). All strains were stored at −80 °C in Brucella broth (Difco) supplemented with 20% glycerol until use.

3.6. Antibacterial screening

The disk-diffusion method was used to screen the anti-*H. pylori* activity of isolated compounds. Sample solutions (ranged from 0.5 to 3.0 mg/ml) were prepared by dissolving each compound in DMSO (Sigma). Positive control used the antibiotics Clarithromycin (Abbot inc) and Metronidazole (Sigma). No activity was considered in the presence of an alone of inhibition zone <12 mm. All strains of *H. pylori* (ATCC43504) were cultured for 4 days at 37 °C in Brucella broth (Difco) containing 5% horse serum (Oxoid) under micro-aerophilic conditions using a disposable O_2 absorbing and CO_2 generating agent, AnaeroGen (Oxoid), with humidity. The culture was then diluted and adjusted to about 1×10^7 CFU/ml with the fresh medium and was uniformly spread with a cotton swab onto the Isosensitest agar (Oxoid) containing 10% horse blood (Oxoid). Sterile blank disks 6 mm (Whatman) were placed on the agar surface. Then, 10 μl of the sample solutions was transfused onto the disks. After 4 days' incubation at 37 °C under the micro-aerophilic conditions with humidity, the plates were screened for growth inhibition zones.

3.7. MIC determination

The MICs of compound **6** and antibiotics were determined by using the 2-fold agar plate-dilution method recommended by the National Committee for Clinical Laboratory Standard (National Committee for Clinical Laboratory Standards, 2003). Bacterial suspensions equivalent to a 2.0 MacFarland standard (containing 1×10^8 CFU/ml) were prepared in saline from a 72 h subculture from a blood agar plate, and the bacteria (5 μl per spot) were applied with an inoculator into the surfaces

of 10 mm agar layers. The plates were read after 4 days' incubation at 37 °C under the micro-aerophilic conditions with humidity.

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