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Triterpenoid Saponins from *Symplocos lancifolia*

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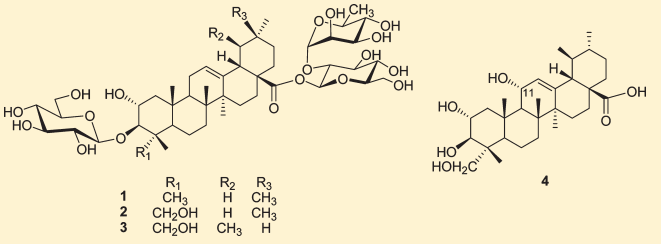
Supporting Information

ABSTRACT: Three new bidesmosidic saponins (**1**−**3**) and a new ursane triterpenoid, 2α,3β,11α,23-tetrahydroxyurs-12-en-28-oic acid (**4**), along with seven known compounds, were isolated from a methanolic extract of the leaves of *Symplocos lancifolia*. The bidesmosidic saponins were found to possess the same sugar unit part, composed of two β-D-glucose moieties and one α-L-rhamnose moiety, linked to maslinic acid, arjunolic acid, and asiatic acid, respectively. Their structures were elucidated by interpretation of their 1D and 2D NMR spectra and completed by analysis of the HRESIMS data. The antibacterial activity of the isolated triterpenoids was evaluated against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and several showed activity against Gram-positive bacteria.



The genus *Symplocos* of the family Symplocaceae comprises 300−500 species distributed in tropical and subtropical Asia and America.[1] A number of plants of this genus are used as traditional herbal medicines for the treatment of diarrhea, dysentery, eye disease, hemorrhagic gingivitis, menorrhagia, and uterine disorders,[2] bowel complaints and ulcers,[3] female diseases,[4] and malaria, tumefaction, enteritis, and snake bites.[5] Antimicrobial activity has also been reported.[6] Recently, much attention has been paid to *Symplocos* species due to their diverse biological activities, particularly anti-HIV, inhibition against phosphodiesterase, and potential antitumor applications.[7] The chemical constituents of *Symplocos* species include triterpenoids, flavonoids, lignans, phenols, alkaloids, sterols, and iridoids.[7] Pentacyclic triterpenoids are the dominant constituents within the genus *Symplocos* and exhibit oleanane, ursane, or lupane skeletons.[7]

Leaves of *S. lancifolia* Siebold & Zucc. were collected in Bac Quang, Ha Giang Province, in Vietnam. This species was selected as part of a systematic screening program of plants growing in Vietnam to find potentially cytotoxic compounds. The ethanolic and ethyl acetate extracts of the leaves, stems, and fruits of *S. lancifolia* were tested in vitro against KB cells, but showed no cytotoxic activity. Nevertheless, the phytochemical study of this *Symplocos* species was pursued in order to establish chemotaxonomic criteria based on the structure of the triterpenoid constituents and to evaluate antibacterial activity of the isolated compounds. This study was carried out under a collaborative framework initiated between the Centre National de la Recherche Scientifique (France) and the Vietnamese Academy of Sciences and Technology (Vietnam). A previous chemical investigation of the aerial parts of *S. lancifolia* led to the identification of ursolic acid, seven flavonoids, two lignans [(+)-isolariciresinol and (+)-pinoresinol], and four phenolic compounds.[8] The present paper reports the isolation and structural elucidation of four new triterpenoid derivatives from the leaves of *S. lancifolia*, among them three bidesmosidic saponins (**1**−**3**), possessing maslinic acid, arjunolic acid, and asiatic acid as aglycones, and 11α-hydroxyasiatic acid (**4**), along with seven known compounds. The antibacterial activity of the isolated triterpenoids and the bidesmosidic saponins (**1**−**3**) was evaluated against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

RESULTS AND DISCUSSION

The dried and powdered leaves of *S. lancifolia* were extracted with boiling 80% methanol. The aqueous methanolic extract was concentrated, and the aqueous residue was partitioned between

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Table 1. ^{13}C and ^{1}H NMR Spectroscopic Data (CD_3OD) for the Aglycones of 1−3 and for 4

	1		2		3		4	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1a	46.6	0.90, m	47.3	0.89, m	47.4	0.90, m	49.0	1.16, tm (13.2)
1b		2.03 (dd, 12.5, 4.4)		2.02, dd (12.7, 4.3)		2.06, dd (12.7, 4.4)		2.49, dd (13.2, 4.4)
2	67.2	3.75, ddd (10.2, 9.3, 5.1)	68.2	3.83, ddd (10.9, 9.5, 4.3)	68.3	3.84, m	69.9	3.73, ddd (11.5, 9.7, 4.3)
3	95.2	3.03, d (9.3)	88.7	3.48, d (9.5)	88.7	3.48, d (9.3)	77.8	3.40, d (9.7)
4	40.5		45.2		45.2		44.2	
5	55.7	0.89, dm (12)	47.8	1.33, dm (13)	47.8	1.33, dm (13)	48.2	1.33, dm (13)
6a	18.2	1.43, m	18.9	1.40, m	18.9	1.41, m	19.0	1.43, m
6b		1.61, m		1.51, m		1.50, m		1. 51, dm (13.4)
7a	33.0	1.49, m	33.3	1.42, m	33.7	1.39, m	34.1	1.29, dm (12.4)
7b		1.60, m		1.61, m		1.65, m		1.66, tdm (12.4, 4.4)
8	39.8		40.7		41.1		43.8	
9	48.2	1.65, m	49.1	1.69, m	49.0	1.62, m	51.3	1.84, d (9.3)
10	38.0		38.6		38.6		39.8	
11	23.5	1.98, m	24.7	1.98, m	24.5	2.01, m	82.1	4.50, dd (9.3, 3.3)
12	122.5	5.30, brt (3.3)	123.4	5.30, brt (3.4)	127.1	5.29, brt (3.5)	127.4	5.59, d (3.3)
13	143.5		145.0		139.1		144.3	
14	42.2		43.2		43.5		43.4	
15a	28.3	1.23, m	29.2	1.22, m	29.2	1.18, d (13)	29.3	1.12, dm (14)
15b		1.63, td (13, 4)		1.65, m		1.87, td (13, 4)		1.89, td (14, 4.7)
16a	22.8	1.68, m	24.0	1.67, m	25.4	1.71, dm (13)	25.2	1.69, m
16b		2.09, td (13, 4)		2.09, td (13.4, 4.2)		2.12, td (13, 4)		2.09, td (13.4, 4.3)
17	nd		48.1		49.9		48.7	
18	41.8	2.85, dd (13.4, 3.5)	42.9	2.85, dd (13, 3.5)	54.3	2.26, d (11.5)	53.7	2.31, d (11.1)
19a	47.2	1.16, m	47.3	1.17, m	40.4	1.42, m	40.3	1.40, m
19b		1.75, t (14)		1.75, m				
20	30.5		31.6		39.8	1.15, m	40.5	1.05, m
21a	33.9	1.25, m	34.9	1.24, m	31.7	1.35, m	31.7	1.38, m
21b		1.42, m		1.42, m		1.54, td (13, 3)		1.54, m
22a	32.9	1.62, m	33.0	1.63, m	37.4	1.68, m	38.1	1.69, m
22b		1.82, td (14.3, 4.2)		1.83, t (13.9, 3.6)		1.80, td (13, 3)		1.74, tm (12.9)
23	28.0	1.14, s	64.0	3.27, d (12)	64.1	3.27, d (11.5)	66.1	3.30, d (11)
				3.74, d (12)		3.74, d (11.5)		3.55, d (11)
24	17.2	0.91, s	14.4	0.76, s	14.5	0.76, s	13.8	0.74, s
25	16.2	1.05, s	17.7	1.07, s	17.9	1.09, s	18.8	1.19, s
26	16.8	0.83, s	17.8	0.83, s	18.2	0.87, s	19.5	0.92, s
27	25.2	1.18, s	26.2	1.20, s	23.9	1.16, s	23.1	1.23, s
28	178.2		178.0		178.0		n.d.[a]	
29	32.7	0.93, s	33.5	0.93, s	17.6	0.92, d (6.3)	17.8	1.05, d (6.4)
30	23.3	0.96, s	24.1	0.96, s	21.5	0.97, d (6.3)	21.6	1.01, d (6.4)

[a] n.d. not detected.

ethyl acetate and water to give an aqueous saponin extract and an organic extract containing triterpenes and sterols. Purification of the aqueous extract was performed using reversed-phase C_{18} column chromatography and semipreparative HPLC and afforded three new triterpenoid saponins (**1**−**3**). The purification of the ethyl acetate extract gave the new ursane triterpenoid 11α-hydroxyasiatic acid (**4**), along with seven known compounds, identified by comparison of their NMR data with those reported in the literature as stigmasterol 3-*O*-β-D-glucoside,[9] three ursane triterpenoids, ursolic acid,[10] corosolic acid,[10a,11] asiatic acid,[10a,12] and three oleanane triterpenoids, oleanolic acid,[10] maslinic acid,[10] and arjunolic acid.[10a,12,13]

Acid hydrolysis of the saponin mixture A gave a mixture of sugars, identified as glucose and rhamnose by TLC, and the absolute configurations as D and L, respectively, were determined by the measurement of their optical rotations after separation by preparative TLC.

Compound **1** showed a $[M + Na]^+$ quasimolecular ion peak at m/z 965.5084 in the positive HRESIMS ($C_{48}H_{78}O_{18}Na$), indicating a molecular mass of 942 Da. The ^{1}H NMR spectra of **1** exhibited signals of an olefinic proton at δ 5.30 (brt, J = 3.3 Hz, H-12) and seven methyl singlets, which were characteristic of a Δ^{12} oleanene skeleton (Table 1). The spectrum showed signals of two oxygen-bearing methine protons at δ 3.03 (d, J = 9.3 Hz,

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Table 2. ^{13}C and ^{1}H NMR Spectroscopic Data (CD_3OD) for the Sugar Units of 1−3

	1		2		3	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
3-β-D-glucose						
1′	105.5	4.36, d (7.7)	105.5	4.43, d (7.8)	105.6	4.43, d (7.8)
2′	74.5	3.28, dd (8.8, 8)	75.4	3.26, dd (8.7, 7.8)	75.4	3.26, t (7.9)
3′	77.3	3.37, t (8.9)	78.2	3.39, m	78.2	3.38, m
4′	70.4	3.35, m	71.3	3.36, m	71.3	3.37, m
5′	77.3	3.34, m	78.1	3.36, m	78.1	3.36, m
6a′	61.5	3.69, m	62.3	3.70, dd (11.7, 5)	62.3	3.70, dm (11.8)
6b′		3.88, dm (11.1)		3.88, dm (11.7)		3.88, dm (11.8)
28-β-D-glucose						
1″	94.9	5.45, d (7.5)	95.1	5.46, d (7.5)	95.0	5.41, d (7.8)
2″	76.3	3.61, t (8.6)	77.2	3.60, dd (8.3, 7.5)	76.4	3.63, t (8)
3″	78.3	3.57, t (8.6)	79.2	3.57, t (8.5)	79.5	3.57, t (8.6)
4″	70.4	3.42, t (9.9)	71.3	3.42, t (9.3)	71.5	3.41, t (9.4)
5″	77.3	3.34, m	78.4	3.35, m	78.4	3.34, m
6a″	61.5	3.69, m	62.3	3.69, dd (12, 4.3)	62.6	3.69, dd (11.8, 4)
6b″		3.81, dd (12, 2.1)		3.81, dd (12, 1.9)		3.79, dd (11.8, 2.2)
2″-α-L-rhamnose						
1‴	101.2	5.39, d (1.5)	101.7	5.38, d (1.3)	101.3	5.47, d (1.3)
2‴	71.0	3.95, dd (3.3, 1.5)	71.9	3.96, m	72.0	3.93, dd (3.1, 1.5)
3‴	71.3	3.67, m	72.2	3.68, dd (9, 3)	72.2	3.66, dd (9.6, 3.3)
4‴	72.7	3.40, t (9.2)	73.7	3.40, t (9.8)	73.7	3.41, t (9.6)
5‴	69.4	3.77, m	70.3	3.77, m	70.2	3.84, m
6‴	17.4	1.26, d (6.2)	18.2	1.26, d (6.2)	18.4	1.29, d (6.2)

H-3α) and 3.75 (ddd, J = 10.2, 9.3, 5.1 Hz, H-2β), suggesting the presence of maslinic acid as the aglycone.[10] Comparison of the spectroscopic data of **1** with maslinic acid indicated that this genin is substituted at positions C-3 (δ_C 95.2) and C-28 (δ_C 178.2). The MS^2 experiment of the $[M + Na]^+$ ion gave positive fragments at m/z 657 $[M + Na - 162 - 146]^+$ and 331 $[162 + 146 + Na]^+$, attributed to the losses of a terminal hexose and a terminal desoxyhexose, and a diglycoside chain consisting of a hexose and a desoxyhexose, respectively. These results suggested that saponin **1** contains three sugar units. The three anomeric carbons were detected at δ_C 94.9, 101.2, and 105.5 in the ^{13}C NMR spectrum, attached to doublets at δ_H 5.45, 5.39, and 4.36, respectively, in the HSQC experiment. Complete assignments of the proton system of each glycosidic unit were achieved by analysis of the COSY, TOCSY, and HSQC spectra. The units with anomeric protons at δ 4.36 (d, J = 7.7 Hz) and 5.45 (d, J = 7.5 Hz) corresponded to two hexoses with their methylene carbons at δ 61.5 and were identified as a terminal β-D-glucopyranosyl (δ_H 4.36) and a β-D-glucopyranosyl ester (δ_H 5.45), substituted at the C-2″ position, as observed by the deshielding of the C-2″ chemical shift (δ_C 76.3) (Table 2). The third glycosidic unit, with an anomeric proton at δ 5.39 (d, J = 1.5 Hz) and a methyl doublet at δ 1.26 (J = 6.2 Hz), was identified as a L-rhamnopyranosyl moiety (Table 2). The small coupling constant of the anomeric proton and the chemical shift of C-5‴ (δ_C 69.4) indicated the usual α-configuration for this sugar.[14] Sequencing of the glycosidic chains in saponin **1** was carried by analysis of HMBC and ROESY experiments. The HMBC spectrum showed cross-peaks between C-28 (δ 178.2) of the maslinic acid aglycone and H-1″ of the glucose ester (δ 5.45). In turn, the ROESY spectrum showed cross-peaks between H-3 of maslinic acid and H-1′ of the terminal glucose (δ 4.36) and between H-2″ (δ 3.61) of the glucose ester and H-1‴ of the terminal rhamnose unit (δ 5.39). Thus, saponin **1** was determined as 3-*O*-[β-D-glucopyranosyl]-28-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]maslinic acid.

The positive HRESIMS of compound **2** gave a quasimolecular ion peak at m/z 981.5053 $[M + Na]^+$, in agreement with a molecular formula of $C_{48}H_{78}O_{19}Na$ and suggesting a M_r of 958. The positive-ion fragments observed at m/z 673 $[M + Na - 162 - 146]^+$ and 331 $[162 + 146 + Na]^+$ indicated the same fragmentation for the sugar part compared to compound **1**, and thus, an additional hydroxy group occurred on the genin. The ^{1}H and ^{13}C NMR data of compound **2** were closely comparable to those of **1**, except for the presence of an oxygen-bearing methylene carbon (δ 64.0) instead of a methyl singlet (Table 1). The additional hydroxy group was placed at the C-23 position, as in arjunolic acid,[12] due to the observation of ROE effects between H_3-23 and H-3α and H_3-23 and H-1′ and of the carbon resonance of C-24 at δ 14.4 (Table 1). Saponin **2** was thus assigned as 3-*O*-[β-D-glucopyranosyl]-28-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]arjunolic acid.

Compound **3** was found to possess the same molecular formula as **2** as observed by the molecular ion at m/z 981.5026 $[M + Na]^+$ in the positive HRESIMS. The identical MS/MS fragmentation suggested an isomeric skeleton for the genins of the two compounds. Analysis of the 2D-NMR (COSY, TOCSY, HSQC, and HMBC) data showed that compound **3** has the same glycosidic part as compounds **1** and **2** (Table 2). The ^{1}H NMR spectrum of **3** exhibited signals of one olefinic proton at δ 5.29 (brt, J = 3.5 Hz), four methyl singlets (δ 0.76, 0.87, 1.09, and 1.16), and two methyl doublets [δ 0.92 (J = 6.3 Hz) and 0.97 (J = 6.3 Hz)], characteristic of the ursene skeleton, which was trihydroxylated in positions C-2α (δ_H 3.84), C-3β [δ_H 3.48 (d, J = 9.3 Hz)], and C-23 [δ_H 3.27, 3.74, d, J = 11.5 Hz)], as in asiatic acid[12] (Table 1). Thus, the structure of saponin **3** was elucidated as 3-*O*-[β-D-glucopyranosyl]-28-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]asiatic acid.

The new triterpenoid **4** gave a molecular formula of $C_{30}H_{48}O_6$ ($[M - H]^-$ at m/z 503) in the negative ESIMS. The ^{13}C NMR spectrum displayed 30 signals, inclusive of six methyl groups (δ 13.8, 17.8, 18.8, 19.5, 21.6, and 23.1), three secondary hydroxy

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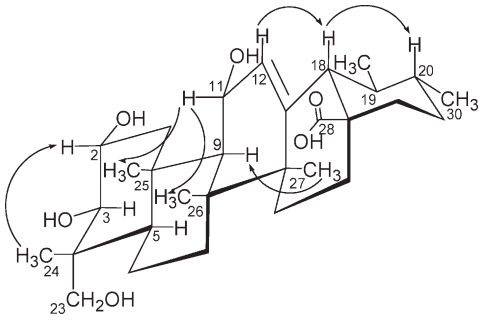
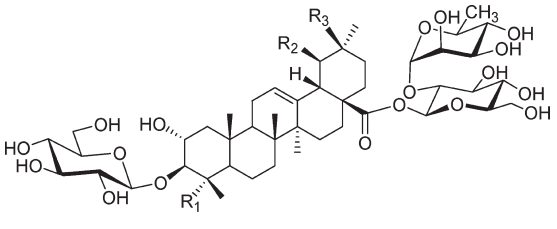


Figure 1. ROE interactions for compound **4**.

groups (δ 69.9, 77.8, and 82.1), a primary carbinol (δ 66.1), and one trisubstituted double bond (δ 127.4, 144.3) (Table 1). The ^{1}H NMR spectrum displayed signals for two secondary methyl groups at δ 1.01 (d, J = 6.4 Hz) and 1.05 (d, J = 6.4 Hz), four tertiary methyls (δ 0.74, 0.92, 1.19, and 1.23), an ethylene proton at δ 5.59 (d, J = 3.3 Hz), and a proton doublet at δ 2.31 (J = 11.1 Hz) attributed to H-18, indicating the presence of a Δ^{12}-ursene skeleton. This compound was substituted by four hydroxy groups, as observed by the presence in the ^{1}H NMR spectrum of three oxygen-bearing methine protons (δ 3.40, 3.73, and 4.50) and one primary hydroxy group (δ 3.30 and 3.55, d, J = 11 Hz), attributed to H-23, as in asiatic acid, due to the carbon resonance of C-24 at δ 13.8 (Table 1). The doublet at δ 3.40 (J = 9.7 Hz) was assigned to axial H-3α, coupled in the COSY spectrum with a signal at δ 3.73 (ddd, J = 11.5, 9.7, 4.3 Hz), assigned to H-2 in a β-axial position. The vinylic proton H-12 appeared as a doublet at δ 5.59 and coupled in the COSY spectrum with the proton signal at δ 4.50 (dd, J = 9.3, 3.3 Hz), indicating the presence of a hydroxy group at C-11. The coupling constants of H-11 suggested a β-axial orientation; therefore the hydroxy was assigned in an α-equatorial position. The H-9 proton appearing at δ 1.84 (d, J = 9.3 Hz) confirmed the axial configuration of H-11. Complete analysis of the COSY, HSQC, and HMBC spectra led to the identification of **4** as 11α-hydroxyasiatic acid. The structure was confirmed by the ROE effects observed between H-12 and H-18β, H-11β-axial, H$_3$-25β-axial, and H$_3$-26β-axial; H-9α-axial and H$_3$-27α-axial; and H-2β-axial and H$_3$-24β-axial



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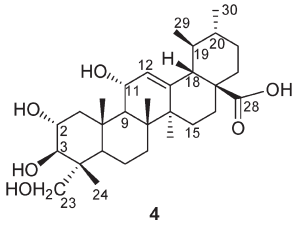
1	CH_3	H	CH_3
2	CH_2OH	H	CH_3
3	CH_2OH	CH_3	H



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(Figure 1). Comparison of the NMR data with 11β-methoxyasiatic acid, isolated from *Shorea robusta*,[15] supported the α-orientation of the hydroxy group at C-11.

The disk diffusion method was used to evaluate the antimicrobial activity of the ethyl acetate extract, the saponin mixture A, fraction II (containing saponins **2** and **3**), and fraction III (containing saponin **1**) against four bacteria, including two Gram-positive (*S. aureus* and *E. faecalis*) and two Gram-negative (*E. coli* and *P. aeruginosa*). Saponin mixture A and fraction III showed good antibacterial activity against *E. faecalis* with inhibition diameters of 26 and 30 mm, respectively. Fraction II showed weak activity with a 12 mm diameter inhibition. Then, TLC bioautography was used to evaluate the antimicrobial activity against *E. faecalis* of saponins **1**−**3** in comparison to the saponin mixture A and fraction III. The results showed in saponin mixture A and fraction III the presence of one active compound characterized with a R_f 0.56 similar to saponin **1**. Nevertheless, no inhibition zone was observed with saponins **1**−**3** at the concentration tested, suggesting that saponin **1** was probably not the active compound in fraction III.

The ethyl acetate extract showed no antibacterial activity, but the six known sapogenins have been previously reported to possess antibacterial activity against various bacteria.[16−21] Ursolic acid, oleanolic acid, and corosolic acid were evaluated in a pure form,[16,19,20] but asiatic acid, maslinic acid, and arjunolic acid were evaluated in a mixture form,[17,18,21] due to difficulties in separating isomeric compounds (corosolic acid/maslinic acid, asiatic acid/arjunolic acid). In order to make a comparison of the antibacterial activity of the six known isolated triterpenoids, their MIC values were determined against four bacteria (Table 3). Due to the limited availability of the new triterpenoid **4**, its antimicrobial activity could not be evaluated. The six sapogenins showed antibacterial activity only against Gram-positive bacteria, as previously observed for ursolic acid and oleanolic acid,[20] with MIC values from 16 to 128 μg/mL. Asiatic acid showed moderate activity against *S. aureus* and *E. faecalis*. Ursolic acid, oleanolic acid, and maslinic acid were the most active against *E. faecalis*, with MIC 16 μg/mL, and corosolic acid was the most active against *S. aureus* (MIC 32 μg/mL).

In conclusion, asiatic acid, 11α-hydroxyasiatic acid (**4**), maslinic acid (also named crataegolic acid), and arjunolic acid were isolated from a plant in the genus *Symplocos* for the first time. This confirms that the presence of pentacyclic triterpenoids and oleanane or ursane saponins are chemotaxonomic criteria of *Symplocos* species. The antibacterial assays showed that these triterpenoids were more active against Gram-positive bacteria *Enterococcus faecalis* and *Staphylococcus aureus*, with MIC values from 16 to 128 μg/mL, as compared to *Escherichia coli* or *Pseudomonas aeruginosa*.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined in MeOH or $CHCl_3$ with a Perkin-Elmer 341 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 NMR spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz). 2D-NMR experiments were performed using standard Bruker microprograms. HRMS, ESIMS and MSMS experiments were recorded on a Finnigan LCQ deca quadripole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA). The samples were introduced by direct infusion in a solution of MeOH at a rate of 5 μL min^{-1}. TLC was performed on precoated silica gel 60 F_{254} plates (Merck), and detection was carried out with 50%

Table 3. Minimal Inhibitory Concentration (MIC) of Triterpenoids Isolated from *Symplocos lancifolia* against Gram-Positive and Gram-Negative Bacteria (μg/mL)[a]

	MIC			
compound	*Staphylococcus aureus*	*Enterococcus faecalis*	*Escherichia coli*	*Pseudomonas aeruginosa*
ursolic acid	128	16	>128	>128
corosolic acid	32	32	>128	>128
asiatic acid	>128	128	>128	>128
oleanolic acid	>128	16	>128	>128
maslinic acid	64	16	>128	>128
arjunolic acid	64	64	>128	>128
vancomycin	2.5	2.5		
ceftazidine			10	10

[a] Each concentration was evaluated in triplicate.

H_2SO_4. Column chromatography (CC) was carried out on Merck Kieselgel 60 (63−200 mesh) or Merck LiChroprep RP-18 (40−63 μm) silica gel. HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, a P580 pump, a UVD 340S diode array detector, and Chromeleon software. A binary solvent gradient was used. Solvent A: H_2O, pH 2.4, with 0.0025% TFA. Solvent B: MeCN. The chromatogram was monitored at 205 nm. A Modulo-cart Uptisphere ODB column (UP5ODB 25M, 250 × 10 mm, 5 μm, Interchrom) was used for semipreparative HPLC of the triterpenes, with a flow rate of 3.5 mL min^{-1}. A C_{18} reversed-phase column (201SP, 250 × 10 mm, 5 μm, Dionex Vydac, Jouyensosap, France) was used for semipreparative HPLC of the saponins, with a flow rate of 4 mL min^{-1}.

Plant Material. Leaves of *Symplocos lancifolia* were collected in Bac Quang, Ha Giang Province, Vietnam, in April 2003 by D. D. Cuong. This plant was identified by Dr. Q. B. Nguyen of the Institute of Ecology, VAST, Hanoi, Vietnam, where a herbarium specimen has been deposited under the reference number VN-1081.

Extraction and Isolation. Dried and finely powdered leaves of *S. lancifolia* (600 g) were macerated for 22 h with petroleum ether (10 L). On drying, the resulting powdered material was macerated in 20% aqueous MeOH (11 L) for 15 h and then boiled for 3 h. The hydromethanolic extract was filtered and evaporated to remove MeOH. The resulting aqueous solution was treated with EtOAc (3 × 1 L), affording an EtOAc-soluble extract (23 g) and a H_2O-soluble extract (51 g). A part (36 g) of the aqueous residue was suspended in MeOH (600 mL) to give an insoluble residue (12 g) and a soluble portion, which was concentrated (to 100 mL) and then poured dropwise into 500 mL of acetone. The resulting precipitate was filtered and dried over KOH in vacuo (10 g). The filtrate was evaporated, dried, suspended in MeOH, and precipitated into Et_2O, and the precipitate was filtered and dried over KOH in vacuo (9 g). The two dried precipitates were each dissolved in H_2O and dialyzed against H_2O in seamless cellulose tubing with agitation for 48 h. The contents of the tubes were freeze-dried to afford 3.3 g of saponin mixture A (from the acetone precipitate) and 0.7 g of saponin mixture B (from the Et_2O precipitate).

The EtOAc extract was fractionated by silica gel CC, using a gradient of $CHCl_3$−MeOH (1:0 to 9:1). Fractions 56−61 (150 mg), eluted with $CHCl_3$−MeOH (98:2), were purified by silica gel CC eluted with $CHCl_3$−MeOH (99:1), and then fractions 5−20 were further purified by silica gel CC using 100% $CHCl_3$ as solvent to give ursolic acid (10 mg) in fractions 4 and 5 and oleanolic acid (2.9 mg) in fractions 11−15. Fractions 6−10 were purified by semipreparative HPLC (column ODB) using 87% B during 20 min to give ursolic acid (t_R 16.47 min, 9.6 mg) and oleanolic acid (t_R 16.97 min, 5.6 mg). Fractions 104−108 (668 mg), eluted with $CHCl_3$−MeOH (95:5), were purified by silica gel CC, using a gradient of hexane−EtOAc (7:3 to 3:7). Fractions 27−31 (52 mg) and fractions 40−68 (57 mg), eluted, respectively, with hexane−EtOAc (5:5 and 4:6), were purified by semipreparative HPLC (column ODB) using isocratic elution with 75% B for 20 min, to give corosolic acid (t_R 13.0 min, 24 mg) and maslinic acid (t_R 12.2 min, 17 mg). Fractions 136−143 (809 mg), eluted with $CHCl_3$−MeOH (9:1), were purified by silica gel CC, using a gradient of $CHCl_3$−MeOH (99:1 to 9:1). Fractions 51−73 (109 mg), eluted with $CHCl_3$−MeOH (97.5:2.5), gave, on concentration in MeOH, a precipitate of stigmasterol 3-*O*-β-D-glucoside (65 mg), and the remaining filtrate contained a soluble mixture of asiatic acid and arjunolic acid. Fractions 91−121 (311 mg) were purified by silica gel CC, using a gradient of hexane−acetone (6:4 to 4:6), to give arjunolic acid (7 mg) in fractions 10−12 and stigmasterol 3-*O*-β-D-glucoside (40 mg) in fractions 43−62. Fractions 16−18, eluted with hexane−acetone (6:4), were purified by semipreparative HPLC with 55% B (0−20 min) to give asiatic acid (t_R 12.61 min, 8 mg), arjunolic acid (t_R 11.72 min, 4 mg), and **4** (t_R 7.48 min, 2.9 mg). Fractions 19−28, eluted with hexane−acetone (6:4), gave asiatic acid (11.6 mg) and arjunolic acid (11.7 mg), after purification by semipreparative HPLC, using the same solvent system as above.

The saponin mixture A (3.3 g) was subjected to C_{18} vacuum-liquid chromatography (6 × 7 cm), eluting successively with 200 mL of MeOH−H_2O (5:5) (fraction I, 2.4 g), (6:4) (fraction II, 375 mg), (7:3) (fraction III, 196 mg), (8:2) (fraction IV, 113 mg), and (100:0) (fraction V, 209 mg). Fraction II was purified by reversed-phase C_{18} CC, using a gradient of MeOH−H_2O (55:45 to 7:3). Fractions 13−18 (82 mg), eluted with MeOH−H_2O (6:4), were purified by silica gel CC, using a mixture of $CHCl_3$−MeOH−H_2O (70:30:2) as eluent. Finally fractions 10−15 (12 mg) were purified by semipreparative HPLC, using a linear gradient of 27% to 30% B for 30 min, to yield saponins **2** (t_R 12.23 min, 6.7 mg) and **3** (t_R 11.52 min, 4.9 mg). Fraction III was purified by silica gel CC, using a gradient of $CHCl_3$−MeOH−H_2O (70:30:0 to 70:30:5), followed by semipreparative HPLC, with the elution program 20−25% B (0−12 min) and 35−40% B (13−25 min), to give saponin **1** (t_R 21.7 min, 3.3 mg).

Saponin ***1****:* white powder; $[\alpha]^{21}_D$ +0.75 (*c* 0.27, MeOH); ^{1}H and ^{13}C NMR (CD_3OD) of the triterpenoid part, see Table 1; ^{1}H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 2; ESIMS (positive-ion mode) *m/z* 965 $[M + Na]^+$; ESIMS-MS MS^2 (965) *m/z* 657 $[M + Na - (162 - 146)]^+$, 331 $[(162 + 146) + Na]^+$; HRESIMS (positive-ion mode) *m/z* 965.5084 $[M + Na]^+$ (calcd for $C_{48}H_{78}O_{18}Na$, 965.5086).

Saponin ***2****:* white powder; $[\alpha]^{21}_D$ −2.2 (*c* 0.55, MeOH); ^{1}H and ^{13}C NMR (CD_3OD) of the triterpenoid part, see Table 1; ^{1}H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 2 ; ESIMS (positive-ion mode) *m/z* 981 $[M + Na]^+$; ESIMS-MS MS^2 (981) *m/z* 673 $[M + Na - (162 - 146)]^+$, 331 $[(162 + 146) + Na]^+$; HRESIMS (positive-ion mode) *m/z* 981.5053 $[M + Na]^+$ (calcd for $C_{48}H_{78}O_{19}Na$, 981.5035).

Saponin ***3****:* white powder; $[\alpha]^{21}_D$ −1.5 (*c* 0.39, MeOH); ^{1}H and ^{13}C NMR (CD_3OD) of the triterpenoid part, see Table 1; ^{1}H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 2; ESIMS (positive-ion mode) *m/z* 981 $[M+Na]^+$; ESIMS-MS MS^2 (981) *m/z* 673 $[M+Na-(162 - 146)]^+$, 331 $[(162 + 146) + Na]^+$; HRESIMS (positive-ion mode) *m/z* 981.5026 $[M + Na]^+$ (calcd for $C_{48}H_{78}O_{19}Na$, 981.5035).

2α,3β,11α,23-Tetrahydroxyurs-12-en-28-oic acid (***4***)*:* white powder; $[\alpha]^{21}_D$ +20.4 (*c* 0.19, MeOH); ^{1}H NMR and ^{13}C NMR (CD_3OD), see Table 1; ESIMS (negative-ion mode) *m/z* 503 $[M - H]^-$; HRESIMS (positive-ion mode) *m/z* 527.3339 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_6Na$, 527.3349) and 543.3096 $[M + K]^+$ (calcd for $C_{30}H_{48}O_6K$, 543.3088).

Acid Hydrolysis of Saponins. The crude saponin mixture A (1 g) was refluxed in 2 N HCl (30 mL) for 4 h. After extraction with EtOAc (3 × 15 mL), the aqueous layer was neutralized with 0.5 M

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NaOH and freeze-dried. Two monosaccharides were identified by comparison with authentic samples by TLC using MeCOEt−*i*-PrOH−Me_2CO−H_2O (20:10:7:6), and their identity was confirmed after preparative TLC in the same solvent and by measurement of their optical rotations as L-rhamnose {$[\alpha]^{21}_D$ +7.3 (*c* 0.15, H_2O); lit.: +8.9 (*c* 10, H_2O)} and D-glucose {$[\alpha]^{21}_D$ +50.3 (*c* 0.12, H_2O); lit.: +52.0 (*c* 10, H_2O)}.

Disk Diffusion Antibacterial Assays. A disk diffusion method was used to determine the antibacterial activities of saponin mixture A and fractions II and III of *S. lancifolia* against two Gram-positive organisms, *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212), and two Gram-negative organisms, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). A 50 μL aliquot of each solution at 10 mg/mL in H_2O was applied to 8 mm diameter paper disks, which were placed in Petri dishes of 9 cm diameter containing nutrient Mueller-Hinton agar previously inoculated with a bacterial suspension (5×10^7 cfu/mL for *S. aureus* and *E. faecalis*; 5×10^6 for *E. coli* and *P. aeruginosa*), prepared by suspending one isolated colony from nutrient agar plates in 10 mL of distilled water. After 18 h of incubation at 37 °C, the inhibition zone for the active extract was measured.[22]

TLC Bioautrographic Antibacterial Assays. Bioautography on thin-layer chromatographie plates[19,23] was used to determinate the antibacterial activity of saponins **1**−**3**, saponin mixture A, and fraction III against *E. faecalis* (ATCC 29212). Test solutions (100 μL) were applied as small spots on TLC plates (silica gel G HPTLC 10 × 20) to give 10, 50, and 100 μg/application zones for saponins **1**−**3** and a 100 μg/application zone for saponin mixture A and fraction III. The organic solvent was evaporated, and plates were eluted in $CHCl_3$−MeOH−H_2O (70/30/5). After migration, the solvent was again evaporated. TLC plates were overlapped homogenously with 20 mL of nutrient agar (Mueller-Hinton) infected by nutrient broth containing bacteria (10^8 cfu/mL). The moist plates were incubated at 37 °C in a humidified chamber for 18 h. The plates were then sprayed with 2 mg/mL of *p*-iodonitrotetrazolium bromide (INT) (Sigma) and incubated for 2 h. The appearance of clear zones on the TLC plates indicated antibacterial activity.

Broth Diffusion Antibacterial Assays. The liquid microdilution growth inhibition method[24] was used to determine the MIC values of the pure triterpenoids (asiatic acid, corosolic acid, ursolic acid, arjunolic acid, maslinic acid, and oleanolic acid) against standard strains of *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa*, as described previously,[25] except that the test samples were resuspended in 256 μg/mL stock solutions in DMSO. Two antibacterial agents were used as positive controls: vancomycin for *S. aureus* and *E. faecalis* and ceftazidine for *E. coli* and *P. aeruginosa*.

■ ASSOCIATED CONTENT

Supporting Information. HSQC and ROESY NMR spectra for compound **1**, ^1H and ^{13}C NMR spectra of compounds **2**−**4**, and chemical structures and NMR data of oleanolic acid, maslinic acid, arjunolic acid, ursolic acid, corosolic acid, and asiatic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

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