A Novel Antibacterial Iridoid and Triterpene from *Caiophora coronata*

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Bioassay-guided fractionation of the antibacterial CH_2Cl_2−MeOH extract obtained from the aerial parts of the Argentinean plant *Caiophora coronata* led to the isolation of a new triterpene, 1β,3β-dihydroxyurs-12-en-27-oic acid, **1**, and a new iridoid, 1α-methoxy-6α,10-dihydroxyisoepiiridomyrmecin (caiophoraenin), **2**, along with the known iridoid isoboonein **3**. Their structures were established by spectroscopic techniques (1D and 2D NMR, HRFABMS, FTIR). The MIC values of isolated compounds were determined against methicillin-sensitive (MSSA) and -resistant (MRSA) strains of *Staphylococcus aureus*, *Bacillus subtilis* (BS), vancomycin-resistant *Enterococcus faecium* (VREF), *Escherichia coli* (EC), *E. coli imp* (ECimp), and *Candida albicans* (CA). Compound **1** was found active against BS, MSSA, MRSA, VREF, and ECimp with MIC values of 2, 4, 4, 4, and 16 μg/mL, respectively.

As a part of the International Cooperative Biodiversity Group, our research program entitled "Bioactive Agents from Dryland Biodiversity of Latin America" explores the dry-land vegetation of South America for the purpose of drug lead discovery. We routinely screen plant extracts for their antibacterial activity against a variety of clinically significant bacterial strains. The CH_2Cl_2−MeOH extract of aerial parts of the Argentinean plant *Caiophora coronata* Hook. et Arn. (Loasaceae) was found to be active against both the methicillin-sensitive and -resistant bacterial strains of *Staphylococcus aureus* in an agar diffusion test. On the basis of this important activity, the extract was selected for bioassay-guided fractionation.

Prior chemical studies on *Caiophora* have led to the isolation of numerous iridoids. An unidentified iridiod, 10-hydroxyoleoside dimethyl ester, as well as a lectin have previously been reported in *C. coronata*.[1,2] Additionally, pentlandioside I, a bis-secoiridoid, was isolated from *Cajophora pentlandii*.[3] The iridoid 10-hydroxyoleoside dimethyl ester is used as a chemotaxonomic marker for this genus.[1]

Here we report the isolation, characterization, and antimicrobial activities of a new iridolactone, a new ursane-type triterpene, and the known iridoid isoboonein.

The crude CH_2Cl_2−MeOH (1:1) extract of the aerial parts of *C. coronata* exhibited activity against both the methicillin-sensitive and -resistant bacterial strains of *S. aureus* in an agar diffusion test with zones of growth inhibition of 11 and 12 mm, respectively. The extract was then fractionated on a silica gel column using ethyl acetate (0 to 90% gradient) in *n*-hexane. Further fractionation of the active fractions afforded two new compounds, the ursane-type triterpene 1β,3β-dihydroxyurs-12-en-27-oic acid, **1**, and the iridoid caiophoraenin, **2**, as well as isoboonein **3**. Isoboonein was identified by comparison of its spectral data with those reported in the literature.[4]

Compound **1** was isolated as a white powder. Its HR-FABMS showed an $[M + H]^+$ ion at *m*/*z* 473.1802 corresponding to the molecular formula $C_{30}H_{48}O_4$. The IR spectrum suggested the presence of hydroxyl (3360 cm^{-1}) and carbonyl (1686 cm^{-1}) groups. The ^{13}C and DEPT spectra indicated seven methyls, eight methylenes, eight methines, and seven quaternary carbons. Of the seven methyls, two were identified as secondary methyls on the basis of HSQC and ^1H spectra, indicating an ursane skeleton.

The quaternary carbon at δ 179.3 was assigned to a free carboxylic group (C-27). The methine at δ 130.6 and quaternary carbon at δ 133.9 could be assigned to a trisubstituted double bond (C-12 and C-13, respectively).

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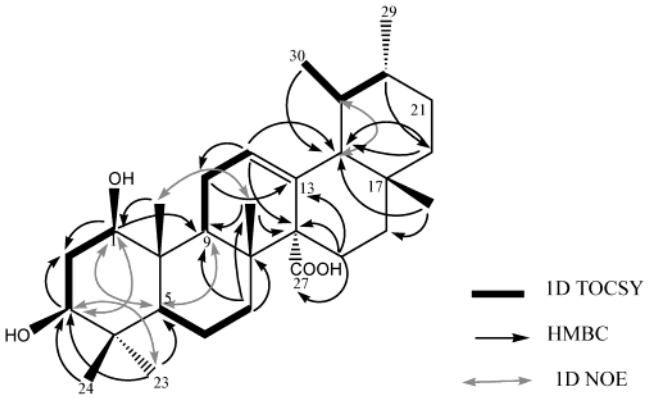

Figure 1. Significant HMBC 1D TOCSY and 1D NOE correlations of **1**.

Two downfield methines at δ 80.9 and 76.8 were assigned to hydroxy-bearing carbons (C-1 and C-3, respectively). The presence of two hydroxyl groups was in addition confirmed by HRFABMS, due to two sequential losses of water giving rise to fragment ions at *m*/*z* 455.18 and 437.18. On close inspection, the ^{13}C NMR data of **1** were similar to those of the ursane-type triterpene karenin ($C_{39}H_{54}O_6$),[5] and the differences could be accounted for by the presence of an additional hydroxy group in the A-ring and the lack of the *p*-coumaroyl moiety. The hydroxy group was assigned to C-1 on the basis of its chemical shift value of δ 80.9. H-1 and H-3 had DQF-COSY cross-peaks to H-2 and were a part of the same spin-system in a 1D TOCSY experiment. H-1 had HMBC correlations with C-2, C-9, and C-25, as shown in Figure 1. This confirmed the position of the hydroxy-bearing carbons in the A-ring. The carboxylic acid was located at C-27 on the basis of the downfield chemical shifts of C-12, C-13, C-14, and C-18. The HMBC correlation of the H-15 multiplet at δ 1.74 with the carboxyl group further supported its location at C-27 (Figure 1). Other DQF-COSY and HMBC data were used to further assign the ^{13}C and ^{1}H spectra as shown in Figure 1.

Relative stereochemistry at C-1 in **1** was established on the basis of observed coupling constants and 1D NOE data. H-1 at δ 3.38 had a diaxial coupling associated with it. Also, H1, H3, H5, and H9 had 1D NOE correlations to each other, establishing that **1** was a 1β,3β-diol. The α-stereochemistry at C-27 was determined by comparison of the chemical shifts of C-12, C-13, C-14, and C-18 with related compounds.[5,6] Thus, the structure of **1** was established as 1β,3β-dihydroxyurs-12-en-27-oic acid.

Caiophoraenin (**2**) was obtained as a pale yellow oil. Its HRFABMS gave the $[M + Cs]^+$ ion at *m*/*z* 363.0214, suggesting a molecular formula of $C_{11}H_{18}O_5$ corresponding to three degrees of unsaturation. The IR spectra indicated the presence of a hydroxyl group (3388 cm^{-1}) and a δ-lactone group (1731 cm^{-1}). The ^{13}C and DEPT spectra showed that there were two methyl, two methylene, six methine, and one quaternary carbon. The quaternary carbon at δ 175.3 indicated the presence of a lactone carbonyl. Thus, an iridolactone skeleton was suggested for **2**.

The carbon at δ 175.3 could be assigned to the lactone carbonyl at C-3. Also, observed in the ^{13}C spectrum were two downfield signals, a methine at δ 75.0 and a methylene at δ 65.2, that could be attributed to hydroxy-bearing carbons. The highly downfield methine signal at δ 96.9 suggested a dioxy-substituted carbon. This signal was correlated in the HMBC to H-9 and was thus assigned as C-1. Also, H-1 and H-9 had a ^3J coupling of 8.6 Hz between them according to a DQFCOSY experiment. The methyl

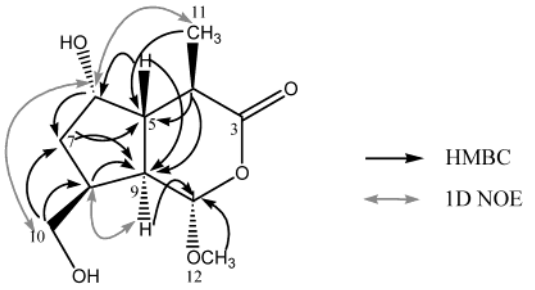

Figure 2. Significant HMBC and 1D NOE correlations of **2**.

signal at δ 52.3 was identified as a methoxy group, on the basis of its correlation in the HSQC spectrum to a three-proton singlet at δ 3.68, and was assigned to the C-12 methoxy because of its $^3J_{CH}$ correlation to C-1 in the HMBC as shown in Figure 2. The oxymethine at δ 75.0 was assigned to C-6 on the basis of the HMBC correlation of the H-6 signal at δ 4.16 to C-5 and C-7. The hydroxy methylene at δ 65.2 could be assigned to C-10 on the basis of the HMBC correlation of H-10a,b to C-8 and C-7. The methyl doublet at δ 0.98 (C-11) was correlated to C-5 in the HMBC experiment. The ^{13}C and ^{1}H NMR data of **2** were largely comparable to gibboside aglucone [$C_{10}H_{16}O_4$][7] except that **2** was a C-9 epimer of the aglucone. Also, the hydroxy group in **2** was located at a different position and there was a methoxy group present in **2**.

A close scrutiny of 1D NOE patterns and coupling constants allowed the determination of the relative stereochemistry of **2** as shown in Figure 2. The characteristic δ-lactone carbonyl at 1731 cm^{-1} suggests a half-chair conformation for the lactone ring.[8] The coupling constant of 8.6 Hz between the H-1 doublet and H-9 indicated a diaxial relationship and suggested a *trans*-fusion at the ring junction.[9] The H-9 double doublet possessed an additional diaxial coupling of 11.85 Hz, indicating a possible diaxial relationship with H-5. These suggested that **2** possessed a conformation similar to that of isoepiiridomyrmecin.[10] Additionally, lack of NOE correlation between H-5 and H-9 in selective 1D NOE experiments further validated this *trans*-relationship. H-8 and H-9 were related in the 1D NOE experiment. The H-6 signal was determined to be in an equatorial position on the basis of its coupling constants. It had 1D NOE peaks to the H-11 methyl protons and also to the H-10a,b oxymethylene protons. Therefore, the structure of **2** was established as 1α-methoxy-6α,10-dihydroxyisoepiiridomyrmecin.

The antimicrobial activity of compounds **1**−**3** was determined against a variety of clinically relevant pathogens, namely, methicillin-sensitive 375 (MSSA) and -resistant 310 (MRSA) strains of *Staphylococcus aureus, Bacillus subtilis* 327 (BS), vancomycin-resistant *Enterococcus faecium* 379 (VREF), *Escherichia coli* 442 (EC), *E. coli imp* 389 (ECimp), and *Candida albicans* 54 (CA). Compounds **2** and **3** were essentially inactive against all the bacterial strains tested with MIC values > 128 μg/mL. However, compound **1** showed appreciable activity against MSSA and BS with MIC values of 4 and 2 μg/mL, respectively. Interestingly, **1** was equally active against MRSA and VREF with MIC values of 4 μg/mL. Also, **1** exhibited moderate activity against ECimp with an MIC of 16 μg/mL. None of the compounds tested were active against EC or CA. Pen G was used as control for the bioassay, and its activities against MSSA, MRSA, BS, VREF, EC, ECimp, and CA were 0.06, 128, 0.25, 32, 32, 4, and >128 μg/mL, respectively.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Jasco P-1020 digital polarimeter. IR (as a film on a diamond cell) was measured on a Thermo Nicolet Avatar 360 FT-IR spectrometer. A JEOL HX110A mass spectrometer was used in recording HRFAB mass spectra. NMR spectra (^{1}H, selective 1D NOE, selective 1D TOCSY, ^{13}C, DEPT-135, DEPT-90, gradient-selected HSQC, gradient-selected HMBC, DQF-COSY) were recorded using either a Bruker DRX-500 or DRX-600 spectrometer in CD_3OD. Chemical shifts were expressed in ppm (δ) using partially deuterated solvent chemical shifts at δ 49 (^{13}C) and δ 3.31(^{1}H) as reference for ^{13}C and ^{1}H NMR signals, respectively. The mixing times used in recording selective 1D NOE were 400 ms for **1** and 700 ms for **2**. Mixing times for selective 1D TOCSY spectra were 60 ms for **1** and 20 ms for **2**. TLC plates were sprayed with 0.5% anisaldehyde, 10% HOAc, and 5% H_2SO_4 in MeOH and heated until colored spots appeared. Open column chromatography was conducted using silica gel (63−200 μm SAI). Preparative TLC was carried out using 20 × 20 cm, 0.5 mm silica gel 60 plates (EM Science) to isolate compounds **2** and **3**. Reversed-phase HPLC was employed for the isolation of **1** using a Varian ProStar semiprep HPLC system equipped with 9012 pump and a 9065 PDA detector, employing a Varian 150 × 4.6 mm C_{18} column and eluting with a gradient of 40% ACN in 0.15% HCOOH−H_2O to 100% ACN in 30 min at a flow rate of 1.8 mL/min, and detection at 200 nm.

Plant Material. Aerial parts of *Caiophora coronata* were collected and identified by Reneé H. Fortunato and R. Kiesling on January 22, 1997, 28 kilometers west of Puesto de Gendarmeria in the province of San Juan, Argentina (30°21′49″ S; 69°41′30″ W). A voucher specimen (RHF 5574) has been deposited at the Instituto Nacional de Tecnologia Agropecuaria (INTA), Buenos Aires, Argentina. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between the University of Arizona and INTA.

Extraction and Isolation. Air-dried and powdered aerial parts of *C. coronata* (684 g) were extracted with 9 L of CH_2Cl_2−MeOH (1:1) to give 31.1 g of crude extract. This extract (30 g) was chromatographed on a Si gel column with a step gradient of EtOAc in *n*-hexane starting with 0% EtOAc in *n*-hexane. Fractions eluting with 35−65% EtOAc in *n*-hexane were determined to contain activity against *B. subtilis* and *S. aureus*.

Fractions eluting with 63% EtOAc in *n*-hexane were subjected to preparative TLC with 80% EtOAc in *n*-hexane, resulting in the isolation of **2** (R_f = 0.54, 8.1 mg) and **3** (R_f = 0.36, 8.8 mg).

Fractions eluting with 49−55% EtOAc in *n*-hexane (5.9 mg) were further fractionated by open column chromatography with Sephadex LH-20 using MeOH. Compound **1** was seen as a characteristic pink spot on TLC. Reversed-phase HPLC was performed leading to the isolation of **1** (2 mg), which had a retention time of 22.4 min.

In Vitro Antimicrobial Activity Testing. The in vitro antibacterial activities were determined by the agar diffusion on microbroth dilution method as previously described.[11] The minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method as previously described.[12] Briefly, microtiter plates containing 2.5 μL per well of 2-fold serial dilutions of each antimicrobial agent were inoculated with 100 μL of bacterial suspension in Mueler-Hinton II broth to yield the appropriate density (1−5 × 10^5 CFU/mL). The plates were incubated for 18 h at 35 °C in ambient air. The MIC was defined as the lowest concentration of a compound that completely inhibited the growth of the organism as determined by the unaided eye.

A modified version of the agar diffusion method was used to evaluate the activities of the crude extract, fractions, and pure compounds against selected bacterial isolates. Assay plates (12 × 12 in. Sumilon) were prepared by pouring 125 mL of agar medium (tempered at 50 °C) inoculated with an overnight broth culture of the test organisms (adjusted to a final inoculum density of approximately 10^6 cells per mL). The medium was allowed to solidify, and 144 wells (5 mm diameter) were bored into the agar layer using an automated plate-welling machine. Twenty microliters (20 μL) of the test compound (10 mg/mL in DMSO) were dispensed into agar wells, and the plates were incubated at 37 °C for 18 h. The zones of growth inhibition were measured using a hand-held digital caliper. Fractions showing zones of inhibition of 8 mm or greater were selected for further purification.

1β,3β-Dihydroxyurs-12-en-27-oic acid (1): white powder; mp 173−174 °C; $[\alpha]^{25}_D$ +62° (CH_3OH, *c* 0.045); IR (KBr) ν_{max} 3360, 2947, 2183, 1686, 788 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) δ 5.52(1H, dd, *J* = 1.8, 5.5, H-12), 3.38(1H, dd, *J* = 6.7, 9.4, H-1), 3.14(1H, dd, *J* = 6.7, 10.1, H-3), 2.75(1H, ddd, *J* = 5.0, 5.5, 18.8, H-11a), 2.48(1H, dd, *J* = 5.0, 11.5, H-9), 2.09(1H, m, H-11b), 1.95(1H, dt, *J* = 3.2, 13.4, H-15a), 1.74(3H, ov, H-2, H-15b), 1.67(1H, td, *J* = 3.2, 13.3, H-7a), 1.56(1H, dt, *J* = 3.7, 13.3, H-6a), 1.47(1H, qd, *J* = 2.3, 13.3, H-6b), 1.41(1H, m, H-16a), 1.33−1.34(2H, ov, H-18, H-21a), 1.24−1.26(2H, m, H-16b, H-21b), 1.19(1H, dt, *J* = 3.5, 12.8, H-7b), 1.06(3H, s, H-26), 1.02(3H, s, H-25), 0.97(1H, m, H-19), 0.91(3H, s, H-23), 0.89(3H, d, *J* = 5.0, H-29), 0.87−0.84(3H, ov, H-20, H-22), 0.84-(3H, d, *J* = 6.0, H-30), 0.83(3H, s, H-28), 0.74(3H, s, H-24), 0.57(1H, d, *J* = 10.5, H-5); ^{13}C NMR (CD_3OD, 125 MHz) δ 179.3(C, C-27), 133.9(C, C-13), 130.6(CH, C-12), 80.9(CH, C-1), 76.8(CH, C-3), 61.6(CH, C-18), 57.3(C, C-14), 54.5(CH, C-5), 48.9(CH, C-9), 44.4(C, C-10), 42.2(CH_2, C-16), 41.2(C, C-8), 40.9(CH, C-20), 39.8(C, C-4), 38.8(CH, C-19), 38.3(CH_2, C-2), 38.0(CH_2, C-7), 34.7(C, C-17), 31.7(CH_2, C-21), 30.3(CH_2, C-22), 29.7(CH_3, C-30), 28.6(CH_3, C-23), 27.3(CH_2, C-11), 23.5(CH_2, C-15), 21.7(CH_3, C-29), 19.4(CH_3, C-26), 18.9(CH_2, C-6), 18.5-(CH_3, C-28), 16.0(CH_3, C-24), 12.9(CH_3, C-25); HRFAB$^+$ *m/z* 473.1802 [M + H]$^+$, 455.18, and 437.18.

Caiophoraenin (2): pale yellow oil; $[\alpha]^{25}_D$ +58° (CH_3OH, *c* 0.31); IR (KBr) ν_{max} 3388, 2954, 1731, 1437, 1261, 783 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) δ 4.71(1H, d, *J* = 8.6, H-1), 4.16-(1H, dt, *J* = 2.8, 6.0, H-6), 3.85(1H, dd, *J* = 4.1, 12.4, H-10a), 3.68(3H, s, H-12), 3.35(1H, dd, *J* = 2.8, 11.9, H-10b), 2.4(1H, m, H-8), 2.16(1H, dd, *J* = 8.6, 11.9, H-9), 1.88(2H, ov, H-4, H-7a), 1.88(1H, dd, *J* = 8.8, 14, H-7a), 1.72(2H, ov, H-5, H-7b), 1.72(1H, dd, *J* = 5.3, 14, H-7b), 0.98(3H, d, *J* = 6.9, H-11); ^{13}C NMR (CD_3OD, 125 MHz) δ 175.3(C, C-3), 96.9(CH, C-1), 75.0-(CH, C-6), 65.2 (CH_2, C-10), 54.2(CH, C-9), 52.3(CH_3, C-12), 43.5(CH, C-5), 40.4(CH_2, C-7), 39.9(CH, C-4), 39.7(CH, C-8), 12.2(CH_3, C-11); *m/z* HRFAB$^+$ *m/z* 363.0214 [M + Cs]$^+$.

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