# Notes

## Bacterial Biofilm Inhibitors from *Diospyros dendo*

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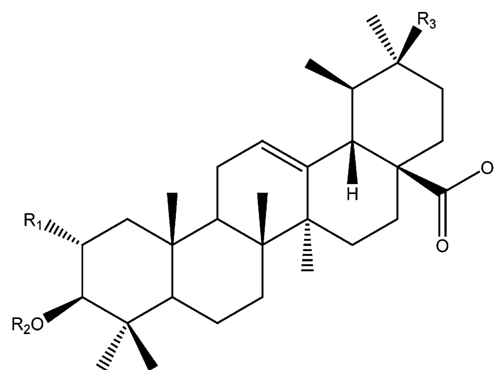
One new (**1**) and four known (**2**−**5**) ursene triterpenes with potent inhibition of the formation of the bacterial biofilm *Pseudomonas aeruginosa* PA01 were obtained from *Diospyros dendo* using a high-throughput natural products chemistry procedure. These compounds were isolated as mass-limited samples. The miniaturization of the structure elucidation and dereplication was performed primarily utilizing a capillary-scale NMR probe.

Free-floating bacteria have the ability to attach onto solid surfaces, creating a complex community of bacteria known as a biofilm. Encased in a complex polysaccharide matrix, a biofilm is protected against antibiotics and therefore is the cause of many recalcitrant infections as well as resistance to antibiotics.[1] The implication of bacterial biofilms in the resistance to antibiotics and chronic bacterial infections is a cause for concern in the medical community. Within bacteria, a biofilm matrix is able to resist antibiotics at concentrations from 1000 to 1500 times higher than are conventionally used.[2] Biofilms are involved in two-thirds of human bacterial infections.[3] No specific biofilm inhibitor is commercially available. *Pseudomonas aeruginosa* is a versatile Gram-negative bacterium that recently has been a very useful model for the study of biofilm formation.[4] People with cystic fibrosis, burn victims, cancer patients, and people requiring extensive stays in intensive care units are particularly at risk of diseases resulting from *P. aeruginosa* infection and are thus susceptible to biofilm formation.[5]

Plants and animals that live a sessile life in nutrient-rich environments, like the coastal sea and the jungle, will become overwhelmed by microbial biofilms if they lack any means of biofilm control. Many have turned to the use of bacterial signal inhibitors to preclude bacterial biofilm formation on their surfaces. In an effort to discover new natural bacterial biofilm inhibitors from plants through high-throughput natural products chemistry procedures,[6] we have investigated the leaves of *Diospyros dendo* Welw. ex Hiern (Ebenaceae). The genus *Diospyros* has about 475 species that are common in tropical regions worldwide. Some of them are commercial fruit-producing trees, such as *D. virginiana*, the common persimmon in North America, and *D. kaki*, the Japanese persimmon.[7] This genus is known to yield triterpenes.[8] In this paper, we report the isolation of one new (**1**) and four known (**2**−**5**) ursene triterpenes from *D. dendo* and their inhibition of the formation of the bacterial biofilm *P. aeruginosa* PA01. The miniaturization of the structure elucidation and dereplication[9] of these mass-limited 12-ursen-28-oic acid triterpenes was performed primarily using a capillary-scale NMR probe and MS data.

The triterpenes located in the ethyl acetate (neat) flash fraction were subjected to preparative HPLC $C_{18}$ chromatography using 30% to 70% acetonitrile in water over 40 min, collecting 1 min fractions. Compounds **1**−**5** resided in preparative HPLC fraction 38, which exhibited a potent inhibition of the formation of bacterial biofilm *P. aeruginosa* PA01. The HPLC-ELSD-MS data acquired from fraction 38 showed that it contained compounds with molecular weights less than 700 that could readily be isolated using reversed-phase chromatography. The initial mobile phase gradient applied to the isolation of compounds **1**−**5** from fraction 38 was based on the elution profile observed during the preparative HPLC separation that afforded this fraction. A semipreparative HPLC method was developed that resulted in an isocratic gradient of 75% acetonitrile in water for 32.0 min followed by 95% acetonitrile in water for 5.0 min to obtain compounds **1**, 3*β*-*O*-*trans*-*p*-coumaroyl-2α-hydroxy-12-ursen-28-oic acid (**2**),[10] 3*β*-*O*-*cis*-*p*-coumaroyl-2α-hydroxy-12-ursen-28-oic acid (**3**),[11] 3*β*-*O*-*trans*-feruloyl-2α-hydroxy-12-ursen-28-oic acid (**4**),[11] and ursolic acid (**5**). Comparing their MS and NMR data with the literature, the known triterpenes **2**−**4** were identified as indicated above. Compound **5** was identical with an authentic sample of ursolic acid purchased from Sigma-Aldrich (St. Louis, MO).




**1** $R_1$ = H, $R_2$ = *cis*-*p*-coumaroyl, $R_3$ = OH
**2** $R_1$ = OH, $R_2$ = *trans*-*p*-coumaroyl, $R_3$ = H
**3** $R_1$ = OH, $R_2$ = *cis*-*p*-coumaroyl, $R_3$ = H
**4** $R_1$ = OH, $R_2$ = *trans*-feruloyl, $R_3$ = H
**5** $R_1$ = $R_2$ = $R_3$ = H

The molecular weight of compound **1** and its elemental formula of $C_{39}H_{54}O_6$ were deduced from the positive-mode HRESIMS, which showed a $[M + Na]^+$ ion peak at *m/z* 641.3816. The $^1$H and COSY NMR ($CD_3OD$) spectra of **1** showed the presence of a *cis*-

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**Table 1.** Bacterial Biofilm Inhibition by Compounds **1**−**5** at 10 μg/mL[a]

| compound | *Pseudomonas aeruginosa* PA01 biofilm inhibition (%) |
|---|---|
| **1** | 62 |
| **2** | 35 |
| **3** | 32 |
| **4** | 48 |
| **5** | 35 |

[a] Positive control: furanone 56 displayed 13% inhibition at 10 μg/mL.[17]

*p*-coumaroyl moiety [δ 7.67 (2H, d, *J* = 8.4 Hz), 6.90 (1H, d, *J* = 12.9 Hz), 6.76 (2H, d, *J* = 8.4 Hz), 5.87 (1H, d, *J* = 12.9 Hz)].[11,12] The presentation of characteristic fragment peaks in the electron ionization (EI) MS at *m*/*z* 207 and 264 resulting from *retro*-Diels−Alder (RDA) cleavage of ring C indicated that one hydroxyl group was present in ring A or B and another hydroxyl group was present in ring D or E.[13] In consideration of the biogenetic pathways, the hydroxyl group in ring A or B was assigned to the C-3 position. Compared with ursolic acid (**5**), the proton signal of H-3 in **1** was shifted to a lower field at δ 4.62 (1H, dd, *J* = 10.0, 2.9 Hz, H-3α). Therefore, the *O*-cis-*p*-coumaroyl moiety was confirmed to be at C-3. According to the molecular formula and the observed $^1$H NMR spectrum, **1** should contain a quaternary hydroxyl group in ring D or E, which could be placed at the C-20 position due to the fact that its $^1$H NMR spectrum showed only one methyl doublet instead of two doublets in ursolic acid (**5**) and a one-proton doublet assigned to H-18 at δ 2.24 (1H, d, *J* = 11.2 Hz, H-18β).[14] This was further supported by an isolated proton spin system of H-18, H-19 [δ 1.41 (1H, dt, *J* = 11.2, 6.3 Hz, H-19α)] and H-29 [δ 0.93 (3H, d, *J* = 6.3 Hz)] in the COSY spectrum. Therefore, the structure of **1** was deduced as 3β-*O*-*cis*-*p*-coumaroyl-20β-hydroxy-12-ursen-28-oic acid.

Among the five purified triterpenes, compound **1** was found to be the best inhibitor against the bacterial biofilm *P. aeruginosa* PA01, with an inhibition of 62% at 10 μg/mL (Table 1). The results presented herein provide preliminary information for the potential use of naturally occurring ursolic acid derivatives as biofilm inhibitors of the Gram-negative bacterium *P. aeruginosa,* which was described as one of the top three causes of opportunistic human infections.[5c]

## Experimental Section

**General Experimental Procedures.** For instrumentation and general methods, see the preceding papers.[9] EIMS were recorded on an Agilent 5973N mass selective detector (direct inlet 70 eV).

**Plant Material.** The leaves of *D. dendo* were collected from the Lope game preserve in Gabon in November 2000. Plant samples were dried on site, then shipped to Sequoia Sciences. The plant was identified by Gretchen Walters (Missouri Botanical Garden Herbarium, St. Louis, MO). A voucher specimen (No. 481) was deposited at the Herbarium of the Missouri Botanical Garden.

**Extraction and Isolation.** Dried leaves (200 g) were extracted with EtOH−EtOAc (50:50) to obtain a 10 g extract. One gram of the extract was loaded onto a flash column. As previously described,[9] the ethyl acetate flash fraction generated 35 mg, which was fractionated by preparative $C_{18}$ HPLC from 30% to 70% acetonitrile in water, collecting 40 1-min fractions. The isolation of individual triterpenoids from preparative HPLC fraction 38 was performed using semipreparative Keystone BetaMax Neutral $C_{18}$ (8 × 250 mm i.d., 5 μm), as described above, to obtain pure compounds **1** (8 μg, $t_R$ = 14.0 min), **2** (11 μg, $t_R$ = 15.6 min), **3** (6 μg, $t_R$ = 17.8 min), **4** (3 μg, $t_R$ = 20.9 min), and **5** (16 μg, $t_R$ = 27.8 min). NMR data for the structure elucidation and dereplication were acquired on a Bruker Avance 600 MHz NMR system (Bruker Instruments, Rheinstetten, Germany) with a 5 μL capillary-scale NMR probe, CapNMR (MRM/Protasis, Savoy, IL), having a 1.5 μL active volume.[6a,9] Purified triterpenoid was dissolved in 6.5 μL of $CD_3OD$ and loaded manually into the probe. For the new compound **1**, 8 μg was diluted with 6.5 μL of $CD_3OD$. Injection: 5 μL, from which 2 μg was in the active volume (1.5 μL). Data acquisition for $^1$H NMR: Number of scans (NS) = 64, 5 min; for $^1$H−$^1$H COSY: NS = 4, 32 min.

**3β-*O*-*cis*-*p*-Coumaroyl-20β-hydroxy-12-ursen-28-oic acid (1).** Insufficient material was available to obtain an optical rotation value or an IR or a UV spectrum. $^1$H NMR ($CD_3OD$) δ 5.27 (1H, t, *J* = 3.4, 3.2 Hz, H-12), 4.62 (1H, dd, *J* = 10.0, 2.9 Hz, H-3α), 2.24 (1H, d, *J* = 11.2 Hz, H-18β), 1.97 (2H, m, H-11), 1.41 (1H, dt, *J* = 11.2, 6.3 Hz, H-19α), 1.17 (3H, s), 1.07 (3H, s), 0.99 (3H, s), 0.93 (3H, d, *J* = 6.3 Hz, H-29), 0.91 (3H, s), 0.88 (3H, s), 0.85 (3H, s), and *cis*-*p*-coumaroyl [δ 7.67 (2H, d, *J* = 8.4 Hz), 6.90 (1H, d, *J* = 12.9 Hz), 6.76 (2H, d, *J* = 8.4 Hz), 5.87 (1H, d, *J* = 12.9 Hz)]; EIMS *m*/*z* (rel int) 618 $[M]^+$ (15), 264 (60), 219 (71), 207 (33), 189 (17), 147 (100); ESIMS *m*/*z* 617 $[M - H]^-$, 619 $[M + H]^+$, 641 $[M + Na]^+$; HRESIMS *m*/*z* 641.3816 $[M + Na]^+$ ($C_{39}H_{54}O_6Na$ requires 641.3818).

**Bacterial Biofilm Inhibition.** Compounds **1**−**5** were bioassayed for their in vitro antibacterial biofilm activity against *Pseudomonas aeruginosa* PA01. This method uses standard presterilized 96-well polystyrene microtiter plates.[15] Each well was filled to a final volume of 200 μL. Initially, a concentrated compound solution was transferred into each well, except those used as controls, to achieve a final test concentration of 10 μg/mL. A 150 μL sample of sterile tryptic soy broth medium was then added and followed by 50 μL of bacterial inoculum appropriately diluted approximately 1 in 3. The plates were covered and then placed on a shaker for 24 h. After the allotted incubation period of compounds **1**−**5**, which had been isolated using semipreparative HPLC, the samples were removed from the shaker and immediately analyzed with a microtiter plate reader at 630 nm and were then rinsed and stained. The absorbance reading taken at 630 nm prior to rinsing the wells was compared to negative controls consisting of medium, inoculum, and diluent. The absorbance readings of wells containing compounds **1**−**5** and without test compounds were similar, demonstrating bacterial growth was not inhibited by compounds **1**−**5**. The rinse involved first draining the wells, filling each well with phosphate-buffered saline (PBS), and draining each well again. The rinse removed any suspended cells from the system. The biofilm was then stained with a 1 g/L Protocol crystal violet solution for 10 min. Each well was rinsed again four times to remove any excess stain from the system and then eluted with 250 μL of ethanol. The elution step improves the detection of the stain during the analyses. The plate was then immediately analyzed with a microtiter plate reader at 540 nm. The samples and controls were analyzed in triplicate. Negative and positive controls are run for every plate. The positive control substance was furanone 56, previously documented to moderately inhibit the formation of biofilms.[16] Negative controls included wells with only an appropriate volume of sample diluent and diluted overnight culture of *P. aeruginosa* PA01. Background absorbance was measured in wells consisting of medium and diluent. The background absorbance was subtracted from the samples and the negative controls. The samples were compared to the negative controls to determine any reduction in the total amount of biofilm.

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(17) Absorbance measurements were taken to ensure bacterial growth occurred in the wells with compounds **1**−**5** prior to analyzing for biofilms. After the wells were rinsed and stained, the wells containing compounds **1**−**5** were compared to the negative controls of inoculum, medium, and solvent without test compounds, demonstrating differences in biofilms attached to the surface in these wells. An accepted positive control for biofilm assays is not available. Researchers have used various membrane detergents and antibacterial compounds. We selected not to use these compounds because they kill bacteria and do not exhibit specific biofilm inhibition. Furanone 56 was used because it has been shown to modulate quorum sensing involved in biofilms and reduce biofilm formation at various stages throughout the development of biofilms. By comparing the wells containing test compounds to wells with only bacteria, specific biofilm inhibition is observed because bacterial growth has occurred in both wells.

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