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Article

Caerulines A and B, Flavonol Diacylglycosides from *Persea caerulea*

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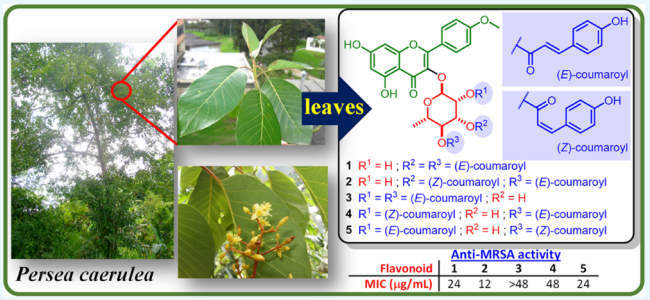
ABSTRACT: Two undescribed 4′-*O*-methylkaempferol-[3″,4″-di-*p*-coumaroyl]-α-L-rhamnopyranosides, caerulines A and B (**1**−**2**), along with three known 4′-*O*-methylkaempferol diacylrhamnosides isomers (**3**−**5**) were isolated from an ethanol extract of the leaves of *Persea caerulea*, a native plant growing on the Colombian Caribbean coast. The chemical structures of **1** and **2** were elucidated by spectroscopic methods. The effect of compounds **1**−**5** against four pathogenic microorganisms [i.e., methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, *Candida albicans*, and *Aspergillus fumigatus*] was tested *in vitro*. The compounds exhibited no activity against these pathogens except MRSA (MIC 12−48 μg/mL). Caeruline B (**2**) was found to be the most active compound with a modest anti-MRSA activity (MIC = 12 μg/mL).



INTRODUCTION

The genus Persea (Lauraceae)[1] consists of approximately 190 species[2] widely used in traditional medicine due to their antihypoglycemic,[3] antidiarrheal,[4] analgesic,[5] and anti-inflammatory properties,[6] which have prompted the identification of various bioactive compounds, including alkaloids,[7] terpenes,[1] flavonoids,[8,9] alkylfurane,[10,11] and lignans,[12] among others. *Persea caerulea* is commonly known as "aguacatillo", a widely distributed tree (*ca.* 500−2000 masl) in South America, mainly in Colombia, Bolivia, Ecuador, Peru, and Venezuela.[13] We previously reported 14 compounds from this plant, including two tannins, two flavanols, four flavonoid glycosides, one flavonol, two coumarins, and a mixture of three sterols.[8,14] In this context, flavonoids are among the main phytoconstituents of the *Persea* genus, and among them, flavanols have been identified in several *Persea* plants.[15−17] In fact, they could be used as taxonomic markers to differentiate species within the *Eriodaphne* and *Persea* subgenera.[8]

In addition, flavonols are also noted due to their antibacterial properties against a broad variety of pathogenic microorganisms.[18] Considering the current increase in difficult-to-treat infections due to the emergence of resistant microorganisms, the search for new antimicrobials, such as bioactive flavonoids, is an ongoing challenge to find antibiotic substitutes.[19] Previous structure−activity relationship surveys disclosed that particular substitution patterns of hydroxy groups and hydrophobic substituents (e.g., alkyl/prenyl, alkylamino, and additional oxygen- or nitrogen-containing heterocyclic moieties) usually positively influence the antibacterial activity of flavonoids, but an activity reduction is often observed if the active hydroxy groups are methylated.[18] Furthermore, flavonoids can exist as glycosides, in which sugar residues act as a decisive factor in their architectural complexity and bioactivity.[20] Such flavonoid transformation is endogenously accomplished by glycosyltransferases, which can create interesting and highly diverse compounds with broad or specialized functions.[21] The sugar moiety can be essential for glycoside bioactivity in some cases, but its pharmacokinetics and bioavailability could usually be enhanced by glycosylation.[22] In this regard, a better understanding of the increased bioactivity of glycoconjugates in comparison to free aglycones could promote the discovery and development of new glycosylated compounds with higher activity and efficacy (e.g., vancomycin).[23,24] Indeed, if the sugar residue is derivatized by acylation or phenylacylation, its chemical and biological diversity might be considerably increased. Such variations have a significant influence on the growth inhibition of both Gram-positive and Gram-negative bacterial strains, especially resistant strains such as vancomycin-resistant Enterococci (VRE) or methicillin-resistant *Staphylococcus aureus* (MRSA).[19,25] Actually, a kaempferol diacylrhamnoside was previously isolated from a Lauraceae plant (i.e., *Laurus*

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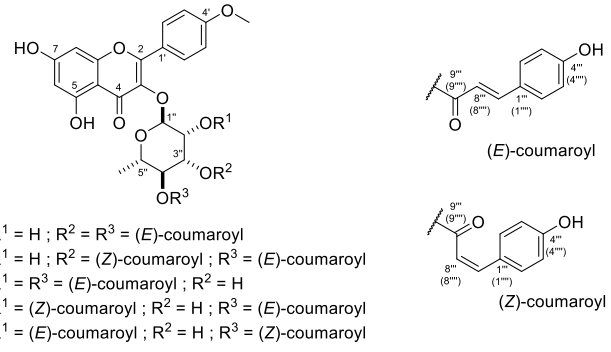
nobilis) and it exhibited antibacterial activity against MRSA (MIC < 8 μg/mL).[26] Thus, as part of our constant efforts to discover undescribed molecules from natural sources with important bioactivity and chemosystematic significance, we continued to explore the chemical potential of *P. caerulea*. The ethanol extract from *P. caerulea* leaves was subjected to sequential fractionation and purification by semipreparative HPLC, yielding two undescribed flavonol diacylglycosides, namely, caerulines A (**1**) and B (**2**), together with three closely related compounds (**3**−**5**). All of the compounds identified in this study are reported for the first time in the *Persea* genus. Diacylglycoside flavonols isolated in this study contribute to this differentiation because they have also been identified in *Persea lingue*, which belongs to the subgenus *Eriodaphne* as *P. caerulea*.[8] The antibacterial and antifungal activities of the isolated compounds **1**−**5** were also tested against a set of microorganisms (MRSA, *Acinetobacter baumannii*, *Candida albicans*, and *Aspergillus fumigatus*).

RESULTS AND DISCUSSION

The ethanolic extract of leaves from *P. caerulea* was fractionated by vacuum liquid chromatography (VLC) into fractions eluted with petroleum ether, *n*-hexane, toluene, chloroform, ethyl acetate, and methanol. The ethyl acetate fraction showed a promising LC/HRMS profile that suggested the presence of potentially undescribed metabolites not included in the Chapman and Hall Dictionary of Natural Products. This fraction was further fractionated by flash chromatography followed by semipreparative reversed-phase HPLC to yield two undescribed flavonol diacylglycosides, caerulines A (**1**) and B (**2**), along with three known compounds (**3**−**5**) (Figure 1). The known compounds were



1 R^1 = H ; R^2 = R^3 = (*E*)-coumaroyl
2 R^1 = H ; R^2 = (*Z*)-coumaroyl ; R^3 = (*E*)-coumaroyl
3 R^1 = R^3 = (*E*)-coumaroyl ; R^2 = H
4 R^1 = (*Z*)-coumaroyl ; R^2 = H ; R^3 = (*E*)-coumaroyl
5 R^1 = (*E*)-coumaroyl ; R^2 = H ; R^3 = (*Z*)-coumaroyl

Figure 1. Structures of isolated diacylglycosylated flavonols from the leaves of *P. caerulea*.

identified by comparisons of their experimental and reported spectroscopic data as 4′-*O*-methylkaempferol-[2,4″-di(*E*)-*p*-coumaroyl]-α-L-rhamnopyranoside (**3**), 4′-*O*-methylkaempferol-[2″(*Z*)-*p*-coumaroyl,4″-(*E*)-*p*-coumaroyl]-α-L-rhamnopyranoside (**4**), and 4′-*O*-methylkaempferol-[2″(*E*)-*p*-coumaroyl,4″-(*Z*)-*p*-coumaroyl]-α-L-rhamnopyranoside (**5**).[27] The structures of the undescribed flavonols **1**−**2** were established by comprehensive spectroscopic analyses (1D and 2D NMR, HRESIMS). The absolute configuration of their sugar residues was secured by HPLC after hydrolysis and derivatization with chiral reagents.

Caeruline A (**1**) was assigned a molecular formula of $C_{40}H_{34}O_{14}$ on the basis of ESI-TOF-MS measurements (*m*/*z* 737.1886 $[M - H]^-$, calcd for $C_{40}H_{33}O_{14}^-$, 737.1870). Its IR spectrum showed characteristic bands of carbonyl (1680 cm^{-1}), hydroxy (3410 cm^{-1}), and aromatic rings (1605 cm^{-1}). The ultraviolet (UV) spectrum exhibited a maxima at 265 and 314 nm, characteristic of the flavonoid nucleus.[28] The ^{1}H NMR spectrum of **1** (Table 1 and Figure S2) displayed signals at δ 8.01 (d, 8.7 Hz, 2H) for H-2′/H-6′ and δ 7.24 (d, 8.8 Hz, 2H) for H-3′/H-5′, of an AA′XX′ system, hydrogens at δ 6.30 (s) and δ 6.51 (d, 1.7 Hz) assigned to H-6/H-8 of an AX system and a methoxy group at C-4′ (δ 3.90, s, 3H), all characteristic of a 4′-*O*-methylkaempferol substructure. The oxygenated hydrogen region of the ^{1}H-NMR spectrum displayed signals of an anomeric hydrogen at δ 5.73 (m, H-1″), four methines at δ 4.54 (s, H-2″), δ 5.38 (dd, 10.2−3.0 Hz, H-3″), δ 5.28 (d, 9.9 Hz, H-4″), and δ 3.45 (m, H-5″) (Figure S3). These signals together with the presence of a doublet methyl group at δ 0.85 (d, 6.2 Hz, H-6″) suggested the presence of an α-rhamnopyranoside moiety whose absolute configuration was established as L by a modified HPLC analysis of the L-cysteine/*o*-tolylisothiocyanate derivative obtained after acidic hydrolysis of **1** (Figure S21). The ^{1}H NMR spectrum of caeruline A (**1**) also exhibits two pairs of AX and AAX systems. The first pair with signals at δ 7.52 (d, 8.5 Hz, H-2‴/H-6‴), δ 6.89 (d, 8.5, H-3‴/H-5‴) and two mutually coupled olefinic protons at δ 7.61 (d, 16.0, H-7‴), and δ 6.28 (d, 16.0, H-8‴) (Table 1 and Figure S2) corresponds to a *trans*-*p*-coumaroyl moiety. The second pair presents similar signals at δ 7.49 (d, 8.6 Hz, H-2‴′/H-6‴′); 6.86 (d, 8.6 Hz, H-3‴′/H-5‴′), δ 7.52 (d, 16.0 Hz, H-7‴′), and 6.22 (1H, d, 16.0 Hz, H-8‴′) corresponding to a second *trans*-*p*-coumaroyl unit. The ^{1}H−^{1}H COSY experiment showed clear couplings around the rhamnopyranoside ring, that is, δ 5.73 (m, H-1″) and δ 4.54 (s, H-2″); δ 5.38 (dd, 10.2, 3.0 Hz, H-3″) and δ 5.28 (d, 9.9 Hz, H-4″); and δ 5.28 (d, 9.9 Hz, H-4″) and δ 3.45 (m, H-5″) (Figure S7). The HMBC spectrum correlated the signal of the anomeric L-rhamnose hydrogen at δ 5.73 (H-1′) with a carbon at δ_C 135.3 (C-3) (Figure S10), establishing that the rhamnopyranosyl unit is attached to C-3 of the 4′-*O*-methylkaempferol (Figure 2). HMBC also displayed correlations between protons at δ 5.38 (H-3″) and δ 7.61 (d, 16.0, H-7‴) with the carbonyl carbons at δ 166.9 (C9‴) and between δ 5.28 (H-4″) and δ 7.52 (d, 16.0 Hz, H-7‴′) with δ 166.6 (C9‴′) (Figure S9), confirming the latter to be attached to carbons C3″ and C4″ of the rhamnose residue. This assignment was confirmed by the low-field shifts of the hydrogens at δ 5.38 (H2″) and δ 5.28 (H4″) of the sugar as a consequence of their participation in an ester bond. Thus, the structure of caeruline A (**1**) was identified as 4′-*O*-methylkaempferol-[3″,4″-di(*E*)-*p*-coumaroyl]-α-L-rhamnopyranoside, which constitutes an undescribed natural product.

The ESI-TOF-MS of caeruline B (**2**) displayed an $[M - H]^-$ ion at *m*/*z* 737.1891, indicating that the compound has the same molecular formula as **1**. The ^{1}H NMR spectroscopic data of **2** (Table1) (Figure S12) were similar to those of **1**, except for the resonances attributed to one of the coumaroyl moieties. Signals of the olefinic hydrogens of this unit at δ 6.86 (1H, d, 12.8 Hz, H-7‴) and 5.72 (1H, d, 12.8 Hz, H-8‴), together with their coupling constant of 12.8 Hz, were in agreement with the *Z*-configuration of the double bond. The HMBC spectrum (Figure S19) showed correlations of signals at δ 5.37 (dd, 10.2−3.0 Hz, H-3″) and δ 6.86 (H-7‴) to δ 166.9 (C-9‴) and from δ 5.23 (t, 10.0 Hz, H-4″) and δ 7.52 (H-7‴′) to δ 166.5 (C-9‴′) via three-bond coupling, demonstrating that the *cis*-*p*-coumaroyl unit is positioned at

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Table 1. NMR Data (500 MHz, Acetone-d_6, at 30 °C) of Caerulines A (1) and B (2)

	1		2	
#	δ ^{1}H (m, J Hz)	δ ^{13}C	δ ^{1}H (m, J Hz)	δ ^{13}C
2		158.2		158.2
3		135.3		131.6
4		179.1		179.1
5		163.2		162.9
6	6.30 (1H, d, 1.7)	99.8	6.30 (1H, d, 1.7)	99.6
7		165.3		166.1
7-OH	12.62 (1H, s)		12.62 (1H, s)	
8	6.51 (1H, s)	94.7	6.52 (1H, d, 2.0)	94.7
9		158.1		158.1
10		105.8		105.8
1′		123.5		123.5
2′/6′	8.01 (2H, d, 8.7)	131.6	8.01 (2H, d, 8.8)	131.6
3′/5′	7.24 (2H, d, 8.7)	115.1	7.24 (2H, d, 8.8)	115.1
4′		162.9		162.9
4′-OCH_3	3.90 (3H,s)	56.0	3.89 (3H,s)	56.0
1″	5.73 (1H, m)	101.8	5.74 (1H, m)	101.8
2″	4.54 (1H, s)	69.4	4.52 (1H, s)	69.3
3″	5.38 (1H, dd, 10.2, 3.0)	72.2	5.37 (1H, dd, 10.2, 3.0)	71.9
4″	5.28 (1H, d, 10.0)	71.0	5.23 (1H, t, 10.1)	71.0
5″	3.45 (1H, m)	69.5	3.42 (1H, m)	69.6
6″	0.85 (3H, d, 6.2)	17.6	0.84 (3H, d, 6.2)	17.7
1‴		126.8		127.1
2‴/6‴	7.52 (2H, d, 8.5)	131.0	7.79 (2H, d, 8.6)	134.0
3‴/5‴	6.89 (2H, d, 8.5)	116.7	6.83 (2H, d, 8.6)	115.7
4‴		160.7		159.9
7‴	7.61 (1H, d, 16.0)	146.0	6.86 (1H, d, 12.8)	145.6
8‴	6.28 (1H, d, 16.0)	115.1	5.72 (1H, d, 12.8)	116.0
9‴		166.9		166.1
1‴′		126.7		126.7
2‴′/6‴′	7.49 (2H, d, 8.6)	131.0	7.51 (2H, d, 8.7)	131.0
3‴′/5‴′	6.86 (2H, d, 8.6)	116.7	6.89 (2H, d, 8.7)	116.7
4‴′		160.8		160.7
7‴′	7.52 (1H, d, 16.0)	146.0	7.52 (1H, d, 15.9)	146.0
8‴′	6.22 (1H, d, 16.0)	114.9	6.21 (1H, d, 15.9)	114.8
9‴′		166.6		166.5

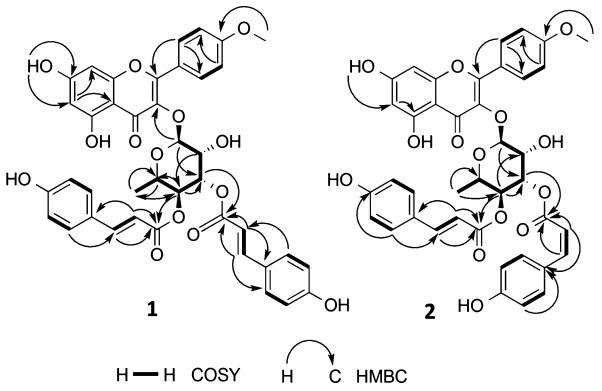

Figure 2. Key HMBC and COSY correlations of compound 1 and 2.

C-3″ and that the *trans*-*p*-coumaroyl unit is located at C-4″ of the rhamnose residue (Figure 2). The L absolute configuration of the latter residue was established by performing the same modified HPLC analysis of the *o*-tolylisothiocyanate derivatives described for 1 (Figure S21). Therefore, the identity of this undescribed compound, named caeruline B (2), is 4′-*O*-methylkaempferol-[3″(*Z*)-*p*-coumaroyl,4″-(*E*)-*p*-coumaroyl]-α-L-rhamnopyranoside.

The biological activities of caerulines A (1) and B (2) and the known 4′-*O*-methylkaempferol diacylrhamnosides (3−5) were tested against a small panel of bacterial and fungal human pathogens, including Gram-positive (MRSA) and Gram-negative (*A. baumannii*) bacteria, *C. albicans* and *A. fumigatus*. Compounds 1−5 exhibited no antibacterial activity against *A. baumannii* and the two fungal strains tested. When tested against MRSA, compounds 1, 2, and 5 exhibited modest antibacterial activity with MIC values of 12 (2) and 24 μg/mL (1 and 5). Compound 4 (MIC 48 μg/mL) was less potent, and compound 3 proved to be inactive at the highest concentration tested (Table 2). Although the purity of the test compounds 1−2 appeared to be not satisfactory by the presence of other signals of closely related compounds in the

Table 2. Anti-MSRA Activity of Compounds 1−5 from *P. caerulea*

compounds	1	2	3	4	5
MIC (μg/mL)	24	12	>48	48	24

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NMR spectra, which could be affecting the observed anti-MRSA activity, we decided to proceed to their biological evaluation because the impurities were below 5% and due to supply problems, it was not possible to increase the isolated amount (<0.8 mg) to improve purity of test compounds. Additionally, several studies of the antibacterial activity of acylated kaempferol glycosides have demonstrated a relationship between their structure and their activity against MRSA.[26,29,30] In this regard, our research contributes to the presence of the methoxy group in C3 (i.e., a 4′-*O*-methylkaempferol aglycone), since this fact could also be related to the bacterial inhibition that 4′-*O*-methylkaempferol exhibits against MRSA.[31] Our findings indicate that the geometric isomerism of both *p*-coumaroyl substitutions, that is, the *Z* and *E* isomeric forms, seems to have an impact on the anti-MRSA activity, with the presence of a *Z*-*p*-coumaroyl moiety at C3 or C4 of the rhamnosyl residue leading to a slight increase in the antimicrobial activity of the compounds. Therefore, with this small set of compounds in hand, a structure−activity (SA) trend can be deduced, which is related to an enhanced activity by a 3,4-diacylated pattern and a combination of an *E* and *Z* isomerism of each *p*-coumaroyl substitution.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were acquired on a Thermo Fisher Scientific Genesys 10S. Infrared (IR) spectra were taken on film in a KBr window on a Perkin Elmer 500 series FTIR Panagon 1000. 1D and 2D NMR spectra were recorded on a Bruker AVANCE III spectrometer at 500/125 MHz (^{1}H/^{13}C NMR, respectively) equipped with a 1.7 mm TCI MicroCryoProbe (Bruker Biospin, Fällanden, Switzerland). All shifts are given in δ (ppm) using the signal of tetramethylsilane as reference. All coupling constants (J) are given in Hz. ESI-TOF spectra and HRESIMS experiments were acquired using a Bruker maXis QTOF (Bruker Daltonik GmbH, Bremen, Germany) mass spectrometer coupled to an Agilent 1200 LC (Agilent Technologies, Waldbronn, Germany). Flash chromatography was performed on a semiautomatic chromatographic system (CombiFlashTeledyne ISCO Rf400x) with a precast reverse-phase column. Semipreparative HPLC separation was performed on a Gilson GX-281 322H2 (Gilson Technologies, USA) coupled to a DAD detector and an automatic fraction collector with a semipreparative reversed-phase column (Zorbax SB-C18, 250 × 9.4 mm, 5 μm).

Plant Material. Plant samples corresponding to the leaves of *P. caerulea* were collected in San Pedro de la Sierra, Magdalena Department, Colombia. The plant material was identified by the botanist Adolfo Jara, and a voucher specimen was deposited at Herbario Nacional Colombiano under code COL 518189.

Extraction and Isolation. Air-dried leaves of *P. caerulea* (250 g) were macerated with 96% ethanol at 20 °C. The ethanolic extract was concentrated by reduced pressure evaporation to yield a crude 98.2 g. A portion of this crude extract (20 g) was fractionated by VLC (80 × 8 cm) on silica gel using petroleum ether (1 L), hexane (1 L), toluene (1 L), chloroform (1 L), ethyl acetate (2 L), and methanol (2 L) as mobile phases producing six fractions, which were additionally monitored by LC−UV−MS. The ethyl acetate fraction (2 g) was loaded onto a reversed-phase C18 (ODS) column (32 × 100 mm) that was eluted at 8 mL/min on an automatic flash-chromatography system (CombiFlash Rf, Teledyne Isco) using a linear gradient from 20 to 100% acetonitrile in water (in 12.5 min) with a final 100% acetonitrile step (for 15 min) collecting 40 subfractions, grouped according to their LC−UV−MS chemical profiles in 10 subfractions: A−H. Subfraction E (12 mg) was further chromatographed by semipreparative reversed-phase HPLC (Zorbax SB-C18, 9.4 × 250 mm, 5 μm; 3.6 mL/min, UV detection at 210 and 280 nm) with an isocratic elution of 35% CH_3CN/65% H_2O with 0.1% trifluoroacetic acid over 37 min, yielding **3** (0.9 mg) and **1** (0.7 mg) eluting at 12 and 16 min, respectively. Subfraction F (10 mg) was subjected to semipreparative reversed-phase HPLC (Zorbax SB-C18, 9.4 × 250 mm, 5 μm; 3.6 mL/min, UV detection at 210 and 280 nm) with an isocratic elution of 40% CH_3CN/60% H_2O with 0.1% trifluoroacetic acid over 37 min, yielding **4** (0.8 mg), **5** (0.7 mg) and **2** (0.6 mg) eluting at 18, 21.5 and 23.2 min, respectively.

*Caeruline A (**1**).* A yellow amorphous solid; UV λ_{max} (MeOH) (log ε) 265 (4.11) and 314 (3.56) nm; IR ν_{max} (film) 3410 (OH), 1680 (C═O), 1605 (aromatic C−C); ^{1}H (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) spectroscopic data, see Table 1. (−)-ESI-TOFMS m/z: 737.1886 $[M - H]^-$ (calcd for $C_{40}H_{33}O_{14}$, 737.1870).

*Caeruline B (**2**).* A yellow amorphous solid; UV λ_{max} (MeOH) (log ε) 266 (4.16) and 315 (3.71) nm; IR ν_{max} (film) 3420 (OH), 1682 (C═O), 1607 (aromatic C−C); ^{1}H (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) spectroscopic data, see Table 1. (−)-ESI-TOFMS m/z, 737.1891 $[M - H]^-$ (calcd for $C_{40}H_{33}O_{14}$, 737.1870).

Antibacterial and Antifungal Assays. The antimicrobial activities of compounds **1**−**5** were evaluated against two bacterial and two fungal strains from MEDINA's Culture Collection. Antibacterial susceptibility of the compounds was tested against methicillin-resistant *S. aureus* (MRSA) MB5393 and *A. baumannii* MB5973, while antifungal activity was tested against *C. albicans* MY1055 and *A. fumigatus* ATCC 46645 (wild-type strain) following previously described methodologies.[32−34] Briefly, each compound was serially diluted in DMSO with a dilution factor of 2 to provide 10 concentrations starting at 48 μg/mL for all antimicrobial assays. The MIC was defined as the lowest concentration of the compound that inhibited ≥95% of the growth of a microorganism after overnight incubation. Genedata Screener software (Genedata, Inc., Basel, Switzerland) was used to process and analyze the data and to calculate the RZ factor, which predicts the robustness of an assay. In all experiments performed in this work, the RZ factor obtained was between 0.87 and 0.98.[33]

Determination of the Absolute Configuration of Rhamnose in 1 and 2. Glycosides **1** and **2** were dissolved in 1 N HCl (aq) (300 μL) at 80 °C for 3 h. The solutions were evaporated to dryness using an N_2 stream. The absolute configuration of the sugars in the residue was determined by the method described by Tanaka et al.[35] Briefly, samples (residues after acid hydrolysis or standards of L-rhamnose and D-rhamnose) were dissolved in pyridine (100 μL) containing L-cysteine methyl ester hydrochloride (6.0 mg) and incubated at 60 °C. After 1 h incubation, *o*-tolylisothiocyanate (0.5 mg) in pyridine solution (200 μL) was added to the mixture and incubated at 60 °C for an additional hour. By comparison of the retention times with the standards and co-HPLC, the absolute configuration of sugars in each hydrolysis was established (Figure S21). The monosaccharides from **1** and **2** were detected as L-rhamnose.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04255.

HRESIMS spectra of compounds **1**−**2**, ^{1}H and ^{13}C spectra (1D and 2D) of compounds **1**−**2**, and HPLC profiles *o*-tolylisothiocyanate derivatives after hydrolysis of compounds **1**−**2** (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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