Antistaphylococcal prenylated acylphloroglucinol and xanthones from Kielmeyera variabilis (Clusiaceae)

Coqueiro, A.; Choi, Y.H.; Verpoorte, R.; Gupta, K.B.S.S.; Mieri, M. de; Hamburger, M.; ... ; Bolzani, V.D.S.

JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Antistaphylococcal Prenylated Acylphoroglucinol and Xanthones from *Kielmeyera variabilis*

Aline Coqueiro,[†,∥,∇] Young H. Choi,[†] Robert Verpoorte,[†] Karthick B. S. S. Gupta,[‡] Maria De Mieri,[§] Matthias Hamburger,[§] Maria C. M. Young,[⊥] Paul Stapleton,[∥] Simon Gibbons,*[,∥] and Vanderlan da S. Bolzani*[,∇]

[†]Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, Leiden, 2333 BE, The Netherlands

[‡]NMR Facility, Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, Leiden, 2333 CC, The Netherlands

[§]Division of Pharmaceutical Biology, University of Basel, Klingelbergstrasse 50, Basel, CH-4056, Switzerland

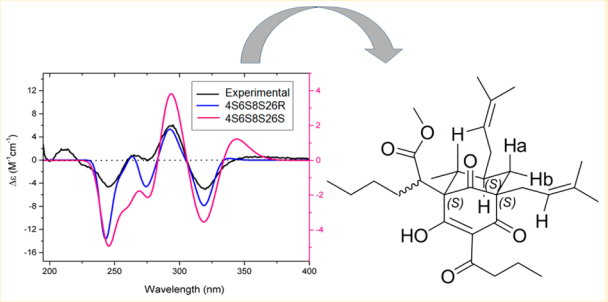
[⊥]Section of Plant Physiology and Biochemistry, Institute of Botany, São Paulo, 01061-970, Brazil

[∥]Research Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy, 29-39 Brunswick Square, London, WC1N 1AX, United Kingdom

[∇]Department of Organic Chemistry, Institute of Chemistry, São Paulo State University, Prof. Francisco Degni 55, Araraquara, 14800-900, Brazil

Supporting Information

ABSTRACT: Bioactivity-guided fractionation of the EtOH extract of the branches of *Kielmeyera variabilis* led to the isolation of a new acylphoroglucinol (**1**), which was active against all the MRSA strains tested herein, with pronounced activity against strain EMRSA-16. Compound **1** displayed an MIC of 0.5 mg/L as compared with an MIC of 128 mg/L for the control antibiotic norfloxacin. The structure of the new compound was elucidated by 1D and 2D NMR spectroscopic analysis and mass spectrometry, and experimental and calculated ECD were used to determine the absolute configurations. The compounds *β*-sitosterol (**2**), stigmasterol (**3**), ergost-5-en-3-ol (**4**), and osajaxanthone (**5**) also occurred in the *n*-hexane fraction. The EtOAc fraction contained nine known xanthones: 3,6-dihydroxy-1,4,8-trimethoxyxanthone (**6**), 3,5-dihydroxy-4-methoxyxanthone (**7**), 3,4-dihydroxy-6,8-dimethoxyxanthone (**8**), 3,4-dihydroxy-2-methoxyxanthone (**9**), 5-hydroxy-1,3-dimethoxyxanthone (**10**), 4-hydroxy-2,3-dimethoxyxanthone (**11**), kielcorin (**12**), 3-hydroxy-2-methoxyxanthone (**13**), and 2-hydroxy-1-methoxyxanthone (**14**), which showed moderate to low activity against the tested MRSA strains.



The genus *Kielmeyera* belongs to the family Clusiaceae and occurs exclusively in South America. Among the 47 known species of this genus, 45 are native to Brazilian ecosystems. These plants grow mainly in the Brazilian Cerrado Biome, specifically in the Midwest region.[1]

Kielmeyera variabilis Mart., commonly known in Brazil as "malva-do-campo", is a medicinal tree used in Brazilian folk medicine to treat several tropical diseases, including schistosomiasis, leishmaniasis, malaria, and fungal and bacterial infections.[2] Previous work has shown that species belonging to the family Clusiaceae produce xanthones and phoroglucinol derivatives that possess antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA).[3,4] Previous studies on *K. variabilis* Mart. have shown that this species contains prenylated xanthones with molluscicidal activity[5,6] and flavonoids with antioxidant properties.[7]

Resistance to methicillin, a form of penicillin, among *S. aureus* began to be noted at the beginning of the 1960s. Since then, these organisms have developed mechanisms of resistance to many different classes of antimicrobial agents, making treatment options severely limited. Infection with MRSA is therefore a serious public health concern that has called for the development of new antibacterial drugs to overcome microbial resistance.[8]

Natural products bearing different structural patterns can be active against resistant strains, so the screening of natural product extracts from microorganisms or plants still constitutes a valid strategy to discover new lead compounds with antibiotic properties.

As part of an ongoing SisBiota CNPq/FAPESP Biodiscovery Program, which aims to discover new sources of bioactive compounds in the Brazilian flora, this investigation evaluated

Special Issue: Special Issue in Honor of John Blunt and Murray Munro

Received: September 26, 2015
Published: February 22, 2016

DOI: 10.1021/acs.jnatprod.5b00858
J. Nat. Prod. 2016, 79, 470−476

the potential of *K. variabilis* Mart. against multidrug-resistant strains. Bioassay-guided fractionation helped to define the bioactive compounds and prove that this plant is applicable as an antimicrobial agent in traditional folk medicine.

RESULTS AND DISCUSSION

Successive extraction of the dried and pulverized *K. variabilis* branches and leaves with EtOH afforded an extract for a preliminary activity screening assay against the multidrug-resistant strain SA-1199B, which overexpresses the NorA efflux pump and has a Gyr-A mutation.[11]

Partitioning of the EtOH extracts of *K. variabilis* branches and leaves by liquid−liquid extraction resulted in four fractions (*n*-hexane, EtOAc, *n*-BuOH, and aqueous MeOH) for each plant part. Tests on all fractions against strain SA-1199B permitted the selection of the most promising fraction to start the bioassay-guided fractionation (Table 1).

Table 1. Antimicrobial Activity of the Extracts and Fractions of *K. variabilis* Branches and Leaves against the Strain SA-1199B

plant part	extract and fractions	MIC (mg/L)
branches	EtOH	32
	n-hexane	16
	EtOAc	64
	n-BuOH	256
	aqueous MeOH	>512
leaves	EtOH	128
	n-hexane	64
	EtOAc	128
	n-BuOH	256
	aqueous MeOH	>512
positive control	norfloxacin	32

The crude EtOH extract of the branches was 4 times more active (MIC of 32 mg/L) than the crude EtOH extract of the leaves (MIC of 128 mg/L) and had activity comparable to the activity of the standard norfloxacin (MIC of 32 mg/L) against the fluoroquinolone-resistant organism. The antibacterial activity of the *n*-hexane fraction of the branches (MIC of 16 mg/L) was higher than the corresponding EtOH extract (MIC of 32 mg/L) and even slightly higher than the activity of the control norfloxacin (MIC of 32 mg/L). This showed that the fractionation process potentiated the activity of the branches extract by concentrating the most active compound(s) in the *n*-hexane fraction.

Bioassay-guided fractionation of the most active fraction permitted identification of the bioactive compound. Fractionation of the *n*-hexane fraction of the branches on a Sephadex LH-20 column led to six subfractions (Fr1 to Fr6). Evaluation of the antibacterial activity of these subfractions showed that Fr1 (756.5 mg) was the most active, with an MIC of 2 mg/L, and therefore Fr1 was 8-fold more potent than the *n*-hexane fraction (MIC of 16 mg/L).

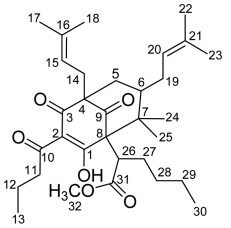
A phytochemical study on the most active subfraction identified compound **1** as the bioactive compound underlying the activity of the EtOH extract of *K. variabilis* branches. The activities of Fr1 and compound **1** were assessed at concentrations ranging from 512 to 0.5 mg/L against a panel of multidrug-resistant *S. aureus* strains with clinical relevance and different mechanisms of resistance. Fr1 also contained the steroids *β*-sitosterol (**2**), stigmasterol (**3**),[16] and ergost-5-en-3-ol (**4**). The nonactive subfraction Fr6 contained the xanthone osajaxanthone (**5**),[17] which we did not test due to the low sample size of this compound.

Among the tested strains were SA-1199B, a multidrug-resistant strain that overexpresses the NorA efflux pump[11] and possesses a gyrase-encoding gene mutation that also confers a high level of resistance to some fluoroquinolones;[18] RN4220, a macrolide-resistant strain;[10] XU212, a clinical MRSA strain that is resistant to tetracycline and bears the TetK efflux pump;[9] ATCC 25923, a standard laboratory strain;[9] and the epidemic methicillin-resistant strains EMRSA-15 and EMRSA-16.[13]

Compound **1** accounted for the activity of the *n*-hexane fraction of the branches. Its activity was similar to or higher than the activity of fraction Fr1. Compared with the positive control norfloxacin, its activity was higher against strains SA-1199B and XU212. It also displayed pronounced activity against the epidemic methicillin-resistant strain EMRSA-16; its MIC was 0.5 mg/L as compared with the MIC of the control (128 mg/L). The activities of compound **1** and the control were similar for the strains ATCC 25923, RN4220, and EMRSA-15.

The mixture of **2** and **3** and of individual compounds **2**, **3**, and **4** did not exhibit any activity against the *S. aureus* strains. Knowing that these compounds are nonactive, their identification was conducted by NMR and GC-MS without isolating them from these mixtures.

Minimum bactericidal concentration (MBC) assays conducted with active compound **1** permitted determination whether this compound exerted a bacteriostatic or bactericidal effect. For these assays, a 10 μL sample was removed from the wells of a plate used to determine the MIC values of compound **1** against SA-1199B (where no bacterial growth had occurred). This volume was plated onto drug-free media. After 24 h of incubation, bacteria grew on the drug-free plates. Hence, compound **1** inhibited the organisms by exerting a bacteriostatic effect.



1

The HRESIMS of compound **1** revealed an $[M + H]^+$ ion *m*/*z* at 529.3547 in the positive mode. Combined with ^{13}C NMR data, this information permitted assignment of the molecular formula of compound **1** as $C_{32}H_{48}O_6$. Two vinyl protons, four vinylic methyl groups, and four allylic protons in the ^1H NMR spectrum suggested the presence of two isopent-2-enyl side chains. HMBC correlations of H-11a, H-11b, H-12a, and H-12b with the carbonyl C-10 and COSY correlations of H-11b to H-11a and H-12b and of H-12b to H_3-13 demonstrated the presence of an *n*-butanoyl chain. A ^{13}C NMR resonance at δ 174.1 (C-31) correlated with H_3-32 (δ 3.65) in the HMBC spectrum is typical of a methoxy group and revealed the presence of an ester carbonyl group. The presence of one methine (δ_C 38.7), three methylene (δ_C 39.9, 37.3, and 22.7), and one methyl carbon (δ_C 15.2) correlated by HMBC reveals the presence of a hexanoyl chain. This chain is confirmed by correlations of H-26 (δ 3.29) and H-27 (δ 2.34) with the ester

DOI: 10.1021/acs.jnatprod.5b00858
J. Nat. Prod. 2016, 79, 470−476

carbonyl C-31 and the correlations of H-26, H-29 (δ 1.43 and 1.52), and H-30 (δ 0.94) with C-28 (δ 37.3).

The ^{13}C NMR spectrum displayed the characteristic resonances of a bicyclic [3.3.1] nonane ring system with three quaternary carbons (δ_C 64.7, 49.2, and 69.5), a methine (δ_C 48.6), a methylene (δ_C 39.5), an enolized 1,3-diketo system (δ_C 114.7, 199.7, and 195.5), and a nonconjugated carbonyl (δ 209.4). Together, these data suggested that compound **1** was a polyisoprenylated phloroglucinol derivative.[4,19−22]

HMBC correlations facilitated establishment of the substitution pattern of the nonane ring system. Correlation of H-26 with C-1, C-9, and C-31 indicated that the ester carbonyl group was connected to the nonane system at C-8. HMBC correlation of H-5a with C-14 and C-19 showed connection of the isoprenyl side chains to C-4 and C-6. By elimination, the 1-butanoyl moiety was linked at C-2. The methyl groups Me-24 (δ 1.20) and Me-25 (δ 1.37) correlated with each other and with C-7 and C-8 (Figure 1).

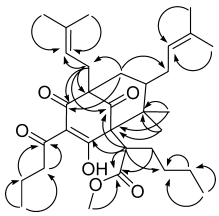


Figure 1. Key correlations observed in the HMBC NMR spectrum of compound **1**.

Proton H-5a had two large coupling constants, i.e., 14.3 Hz, consistent with its geminal coupling to H-5b, and 10.4 Hz, due to an axial−axial coupling with H-6. These data indicated that H-6 was in the axial position, with the C-6 isoprenyl side chain equatorially oriented.[23]

NOESY studies aided the determination of the relative configuration assignments and supported the delineated stereochemistry. Key NOE correlations between H-6 (δ 1.41), the methyl protons at δ 1.20 (H_3-24), and H-5b (δ 2.12) shown in Figure 3a demonstrated that they were all oriented on the same face of the bicyclic [3.3.1] nonane ring,

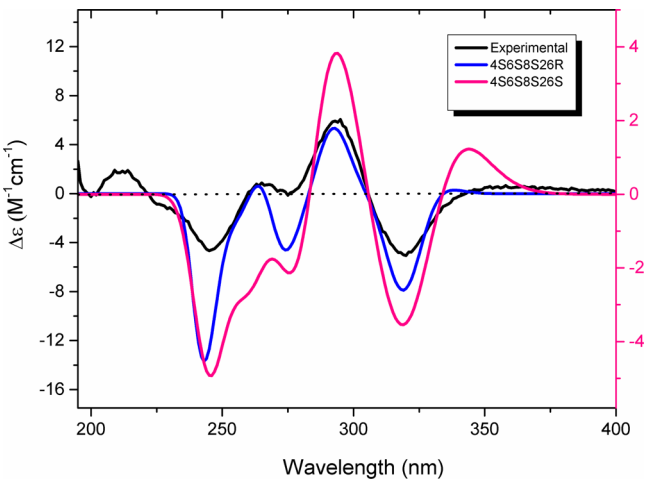


Figure 2. Comparison of experimental and calculated (weighted) ECD spectra of **1** for the two stereoisomers 4*S*,6*S*,8*S*,26*S*, 4*S*,6*S*,8*S*,26*R*. The calculations were performed with TDDFT at the cam-B3LY/6-31G** level in MeOH.

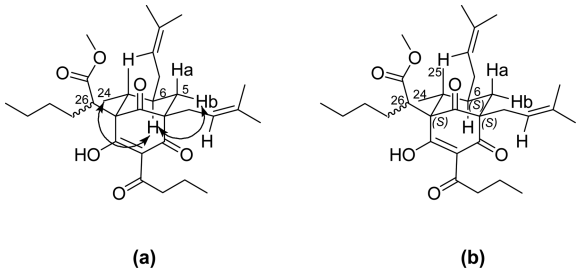


Figure 3. Key correlations observed in the NOESY NMR spectra of compound **1** (a) and absolute configuration of the bicyclic [3.3.1] nonane ring determined by ECD (b).

which placed H-5b and H_3-24 in an 1,3-diequatorial position relative to each other and in a chair conformation in relation to the cyclohexanone ring.

The absolute configuration of **1** was stablished by comparison of its experimental electronic circular dichroism (ECD) curve with those predicted using TDDFT theory. On the basis of geometrical considerations, four possible isomers were conceivable: 4*S*,6*S*,8*S*,26*S*, 4*S*,6*S*,8*S*,26*R*, 4*R*,6*R*,8*R*,26*S*, and 4*R*,6*R*,8*R*,26*R*.

The 4*S*,6*S*,8*S* absolute configuration of the bicyclic [3.3.1] nonane ring, was assigned by the similarity of the experimental and calculated spectra at the cam-B3LY/6-31G** level in MeOH (Figure 2). However, this method was not able to clearly discriminate between the 26*R* and 26*S* configurations, since the calculated spectra for the 4*S*,6*S*,8*S*,26*S* and 4*S*,6*S*,8*S*,26*R* epimers are very similar.

Therefore, the structure of kielmeyeracin (**1**) was established as shown in Figure 3b.

Phytochemical investigation of the EtOAc fraction, i.e., the second most active fraction of *K. variabilis* branches, resulted in the identification of nine xanthones: 3,6-dihydroxy-1,4,8-trimethoxyxanthone (**6**), 3,5-dihydroxy-4-methoxyxanthone (**7**),[24] 3,4-dihydroxy-6,8-dimethoxyxanthone (**8**),[25] 3,4-dihydroxy-2-methoxyxanthone (**9**),[26] 5-hydroxy-1,3-dimethoxyxanthone (**10**),[25,26] 4-hydroxy-2,3-dimethoxyxanthone (**11**),[27,28] kielcorin (**12**),[26] 3-hydroxy-2-methoxyxanthone (**13**),[29] and 2-hydroxy-1-methoxyxanthone (**14**).[30] Although these xanthones were previously isolated from the genus *Kielmeyera*, no studies on the antimicrobial action of these compounds exist. Kielcorin is the only xanthone that has been previously reported for the species *K. variabilis*.[5,6] The amount of xanthones **6**, **7**, and **8** obtained here permitted their testing and identification in a mixture. 1D and 2D NMR techniques, ESIMS, and comparison with literature data enabled the identification of all of the xanthones. The Supporting Information shows the NMR and MS data.

Microbial infections stimulate higher plants to synthesize xanthones related to phytoalexins via a passive defense system. Natural xanthones are potent MRSA inhibitors,[31] and prenylated xanthones show the highest antimicrobial activity.[32,33]

The antimicrobial potential of the isolated oxygenated xanthones was evaluated against the same panel of *S. aureus* strains (Table 4).

The xanthones displayed moderate to weak antibacterial action. Xanthone **9** had the highest activity: MICs varied between 16 and 64 mg/L. The most pronounced activity was observed against the most resistant strain, EMRSA-16. Among the pure tested compounds, xanthone **9** is the only one bearing

DOI: 10.1021/acs.jnatprod.5b00858
J. Nat. Prod. 2016, 79, 470−476

Table 2. In Vitro Antibacterial Activity (MIC in mg/L) of Subfractions and Compounds Isolated from the *n*-Hexane Fraction of *K. variabilis* Branches against Different *S. aureus* Strains

fraction and compounds	SA-1199B	XU212	ATCC 25923	RN4220	EMRSA-15	EMRSA-16
Fr1	2	0.5	1	0.5	1	0.5
1	2	0.25	1	0.25	1	0.5
2 + **3**	>512	>512	>512	>512	>512	>512
2 + **3** + **4**	>512	>512	>512	>512	>512	>512
norfloxacin	32	8	0.5	0.5	0.5	128

Table 3. NMR Spectroscopic Data (600 MHz, Pyridine-d_5) for Compound 1

position	δ_C, type	δ_H (J in Hz)	HMBC
1	199.7, C		
2	114.7, C		
3	195.5, C		
4	64.7, C		
5	39.5, CH_2	a: 2.26 dd (14.3, 10.4)	3, 4, 6, 14, 19
		b: 2.12 overlapped	
6	48.6, CH	1.41 m	
7	49.2, C		
8	69.5, C		
9	209.4, C		
10	203.7, C		
11	41.7, CH_2	a: 3.27 m	10, 13
		b: 3.05 ddd (15.2, 8.9, 6.2)	10, 13
12	19.4, CH_2	a: 1.71 m	10, 11, 13
		b: 1.78 m	10, 11, 13
13	14.5, CH_3	0.95 t (7.5)	11, 12
14	29.4, CH_2	a: 2.80 dd (14.6, 6.8)	3, 4, 5, 9, 15, 16
		b: 2.89 dd (14.6, 8.0)	3, 4, 5, 9, 15, 16
15	121.2, CH	5.64 br t	14, 17, 18
16	134.7, C		
17	26.7, CH_3	1.74 s	15, 16, 18
18	18.3, CH_3	1.67 s	15, 16, 17
19	29.7, CH_2	a: 1.88 br	
		b: 2.10 overlapped	
20	124.3, CH	4.93 br t	19, 22, 23
21	133.5, C		
22	26.5, CH_3	1.69 s	20, 21, 23
23	18.3, CH_3	1.55 s	20, 21, 22
24	25.4, CH_3	1.20 s	7, 8, 25
25	22.6, CH_3	1.37 s	7, 8
26	38.7, CH	3.29 m	1, 8, 9, 27, 28, 31
27	39.9, CH_2	2.34 br	31
28	37.3, CH_2	a: 2.01 br	
		b: 1.81 m	
29	22.7, CH_2	a: 1.52 m	28, 30
		b: 1.43 m	28, 30
30	15.2, CH_3	0.94 t (7.5)	28, 29
31	174.1, C		
32	52.0, OCH_3	3.65 s	31

a catechol group in the structure. In the present work, the activity of oxygenated xanthones was lower than the activities reported for some prenylated xanthones. Indeed, the prenyl groups can serve as modulators of lipid affinity and cellular bioavailability. The presence of a nonpolar group enhances membrane permeability, which accounts for the higher antimicrobial potential of prenylated xanthones[32,33] as compared with the oxygenated xanthones investigated in this study.

The bioassay-guided fractionation applied in this study led to the isolation of the new compound kielmeyeracin (**1**), which displayed strong antibacterial activity against methicillin-resistant *S. aureus* strains. This compound exhibited activity similar to or higher than the activity of the control norfloxacin, mainly against EMRSA-16. *K. variabilis* Mart. proved to have a high antimicrobial potential, which correlated with the use of this plant in folk medicine. Isolation of the bioactive compound makes both this molecule and the plant of interest for further studies on the mechanism of anti-MRSA action and for future in vivo investigations.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured on a PerkinElmer polarimeter (model 341) equipped with a sodium lamp (589 nm) and a 10 cm microcell. UV spectra were obtained on a Shimadzu UV-1800 instrument. The ECD spectra were recorded in MeOH with a Chirascan spectrometer. NMR spectra of compound **1** were recorded on a Bruker 600 Avance II NMR spectrometer (Bruker, Bellerica, MA, USA) in pyridine-d_5 (298 K), at 600 and 150 MHz for ^{1}H and ^{13}C NMR, respectively. 1D and 2D NMR spectra of xanthones and steroids were recorded on a Varian INOVA 500 (11.7 T) spectrometer with TMS as the internal standard. The HRESIMS of compound **1** was performed on a Bruker UPLC system (Dionex 3000) coupled to a Bruker (micrOTOF II) time-of-flight mass spectrometer, equipped with an electrospray interface (ESI). A Bruker Daltonics utrOTOFQ with ESI operating in the positive and negative mode was used to confirm the molecular weight of the xanthones. A Varian ProStar chromatography system with diode array detector was employed to profile the extracts. A Varian ProStar chromatography unit operating at λ = 254 nm was used to accomplish the preparative isolation. The following columns were employed: a Phenomenex Luna Phenyl-Hexyl and a Phenomenex Luna C_{18} column (5 μm, 250 × 4.6 mm, analytical), a Phenomenex Luna Phenyl-Hexyl preparative column (10 μm, 250 × 21.2 mm), and a preparative Phenomenex C_{18} column (10 μm, 250 × 21.2 mm). The mixtures of steroids were analyzed by gas chromatography coupled to a mass detector (GC-MS) with split injector 20:1; the injected volume was 1 μL. The injector temperature was 280 °C, and the temperature of the interface was 300 °C. The initial column temperature was 50 °C, held for 3 min, which was followed by a temperature rise to 295 °C at 2 °C/min. The temperature was kept at 295 °C for an additional 20 min. The total run time was 145.5 min. Helium was employed as the carrier gas at an average linear velocity of 1 mL/min. The ionization mode was electron ionization (EI) in the positive mode with an impact energy of 70 eV. The identities of the isolated compounds were confirmed by comparing ^{1}H and ^{13}C NMR signals with literature values and high-resolution MS data (Supporting Information).

Plant Material. The leaves and branches of *K. variabilis* were collected in Fazenda Campininha in Mogi-Guaçú, state of São Paulo, Brazil, in January 2007. The plant was identified by Dr. Inês Cordeiro (IBt-SMA). A voucher specimen (SP 346310) was deposited in the herbarium "Maria E. P. Kauffman" of the Botanic Institute of São Paulo, state of São Paulo, Brazil.

Extraction. Air-dried and ground leaves and branches were exhaustively extracted by maceration with EtOH at room temperature separately. After filtration, the solvent was evaporated at low

DOI: 10.1021/acs.jnatprod.5b00858
J. Nat. Prod. 2016, 79, 470−476

Table 4. In Vitro Antibacterial Activity (MIC in mg/L) of Xanthones Isolated from *K. variabilis*

compounds	SA-1199B	XU212	ATCC 25923	RN4220	EMRSA-15	EMRSA-16
6 + **7** + **8**	64	64	128	64	128	32
9	32	32−16	64	32	64	16
10	128−64	>512	128	>512	>512	64
11	128−64	128	128	>512	>512	64
12	>512	>512	>512	>512	>512	>512
13	64	64	64	64	64	32
14	64	128	64	64	64	32
norfloxacin	32	8	0.5	0.5	0.5	128

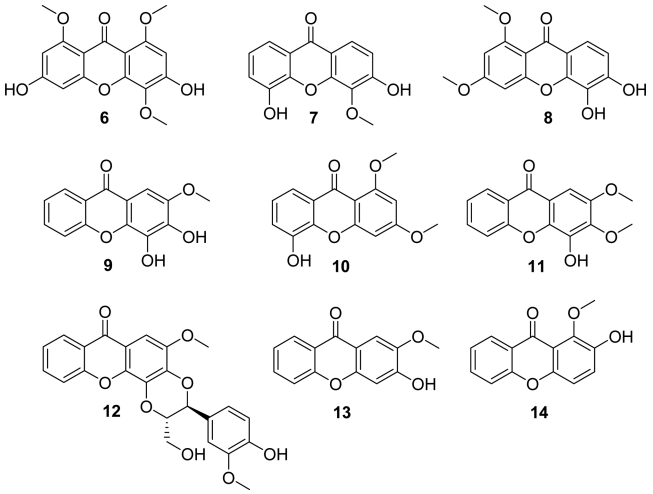


Figure 4. Chemical structure of xanthones **6**−**14**.

temperature (<40 °C) under reduced pressure, to yield a thick syrup. The EtOH extracts were dispersed in MeOH/H_2O (4:1) and successively partitioned with *n*-hexane, EtOAc, and *n*-BuOH. Samples of the EtOH extracts, the *n*-hexane, EtOAc, and *n*-BuOH fractions, and the lyophilized aqueous MeOH fractions were further used in the antimicrobial tests.

Isolation of the Compounds in the *n*-Hexane Fraction of *K. variabilis* Branches. The *n*-hexane fraction (1.0 g) was subjected to a Sephadex LH-20 column, eluted with a gradient of *n*-hexane/$CHCl_3$ from 90:10, v/v, to 0:100, v/v, followed by isocratic elution with $CHCl_3$/MeOH (50:50, v/v). The fractions were analyzed by analytical TLC and grouped into seven subfractions (Fr1−Fr7); part of fraction Fr1 (740 mg) was purified on a silica gel column using a gradient of *n*-hexane/EtOAc (100:0 to 0:100, v/v) and EtOAc/MeOH (100:0 to 0:100, v/v). Fractions were grouped on the basis of the TLC profile, to give 17 subfractions (Fr1.1−Fr1.17). Subfraction Fr1.8 (222.7 mg) was repurified by solid-phase extraction (SPE) on silica gel; a gradient system of *n*-hexane/EtOAc (0:100 to 100:0, v/v) as eluent yielded four subfractions (Fr1.8.1−Fr1.8.4). Part of subfraction Fr1.8.1 (50 mg) was subjected to preparative TLC with *n*-hexane/EtOAc (95:5, v/v) as eluent, which afforded compound **1** (23.5 mg). The other part of subfraction Fr1.8.1 (172.7 mg) was purified by preparative HPLC on a C_{18} column with an isocratic solvent system of MeCN/H_2O (97:3, v/v), which also yielded compound **1** (28.3 mg). Purification of fraction Fr1.10 (48.3 mg) by normal-phase PTLC with *n*-hexane/EtOAc (90:10, v/v) as eluent gave four subfractions (Fr1.10.1−Fr1.10.4). Subfraction Fr1.10.3 (26.6 mg) was subjected to normal-phase PTLC again using $CHCl_3$/MeOH (99:1, v/v) as eluent. This procedure afforded six subfractions (Fr1.10.3.1−Fr1.10.3.6). Fr1.10.3.2 (11.9 mg) consisted of a mixture of compounds **2** and **3**. Subfraction Fr1.11 (17.9 mg) was also subjected to normal-phase PTLC with *n*-hexane/EtOAc (85:15, v/v) as eluent, to give four subfractions (Fr1.11.1−Fr1.11.4). Fr1.11.2 (6.9 mg) comprised a mixture of compounds **2**, **3**, and **4**. Purification of Fr6 by normal-phase PTLC with *n*-hexane/EtOAc (30:70, v/v) as eluent resulted in 10 subfractions (Fr1.6.1−Fr1.6.10). Subfraction Fr1.6.4 (1.0 mg) was identified as compound **5**.

Isolation of the Xanthones in the EtOAc Fraction of *K. variabilis* Branches. The ethyl acetate fraction was subjected to HPLC separation. The compounds were purified on a preparative column (Phenomenex C_{18} column, 10 μm, 250 × 21.2 mm; flow rate of 1 mL/min, 80 min), eluted with MeOH/H_2O (50:50, v/v) + 0.1% HOAc, to yield 12 fractions (Fr1−Fr12). All fractions were analyzed by 1D and 2D NMR and MS. Fraction Fr1 (23.7 mg) consisted of a mixture of three compounds identified as compounds **6**, **7**, and **8**. Fr3 (25.6 mg), Fr5 (50.1 mg), Fr7 (30.2 mg), and Fr11 (30.4 mg) were pure and comprised compounds **9**, **10**, **11**, and **12**, respectively. Fraction Fr9 (26.7 mg) was repurified by semipreparative HPLC (Phenomenex Luna Phenyl-Hexyl column; 10 μm, 250 × 21.2 mm; flow rate of 12 mL/min, 60 min) eluted with MeOH/H_2O (50:50, v/v) + 0.1% HOAc, to afford compounds **13** and **14**.

*Kielmeyeracin (**1**):* yellow oil; $[\alpha]^{25}_{D}$ −70 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.20), 284 (4.15); ECD (*c* = 0.5 mM; MeOH) λ_{max} ($\Delta\varepsilon$) 214 (0.78); 225 sh (−0.35); 248 (−1.75); 265 sh (0.35); 293 (2.39); 320 (−2.06); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz), see Table 3; ESIMS (positive) *m*/*z* 529.3547 $[M + H]^+$ (calcd for $C_{32}H_{49}O_6$, 529.3537).

Antibacterial Activity Testing. *Bacterial Strains.* The susceptibility test involved the standard *S. aureus* reference strain ATCC 25923 and the clinical MRSA isolate XU212 bearing the TetK efflux pump, obtained from Dr. E. Udo.[9] The MsrA macrolide-resistant strain RN4220 was provided by Dr. J. Cove.[10] Strain SA-1199B, which overexpresses the NorA MDR efflux pump, was a gift from Prof. Glenn Kaatz.[11] The strains EMRSA-15[12] and EMRSA-16[13] were supplied by Dr. Paul Stapleton.

Minimum Inhibitory Concentration (MIC). To determine the MIC, the strains were first cultured on nutrient agar (Oxoid) and incubated for 24 h at 37 °C. An inoculum density of 1 × 10^6 cfu/mL of each *S. aureus* strain was prepared in normal saline (9 g/L) by comparison with a 0.5 MacFarland turbidity standard and appropriate dilution. The positive control antimicrobial agent norfloxacin (Sigma-Aldrich Chemical Co. LLC) and the samples were dissolved in DMSO and diluted in cation-adjusted Mueller-Hinton broth (MHB) to give a starting concentration of 1024 mg/L. With the aid of Nunc 96-well microtiter plates, 125 μL of MHB was dispensed into wells 1−11. Then, 125 μL of the test compound or the appropriate antibiotic was dispensed into well 1 and serially diluted across the plate, leaving well 11 empty as a growth control. The remaining volume from well 10 was dispensed into well 12, which served as the sterility control for the prepared samples. Finally, the bacterial inoculum (125 μL) was added to wells 1−11, and the plate was incubated at 37 °C for 18 h. A DMSO control was also included. For MIC determination, 20 μL of a 5 mg/mL MeOH solution of 3-[4,5-dimethylthiazol-2 yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each of the wells and incubated for 20 min. A color change from yellow to dark blue indicated bacterial growth. The lowest concentration at which no growth was observed was determined as the MIC.[11] Mueller-Hinton broth (Oxoid) was adjusted to contain Ca^{2+} at 20 mg/L and Mg^{2+} at 10 mg/L.

Minimum Bactericidal Concentration (MBC). Sample preparation followed the same protocol used for the MIC assays, and the samples were added to the same inoculum density of the SA-1199B strain. The

DOI: 10.1021/acs.jnatprod.5b00858
J. Nat. Prod. 2016, 79, 470−476

96-well plates were incubated at 37 °C for 24 h. After incubation, 10 μL of each solution at different concentrations of the tested compound was transferred to Petri dishes containing drug-free culture medium (Mueller-Hinton agar). The Petri plates were then incubated for a further 24 h. MBC was obtained by observing the growth of colonies in Petri dishes after 24 h of incubation; ≥99.9% reduction in the number of bacterial cells as compared with the starting inoculum was considered as the bactericidal end point. MBC values equivalent to or exhibiting no more that a 2-fold difference from the MIC of the agent indicated a bactericidal drug. An MBC value 8-fold higher than the MIC indicated a bacteriostatic drug.

Computational Methods. Conformational analysis of **1** was performed with Schrödinger MacroModel 9.8 (Schrödinger, LLC, New York) employing the OPLS2005 (optimized potential for liquid simulations) force field in H_2O. Ten conformers within a 2 kcal/mol energy window from the global minimum were selected for geometrical optimization and energy calculation applying DFT with the Becke's nonlocal three-parameter exchange and correlation functional and the Lee−Yang−Parr correlation functional level (B3LYP) using the B3LYP/6-31 G** basis set in the gas phase with the Gaussian 09 program package.[14] Vibrational evaluation was done at the same level to confirm minima. Excitation energy (denoted by wavelength in nm), rotator strength dipole velocity (R_{vel}), and dipole length (R_{len}) were calculated in MeOH by TD-DFT/CAM-B3LYP/6-31G**, using the SCRF method, with the CPCM model. ECD curves were obtained on the basis of rotator strengths with a half-band of 0.3 eV using SpecDis v1.61.[15] ECD spectra were calculated from the spectra of individual conformers according to their contribution calculated by Boltzmann weighting.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00858.

Bioguided fractionation scheme; ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, ^{1}H−^{1}H COSY, NOESY, and HRESIMS spectra for compound **1**; chromatograms of isolation and the spectroscopic and spectrometric data of xanthones (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel: +44 207 7535913. Fax: +44 207 7535964. E-mail: simon.gibbons@ucl.ac.uk.

*Tel: +55 (16)3301-9660. Fax: +55 16 3322-2308. E-mail: bolzaniv@iq.unesp.br.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by grants 06/61187-7 from the São Paulo Research Foundation (FAPESP) as part of the Biodiversity Virtual Institute Program (www.biota.org.br) grant 03/02176-7, and BEX 5285/12-7, from Ciências sem Fronteiras, CAPES Foundation, Ministry of Education of Brazil. The authors also thank Dr. A. Camilo Alécio for GC-MS analysis of the steroids, Dr. M. Pivatto for processing of the LC-MS data of xanthones, and Dr. M. Smiesko for computational facilities and technical assistance in interpreting calculated results.

■ DEDICATION

Dedicated to Professors John Blunt and Murray Munro, of the University of Canterbury, for their pioneering work on bioactive marine natural products.

■ REFERENCES

(1) Barros, M. A. G. *Acta Bot. Bras.* **2002**, *16*, 113−122.

(2) Alves, T. M. A.; Silva, A. F.; Brandão, M.; Grandi, T. S. M.; Smânia, E. F.; Smânia, A., Jr.; Zani, C. L. *Mem. Inst. Oswaldo Cruz* **2000**, *95*, 367−373.

(3) Klaiklay, S.; Sukpondma, Y.; Rukachaisirikul, V.; Phongpaichit, S. *Phytochemistry* **2013**, *85*, 161−166.

(4) Xiao, Z. Y.; Mu, Q.; Shiu, W. K. P.; Zeng, Y. H.; Gibbons, S. *J. Nat. Prod.* **2007**, *70*, 1779−1782.

(5) Pinheiro, L.; Cortez, D. A. G.; Vidotti, G. J.; Young, M. C. M.; Ferreira, A. G. *Quim. Nova* **2003**, *26*, 157−160.

(6) Pinheiro, L.; Nakamura, C. V.; Filho, B. P. D.; Ferreira, A. G.; Young, M. C. M.; Cortez, D. A. G. *Mem. Inst. Oswaldo Cruz* **2003**, *98*, 549−552.

(7) Coqueiro, A.; Regasini, L. O.; Skrzek, S. C. G.; Queiroz, M. M. F.; Silva, D. H. S.; Bolzani, V. S. *Molecules* **2013**, *18*, 2376−2385.

(8) http://www.niaid.nih.gov/topics/antimicrobialresistance/examples/mrsa/Pages/history.aspx.

(9) Gibbons, S.; Udo, E. E. *Phytother. Res.* **2000**, *14*, 139−140.

(10) Ross, J. I.; Farrell, A. M.; Eady, E. A.; Cove, J. H.; Cunliffe, W. J. *J. Antimicrob. Chemother.* **1989**, *24*, 851−862.

(11) Kaatz, G. W.; Seo, S. M.; Ruble, C. A. *Antimicrob. Agents Chemother.* **1993**, *37*, 1086−1094.

(12) Richardson, J. F.; Reith, S. *J. Hosp. Infect.* **1993**, *25*, 45−52.

(13) Cox, R. A.; Conquest, C.; Mallaghan, C.; Maples, R. R. *J. Hosp. Infect.* **1995**, *29*, 87−106.

(14) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, N. J.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. *Gaussian 09*; Gaussian, Inc.: Wallingford, CT, USA, 2009.

(15) Bruhn, T.; Hemberger, A. S. Y.; Bringmann, G. *Spec Dis* version 1.61; University of Wuerzburg: Germany, 2014.

(16) Matida, A. K.; Rossi, M. H.; Blumenthal, E. E. A.; Schuquel, I. T. A.; Malheiros, A.; Vidotti, G. J. *Anais Assoc. Bras. Quim.* **1996**, *45*, 147−151.

(17) Mondal, M.; Puranic, V. G.; Argade, N. P. *J. Org. Chem.* **2006**, *71*, 4992−4995.

(18) Appendino, A.; Gibbons, S.; Giana, A.; Pagani, A.; Grassi, G.; Stavri, M.; Smith, E.; Rahman, M. M. *J. Nat. Prod.* **2008**, *71*, 1427−1430.

(19) Bokesh, H. R.; Groweiss, A.; McKee, T. C.; Boyd, M. R. *J. Nat. Prod.* **1999**, *62*, 1197−1199.

(20) Liu, X.; Yang, X.-W.; Chen, C.-Q.; Wu, C.-Y.; Zhang, J.-J.; Ma, J.-Z.; Wang, H.; Y, L.-X.; Xu, G. *J. Nat. Prod.* **2013**, *76*, 1612−1618.

(21) Cuesta-Rubio, O.; Padron, A.; Castro, H. V.; Pizza, C.; Rastrelli, L. *J. Nat. Prod.* **2001**, *64*, 973−975.

(22) Piccinelli, A. L.; Cuesta-Rubio, O.; Chica, M. B.; Mahmood, N.; Pagano, B.; Pavone, M.; Barone, V.; Rastrelli, L. *Tetrahedron* **2005**, *61*, 8206−8211.

(23) Fukuyama, Y.; Minami, H.; Kuwayama, A. *Phytochemistry* **1998**, *49*, 853−857.

DOI: 10.1021/acs.jnatprod.5b00858
J. Nat. Prod. 2016, 79, 470−476

(24) Zhang, Z.; ElSohly, H. N.; Jacob, M. R.; Pasco, D. S.; Walker, L. A.; Clark, A. M. *Planta Med.* **2002**, *68*, 49−54.
(25) Walia, S.; Mukerjee, S. K. *Phytochemistry* **1984**, *23*, 1816−1817.
(26) Pinto, M. M. M.; Mesquita, A. A. L.; Gottlieb, O. R. *Phytochemistry* **1987**, *26*, 2045−2048.
(27) Corrêa, D. B.; Silva, L. G.; Gottlieb, O. R.; Gonçalves, S. J. *Phytochemistry* **1970**, *9*, 447−451.
(28) Gottlieb, O. R.; Magalhães, M. T.; Pereira, O. S.; Mesquita, A. A. L.; Corrêa, D. B.; Oliveira, G. G. *Phytochemistry* **1970**, *9*, 2537−2544.
(29) Habib, A. M.; Reddy, K. S.; McCloud, T. G.; Chang, C.-J.; Cassady, J. M. *J. Nat. Prod.* **1987**, *50*, 141−145.
(30) Sobral, I. S.; Souza-Neta, L. C.; Costa, A. N.; Guedes, M. L. S.; Martins, D.; Cruz, F. G. *Rev. Bras. Farmacogn.* **2009**, *19*, 686−689.
(31) Saleem, M.; Nazir, M.; Ali, M. S.; Hussain, H.; Lee, Y. S.; Riaz, N.; Jabbar, A. *Nat. Prod. Rep.* **2010**, *27*, 238−254.
(32) Panthong, K.; Pongcharoen, W.; Phongpaichit, S.; Walter, C. T. *Phytochemistry* **2006**, *67*, 999−1004.
(33) Boonsri, S.; Karalai, C.; Ponglimanont, C.; Kanjana-opas, A.; Chantrapromma, K. *Phytochemistry* **2006**, *67*, 723−727.

DOI: 10.1021/acs.jnatprod.5b00858
J. Nat. Prod. 2016, 79, 470−476