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Note

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Branimycins B and C, Antibiotics Produced by the Abyssal Actinobacterium *Pseudonocardia carboxydivorans* M-227

Alfredo F. Braña,[†,#] Aida Sarmiento-Vizcaíno,[†,#] Ignacio Pérez-Victoria,[‡,#] Luis Otero,[§] Jonathan Fernández,[⊥] Juan José Palacios,[⊥] Jesús Martín,[‡] Mercedes de la Cruz,[‡] Caridad Díaz,[‡] Francisca Vicente,[‡] Fernando Reyes,[*,‡] Luis A. García,[∥] and Gloria Blanco[*,†]

[†]Departamento de Biología Funcional, Área de Microbiología, e Instituto Universitario de Oncología del Principado de Asturias, Universidad de Oviedo, 33006 Oviedo, Spain

[‡]Fundación MEDINA, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Avenida del Conocimiento 34, Parque Tecnológico de Ciencias de la Salud, E-18016 Granada, Spain

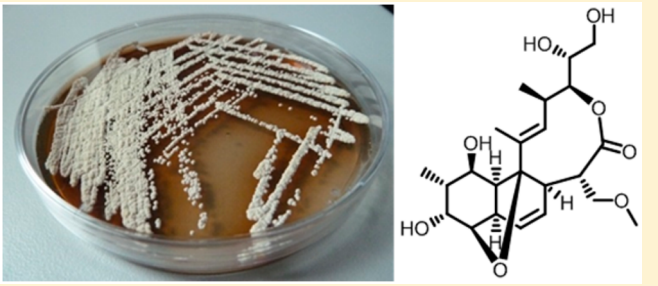
[§]Servicio de Microbiología Hospital de Cabueñes, Gijón, Spain

[⊥]Servicio de Microbiología, Hospital Universitario Central de Asturias (HUCA), Oviedo, Spain

[∥]Departamento de Ingeniería Química y Tecnología del Medio Ambiente, Área de Ingeniería Química, Universidad de Oviedo, Oviedo, Spain

Supporting Information

ABSTRACT: Two new antibiotics, branimycins B (**2**) and C (**3**), were produced by fermentation of the abyssal actinobacterium *Pseudonocardia carboxydivorans* M-227, isolated from deep seawater of the Avilés submarine Canyon. Their structures were elucidated by HRMS and NMR analyses. These compounds exhibit antibacterial activities against a panel of Gram-positive bacteria, including *Corynebacterium urealyticum*, *Clostridium perfringens*, and *Micrococcus luteus*, and against the Gram-negative bacterium *Neisseria meningitidis*. Additionally, branimycin B displayed moderate antibacterial activity against other Gram-negative bacteria such as *Bacteroides fragilis*, *Haemophilus influenzae*, and *Escherichia coli*, and branimycin C against the Gram-positive *Enterococcus faecalis* and methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*.



The branimycins are compounds structurally related to a family of macrolide antibiotics known as nargenicins, which exhibit antimicrobial activity mainly against *Staphylococcus aureus*. Nargenicins and branimycins have a tricyclic structure with either a 9- or 10-membered lactone ring and contain a unique ether bridge. In 1977, the first members of the nargenicin family were isolated by Pfizer and Upjohn after the aerobic fermentation of *Nocardia argentinensis* ATCC 31306. This family of compounds and their antibacterial activity were later patented,[1] and the structure of one of them, nargenicin A1, was elucidated.[2] Although it showed antibacterial activity *in vitro*, this was restricted to Gram-positive bacteria, particularly methicillin-resistant *S. aureus* (MRSA). It was also described that nargenicin A1 induces cell differentiation and can be used, therefore, as a possible treatment for neoplastic diseases.

The first branimycin (**1**) was isolated in 1998 from the Actinomycete GW 60/1571 and its structure determined by the Laatsch group through NMR analysis and comparison with nargenicin A1.[3] Since then, there was a great interest in this new molecule, and a couple of organic syntheses have been developed.[4,5] Recently, the semisynthesis of branimycin derivatives has been reported in a patent,[6] demonstrating distinct antibiotic activities against *Bacillus subtilis*, *S. aureus*, *E. coli*, and *Streptomyces viridochromogenes*. The patent also reports that branimycin and derivatives exhibit *in vivo* activity in animal models of infection, efficacious in treating infections *in vivo*, particularly via the oral route, increasing the interest of this family of natural products. Very interestingly, this year the configuration of branimycin at position C-17 has been revised after X-ray crystallography and NMR studies in DMSO-d_6.[7]

Oceans constitute more than 70% of our planet's surface, of which 92−93% is deep sea (where 60% is covered by water more than 2000 m deep).[8] The deep sea constitutes an extreme environment with high pressure, low temperature, darkness, high salinity, and low oxygen concentration, which has been revealed to be a worthy source for the discovery of new antibiotics.[9]

Previous work in the Cantabrian Sea (Biscay Bay), Northeast Atlantic, has revealed that bioactive Actinobacteria, displaying a wide repertoire of chemically diverse molecules with different antibiotic or antitumor activities, were isolated in the submarine

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J. Nat. Prod. XXXX, XXX, XXX−XXX

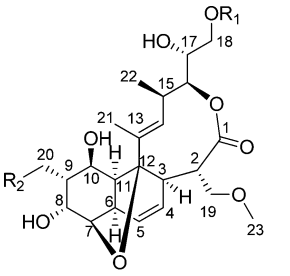
Table 1. ^{1}H and ^{13}C NMR Spectra of Compounds 2 and 3 (^{1}H 500 MHz, ^{13}C 125 MHz, $CDCl_3$)

	2		3	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	179.6, C		179.5, C	
2	46.1, CH	3.10, ddd (10.9, 9.6, 5.0)	46.1, CH	3.08, ddd (10.6, 9.6, 5.1)
3	51.5, CH	3.03, br d (9.6)	51.5, CH	3.02, br d (9.6)
4	126.4, CH	5.37, dd (9.8, 1.5)	126.4, CH	5.36, dd (9.7, 2.0)
5	131.5, CH	6.06, dd (9.8, 6.9, 1.3)	131.5, CH	6.05, dd (9.7, 6.9, 1.0)
6	38.6, CH	2.67, br d (6.9)	38.4, CH	2.71, br d (6.9)
7	84.0, CH	4.14, d (4.8)	83.8, CH	4.09, d (4.8)
8	72.4, CH	3.85, dd (4.8, 4.0)	71.5, CH	4.06, dd (4.8, 4.2)
9	35.7, CH	2.15, ddq (10.8, 4.0, 6.8)	40.1, CH	2.31, m
10	75.5, CH	3.56, m	71.9, CH	4.00, d (10.9)
11	48.6, CH	2.47, br s	48.1, CH	2.46, d (1.9)
12	88.4, C		88.5, C	
13	133.4, C		133.8, C	
14	138.9, CH	5.74, br d (7.6)	138.4, CH	5.70, br d (7.6)
15	32.2, CH	2.91, m	32.1, CH	2.90, m
16	79.8, CH	5.03, dd (9.9, 6.6)	79.8, CH	5.02, dd (10.0, 6.5)
17	69.3, CH	3.91, br d (9.8)	69.3, CH	3.90, br d (9.7)
18	64.2, CH_2	3.81, dd (12.1, 2.5)	64.2, CH_2	3.79, dd (12.2, 2.9)
		3.71, br d (12.1)		3.70, br d (11.5)
19	73.5, CH_2	3.55, m	73.5, CH_2	3.55, dd (10.6, 8.6)
		3.48, dd (11.2, 5.0)		3.47, dd (8.2, 4.8)
20	12.9, CH_3	1.03, d (6.8)	73.3, CH_2	3.76, dd (9.5, 4.6)
				3.63, dd (9.5, 5.6)
21	16.8, CH_3	1.68, s	16.8, CH_3	1.70, s
22	15.1, CH_3	1.26, d (6.9)	15.1, CH_3	1.25, d (6.8)
23	59.2, CH_3	3.31, s	59.1, CH_3	3.30, s
24			59.1, CH_3	3.35, s

Avilés Canyon up to 4700 m depth.[10−13] One of these strains, *Pseudonocardia carboxydivorans* M-227, isolated at 3000 m depth in the water column, was further studied.[13] We report herein the discovery from cultures of this strain of two new antibiotics of the branimycin family with antibacterial activity against Gram-positive and Gram-negative clinical pathogens.

A culture of *P. carboxydivorans* M-227, isolated and identified as previously described,[13] in R5A medium was solid-phase extracted and subsequently eluted with a MeOH gradient. After bioassay-guided identification of the active fractions these were pooled and further purified by semipreparative reversed-phase HPLC, leading to the isolation of the two compounds responsible for the antibacterial activity. These natural products were not included in our in-house dereplication library[14] and turned out to be new, being designated as branimycins B (**2**) and C (**3**) based on their structure.

A protonated molecule at m/z 439.2326 together with the presence of 23 signals in its ^{13}C NMR spectrum assigned a molecular formula of $C_{23}H_{34}O_8$ to branimycin B (**2**). According to the analysis of its ^{1}H, ^{13}C (Table 1), and HSQC spectra, these 23 carbon atoms accounted for the presence in the molecule of one 1,2-disubstituted and one trisubstituted double bond, one carbonyl group, five oxygenated methines, two oxygenated methylenes, one nonprotonated carbon attached to oxygen, six aliphatic methines, and one oxygenated and three aliphatic methyl groups. Taking into account the seven degrees of unsaturation that could be deduced from its molecular formula and the three unsaturations due to the presence of two double bonds and one carbonyl group, compound **1** must be a tetracyclic molecule.



1 R_1=CH_3; R_2=OCH_3
2 R_1=R_2=H
3 R_1=H; R_2=OCH_3

Correlations observed in the COSY spectrum (Figure 1) established the following spin systems: H-19−H-2−H-3−H-4−

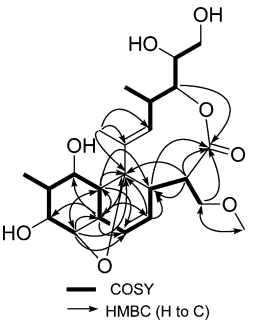


Figure 1. Key COSY and HMBC correlations observed in the spectra of branimycin B.

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J. Nat. Prod. XXXX, XXX, XXX−XXX

H-5−H-6, H-7−H-8−H-9−H-10−H-11, and H-14 to H-18. Additional cross-peaks in this spectrum also revealed the attachment of the methyl groups CH_3-20 to C-9 and CH_3-22 to C-15, and a low-intensity signal between H-6 and H-11 also secured the connection between C-6 and C-11. Although no correlation was observed in the COSY spectrum between H-6 and H-7, the linkage between their corresponding carbons was based on HMBC correlations observed between H-7 and C-5, C-6, and C-11, H-6 and C-4, C-5, C-8, C-10, C-11, and C-12, and H-11 and C-5, C-6, and C-7 (Figure 1). A second ring closure was established between C-3 and the nonprotonated oxygenated carbon C-12 on the basis of HMBC cross-peaks between H-2 and C-3 and C-12, H-4 and C-3 and C-12, and H-11 and C-3 and C-12 (Figure 1). The third ring was due to the existence of an ether bridge between C-7 and C-12 and was evidenced by an intense HMBC correlation between H-7 and C-12 (Figure 1) and by the deshielded chemical shift of carbons C-7 and C-12 (84.0 and 88.4 ppm, respectively). The placement of methyl CH_3-21 at the quaternary olefinic carbon C-13 was revealed by HMBC correlations between the H-21 protons and C-12, C-13, and C-14 (Figure 1). C-2 was attached to the carbonyl carbon C-1 at δ_C 179.6 ppm on the basis of HMBC correlations from H-2, H-19, and H-3 to C-1 and an additional correlation of this carbon to H-16, and the deshielded chemical shift of the latter proton (δ_H 5.03 ppm) indicated the existence of a lactone ring between C-1 and C-16, accounting for the last unsaturation of the molecule. Finally, the oxygenated methyl group C-23 present in the molecule was placed at C-19 based on the presence of HMBC correlations between the H-23 protons and C-19 and both H-19 protons and C-23.

Once the planar structure of compound **1** was established, a literature search revealed the existence of a molecule having the same structural core, branimycin.[3−5,7] A detailed comparison between the NMR spectra of both molecules confirmed their similarity and established the differences in the structures as the absence in compound **2** of the methyl ether at C-18, leaving a free hydroxy group at this position, and the absence of the methoxy group at C20 present in the structure of branimycin (**1**). Interestingly, the above-mentioned patent[6] reports a branimycin analogue named baleomycin, which likewise lacks the methoxyl group at C-20 but keeps the methyl ether at C-18, being thus closely related to **2**. On the other hand, a detailed comparison of the NMR chemical shifts, multiplicities, and NOESY correlations of **2** and branimycin[7] also revealed the same relative configuration for both molecules, and the same absolute configuration could also be proposed on the basis of the similar magnitudes and positive values of their specific rotations ($[\alpha]^{25}_D$ +80, *c* 0.045, $CHCl_3$, for branimycin).[5] The absence of NOESY correlations between the H-18 protons and the methyl H-22 is in favor of an (*R*)-C-17 configuration as recently described for branimycin.[7]

A molecular formula of $C_{24}H_{36}O_9$ was determined for compound **3** based on the existence of 24 signals in its ^{13}C NMR spectrum and ions detected in the HRESIMS spectrum corresponding to the proton and ammonium adducts. Analysis of its ^1H and ^{13}C NMR spectra revealed a close similarity with those of compound **1**, the most significant differences being the absence of the signal of the aliphatic methyl doublet group at δ_H 1.03/δ_C 12.9 ppm, corresponding to CH_3-20, present in the spectra of **1**, replaced by the presence of an extra oxygenated methylene (δ_H 3.76 and 3.63/δ_C 73.3 ppm, CH_2-20) and an oxygenated methyl group at δ_H 3.35/δ_C 59.1 ppm (C-24) in those of **2**. These changes were in agreement with the replacement of the C-20 methyl group in the structure of compound **2** by a methoxymethyl group in that of compound **3**. COSY correlations between H-9 at δ_H 2.31 ppm and both H-20 protons at δ_H 3.76 and 3.63 ppm and HMBC correlations between both H-20 protons and C-24 at δ_C 59.1 ppm and between H-24 at δ_H 3.35 ppm and C-20 at δ_C 73.2 ppm corroborated this proposal. Once again, the same absolute configuration as in branimycin was proposed for branimycin C based on similar chemical shifts and multiplicities, NOESY correlations, and the magnitude and positive value of its specific rotation.

Antimicrobial activities of compounds **2** and **3** were tested against a panel of human pathogens (Table S1). Some of these pathogens were isolated and identified in clinical microbiology laboratories from samples obtained from patients with clinical infections.

Table 2 shows the minimum inhibitory concentrations (MIC) obtained in these antibacterial tests. Both compounds exhibited moderate activities against Gram-positive bacteria (*C. urealyticum*, *C. perfringens*, and *M. luteus*). In addition, compound **2** displayed moderate activities against *E. faecalis* 10544, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538P, and the methicillin resistant *S. aureus* (MRSA MB5393). Concerning Gram-negative bacteria, both compounds showed moderate activities against *N. meningitidis*. Compound **1** also displayed

Table 2. Minimum Inhibitory Concentrations (MIC, μg/mL) against Clinic Bacterial Pathogens

microorganism	2	3
Gram-Positive		
Clostridium perfringens 103281	32	16
Corynebacterium urealyticum 1492	8	16
Enterococcus faecalis 10544	>64	64
Enterococcus faecalis ATCC 29212	>64	>64
Enterococcus faecalis ATCC 51299	>64	>64
Enterococcus faecium 10701	>64	>64
Micrococcus luteus ATCC 14452	1	16
Mycobacterium tuberculosis H37Rv	>32	>32
Mycobacterium tuberculosis MDR-1	>32	>32
Mycobacterium tuberculosis MDR-2	>32	>32
Staphylococcus aureus ATCC 25923	>64	64
Staphylococcus aureus ATCC 6538P	>128	32
Staphylococcus aureus ATCC 43300	>64	>64
Staphylococcus aureus 11497	>64	>64
Staphylococcus aureus MRSA MB5393	>160	20−40
Staphylococcus aureus MSSA MB2865	>160	80
Streptococcus pneumoniae 64412	>128	>128
Streptococcus pyogenes 81293	>128	>128
Gram-Negative		
Acinetobacter baumannii MB5973	>80	>80
Bacteroides fragilis ATCC 25285	32	128
Bacteroides fragilis 61592	128	>128
Escherichia coli MB2884	>80	>80
Escherichia coli ESS	64	128
Haemophilus influenzae ATCC 49247	32	>64
Haemophilus influenzae 10996	>64	>64
Klebsiella pneumoniae ATCC 700603	>80	>80
Neisseria meningitidis 71327	32	64
Pseudomonas aeruginosa PAO1	>80	>80

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moderate bioactivity against *B. fragilis*, *H. influenzae* ATCC 49247, and *E. coli*.

In conclusion, two new antibiotics, branimycins B (**1**) and C (**2**), were isolated and characterized from the deep-sea-derived *P. carboxydivorans* M-227 isolated from the water column at 3000 m depth in the Cantabrian Sea. These compounds exhibited significant inhibitory activities against diverse pathogenic bacteria, both Gram-positive and Gram-negative isolated at two main Hospitals (HUCA and Cabueñes) located in the same geographical region where the microorganism was isolated. Our findings constitute another example of the relevance of marine natural products as a source of new bioactive molecules and candidates for the treatment of pathogenic antibiotic-resistant bacteria.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined with a JASCO P-2000 polarimeter. IR spectra were measured with a JASCO FT/IR-4100 spectrometer equipped with a PIKE MIRacle single-reflection ATR accessory. NMR spectra were recorded on a Bruker Avance III spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm TCI MicroCryoProbe, using the signal of the residual solvent as internal reference (δ_H 7.27 and δ_C 77.0 ppm for $CDCl_3$). HRESIMS spectra were acquired using a Bruker maXis QTOF mass spectrometer. Semipreparative HPLC analyses and separations were conducted using an Alliance chromatographic system equipped with a SunFire C18 column (10 μm, 10 × 250 mm). For UPLC analysis an Acquity UPLC equipped with a BEH C18 column (1.7 μm, 2.1 × 100 mm) was used.

Microorganism and Fermentation Conditions. Strain M-227 was isolated from a deep-water sample collected from the Cantabrian Sea at a depth of 3000 m as previously described.[13] A seed culture was prepared by inoculating spores of this strain in 50 mL of GCM medium (1.5% glucose, 2% soy peptone, 0.15% yeast extract, 1% MOPS, 0.01% $CaCl_2$, pH 6.7) in a 250 mL Erlenmeyer flask. This culture was incubated in an orbital shaker for 4 days at 28 °C and 250 rpm and used to inoculate (at 2%, v/v) 20 × 250 mL Erlenmeyer flasks, each containing 50 mL of R5A medium, which were incubated for 10 days in the above conditions.

Bioassay-Guided Isolation and Purification. The cultures were centrifuged, the pellets were discarded, and the supernatants were filtered and applied to a solid-phase extraction cartridge (Sep-Pak Vac C18, 10 g). The retained material was eluted with a mixture of MeOH and 0.05% trifluoroacetic acid (TFA) in H_2O. A linear gradient from 0 to 100% MeOH in 60 min, at 10 mL/min, was used. Fractions were collected every 5 min, and their antibiotic activity was detected by disk diffusion bioassay, using *Micrococcus luteus* as indicator microorganism. Most of the activity was located in the two fractions taken between 15 and 25 min, which were evaporated *in vacuo*, and the dry material was subsequently redissolved in 3 mL of DMSO and MeOH (1:1). Aliquots (100 μL) of these active fractions were chromatographed in a SunFire C18 column (10 μm, 10 × 250 mm), with CH_3CN and 0.05% TFA in H_2O as solvents. Elution was performed with a linear gradient from 20% to 100% CH_3CN in 10 min, at 5 mL/min, and the eluate was collected in fractions taken every 10 s. Once more, the antibiotic activity in these fractions was located by bioassay, and subsequently all the active material was chromatographed in multiple injections in the same conditions. The collected active fractions corresponding to the same retention times were pooled, diluted 4-fold with H_2O, desalted, and concentrated by solid-phase extraction (Sep-Pak C18). UPLC analysis of these fractions[15] indicated that the antibiotic activity appeared to correlate with the presence of two major peaks. These peaks were further purified using the same column and solvents, but this time an isocratic elution with 20% CH_3CN was employed. The purified compounds were diluted with H_2O and solid-phase extracted as above. They were finally dissolved in a mixture of *tert*-butanol and H_2O (1:1) and lyophilized. The resulting yields were 58.6 mg of **1** and 61.7 mg of **2** from a 1 L culture.

*Branimycin B (**2**):* white, amorphous solid; $[\alpha]^{20}_D$ +110 (*c* 0.1, $CHCl_3$); IR (ATR) ν_{max} 3419, 3038, 2961, 2929, 2878, 2832, 1719, 1457, 1378, 1248, 1147, 1117, 1082, 1030, 984, 944, 890 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRESIMS *m*/*z* 456.2596 $[M + NH_4]^+$ (calcd for $C_{23}H_{38}NO_8$, 456.2592), 439.2326 $[M + H]^+$ (calcd for $C_{23}H_{35}O_8$, 439.2326), 421.2222 $[M - H_2O + H]^+$ (calcd for $C_{23}H_{33}O_7$, 421.2221).

*Branimycin C (**3**):* white, amorphous solid; $[\alpha]^{20}_D$ +100 (*c* 0.1, $CHCl_3$); IR (ATR) ν_{max} 3422, 3038, 2961, 2931, 2879, 2833, 1722, 1457, 1386, 1248, 1140, 1118, 1083, 1030, 979, 945, 889 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRESIMS *m*/*z* 486.2709 $[M + NH_4]^+$ (calcd for $C_{24}H_{40}NO_9$, 486.2698), 469.2432 $[M + H]^+$ (calcd for $C_{24}H_{37}O_9$, 469.2432), 451.2333 $[M - H_2O + H]^+$ (calcd for $C_{24}H_{35}O_8$, 451.2326).

Antimicrobial Activities of Compounds 2 and 3 against Clinic Pathogens. Antimicrobial activities of compounds **2** and **3** were evaluated, and the MICs were determined against a panel of human pathogens (Table S1). Some of these pathogens were isolated and identified in clinical microbiology laboratories from samples obtained in patients with clinical infections. Mueller-Hinton agar (Biomedics) was the culture medium used in bioassays against *E. coli*, *S. aureus*, *E. faecalis*, *E. faecium*, *M. luteus*, and *H. influenzae*, being supplemented according to the CLSI conditions for *S. pneumoniae*, *S. pyogenes*, and *N. meningitidis*. Trypticasein soy agar w/5% sheep blood (DIFCO) was used for *C. urealyticum*. Brucella Broth (Sigma) supplemented with hemin (5 μg/mL), vitamin K_1 (1 μg/mL), and lysed horse blood (5% v/v) was used for *B. fragilis* and *C. perfringens*. Assays against methicillin-resistant *S. aureus* MRSA MB5393 and methicillin-sensitive *S. aureus* MSSA MB2865 were performed by mixing a volume of 90 μL of the appropriate diluted inocula with 8.4 μL of medium (LB or BHI) and 1.6 μL of each compound dilution. Assays were performed in triplicate using 2-fold serial dilutions from 160 to 0.31 μg/mL. Vancomycin (32 to 4 μg/mL) and penicillin G (0.312 to 0.039 μg/mL) were used as positive controls for MRSA and MSSA, respectively. Amphotericin B (16 to 0.25 μg/mL) was used as negative control in both cases. Antimicrobial activity tests against *A. baumannii* MB5973, *E. coli* MB2884, and *P. aeruginosa* PAO1 were performed as previously described.[16]

For the rest of the Gram-positive and Gram-negative bacteria, the antimicrobial assays were performed according to CLSI performance standards.[17] For *Mycobacterium tuberculosis*, susceptibility testing was done in Middlebrook 7H10 agar medium supplemented with 10% OADC and 0.5% glycerol according to the agar proportion method for slowly growing mycobacteria.[18]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b01107.

UV (DAD), HRMS, and 1D and 2D NMR spectra of branimycins B and C, pictures and phylogenetic analysis of the microorganism, and description of the pathogenic strains used in the antibacterial tests (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail (F. Reyes): fernando.reyes@medinaandalucia.es. Tel: +34 958993965.

*E-mail (G. Blanco): gbb@uniovi.es. Tel: +34 985103205.

ORCID

Ignacio Pérez-Victoria: 0000-0002-4556-688X

Fernando Reyes: 0000-0003-1607-5106

Author Contributions

#A. F. Braña, A. Sarmiento-Vizcaíno, and I. Pérez-Victoria contributed equally to this work

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Notes

The authors declare no competing financial interest.

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