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Meroterpenes from *Penicillium* sp found in association with *Melia azedarach*

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Abstract

A *Penicillium* sp was isolated from the root bark of *Melia azedarach* and cultivated over sterilized rice. After chromatographic procedures, two meroterpenes, named preaustinoid A and B, were obtained in addition to the known alkaloid verruculogen. Their structures were identified by extensive spectroscopic studies, and they exhibited moderate bacteriostatic effects on *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus* sp.

Keywords: *Melia azedarach*; Meliaceae; *Penicillium*; Roots; Endophytic; Meroterpene; Alkaloid

1. Introduction

Melia azedarach (Meliaceae) (Pennington and Styles, 1975) is native to northwestern India but is now naturalized throughout the tropics. Our research group has long been studying the chemistry of Meliaceae plants looking for new biologically active compounds and secondary metabolites that can be used to aid botanical classification. The main class of small molecule metabolites produced by these plants consists of modified triterpenoids known as limonoids, of which azadiractin is the most well known owing to its insecticidal properties (Pennington and Styles, 1975; Nakatani et al., 1981). The production of limonoids by triterpene degradation in plants appears to occur in a series of steps, including a Bayer-Villiger-like oxidation, and structural rearrangements (Waterman and Grundon, 1983). Many of the structural features present in the limonoid molecules may also be found in fungal secondary metabolites, with austin and similar meroterpenes being good examples (Chexal et al., 1976; Scott et al., 1986; Ahmed et al., 1989; Hayashi et al., 1994; Simpson et al., 1997). The A and B rings present in these meroterpenes are identical to those of some limonoids produced by *Carapa* species (Meliaceae). Three more compelling examples are the production of gibberellin derivatives by fungi (Stowe and Yamaki, 1957; Harborne, 1993; MacMillan, 1996), trichotecene macrocyclic lactones by the female plant *Baccharis megapotanea* (Jarvis et al., 1988), and more recently, the production of taxol™, an anticancer diterpene formerly obtained from the host plant *Taxus brevifolia*, by fungi in artificial culture medium (Stierle et al., 1993). Thus, the production of similar micromolecular metabolites by fungi and plants is being investigated with increasing interest and effort is being made to better understand the possible ability of endophytic microorganisms to exchange genetic information during their association with the host plants, resulting in the codification of specific enzymes that may be involved in the biosynthesis of some secondary metabolites similar, or identical with those produced by the isolated organisms (Stierle et al. 1993; Horr, 1997; Rizzo et al., 1997).

During our studies on the chemical constituents produced by endophytic fungi isolated from *M. azedarach*, we found that *Penicillium* sp produced two new meroterpenoids (**1** and **2**) and the known alkaloid verruculogen (**3**). Based on chemical structure comparisons

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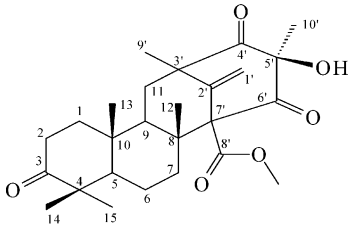
compounds **1** and **2** are probably precursors of the austinoid meroterpenes. In this paper, the production, isolation, structural determination and antibacterial activity of these three compounds are described.

2. Results and discussion

Substances **1**, **2** and **3** were produced during cultivation of *Penicillium* sp over sterilized rice, and isolated as amorphous solids following silica gel column and preparative TLC separations of the methanol extract from the culture. Alkaloid **3** was identified by comparison of its physical data (NMR, m.p., etc) with those reported in the literature (Uramoto et al., 1982; Horak and Ulegaar, 987).

The positive APCIMS of **1** showed an abundant ion $[M+H]^+$ at m/z 445, from which the molecular formula $C_{26}H_{36}O_6$ was deduced and confirmed by HREIMS (calc. 444.2512; experimental 444.2508). The IR spectrum showed strong absorptions at 3391 cm^{-1} (hydroxyl group), 1746 cm^{-1} (carboxymethyl ester group), 1728 and 1701(ketones) and 1692 cm^{-1} (α,β-unsaturated δ-lactone group) and 1637 cm^{-1} (vinyl group), respectively.

An analysis of the NMR spectroscopic data (Table 1) and their comparison with model compounds indicated the presence of a partial 4,4,8,10-tetramethyldecalin structure in compound **1** similar to that observed in many other terpenoids (Ammann et al., 1982). The signals at δ 2.38 (H-2α, *m* and H-2β, *m*) and the ^1H–^{13}C long-range correlations between two methyl groups [δ 1.00 (3H-14) and 1.05 (3H-15)] with a carbonyl carbon (δ 217.0) in the HMBC spectrum established the position of a keto group at C-3. Other ^1H–^{13}C long-range correlations detected for **1** are summarized in Fig. 1. The ^{13}C NMR spectrum also showed signals for two additional ketone carbonyls (δ 207.8 and 203.9), one carboxymethyl ester (δ 168.5), an exocyclic carbon-carbon double bond (δ 112.7 and 145.0), two methyl groups (δ 22.1 and 15.1) and a quaternary carbinolic carbon (δ 80.1). The connectivity of these functions was based on analysis of the HMBC spectrum. The methyl hydrogens at δ 1.38 were correlated with the quaternary carbonylic carbon at δ 80.1 (which was correlated with a hydroxyl hydrogen at δ 3.30) and with two carbonyl carbons at δ 207.8 and 203.9 establishing the partial 2-hydroxy-2-methyl-1,3-dioxo structure. The correlation of the 3H at δ 1.48 (3H-9) with the carbonyl carbon at δ 207.8 (C-4′) and the presence of a highly deshielded non-oxygenated carbon at δ 72.5 (C-7′), in correlation with the vinyl hydrogen at δ 5.40 (H-1′b) in the HMBC spectrum, suggested a [3:3:1]-bicyclo system as a partial structure of **1**. Finally, the identification of the methyl ester group and the relative configurations at the stereocenters were made through an analysis of the following 1D-NOE data. Irradiation of the signal at δ 3.73 (OCH_3) in the ^1H NMR spectrum enhanced the signals at δ 4.89 (H-1′a), 3.30 (OH) and 1.26 (3H-12), while irradiation of the signal at δ 1.38 (3H-10′) enhanced the doublet-of-doublets at 0.61 (H-9). These data suggest that the methyl group 3H-10′ is located at the *endo* face in the [3:3:1]-bicyclo system while the hydroxyl is at the *exo* face. Other important NOE data are represented in Fig. 2. Thus, the structure of this



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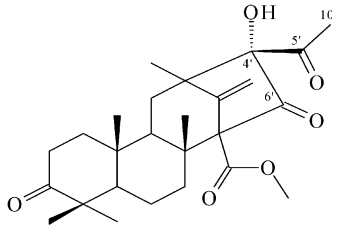

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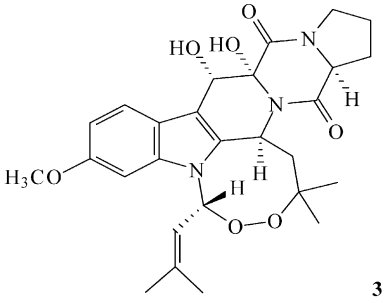

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meroterpene, named preaustinoid A, was identified as **1**, and is apparently a new natural product.

The mass spectrum obtained by HREIMS and APCIMS for compound **2** was consistent with an isomer of compound **1** [$[M]^{+\cdot}$ at m/z 444.2505 (calc. 444.2512) and $[M+H]^+$ at m/z 445, respectively]. Carbonyl groups [1738 cm^{-1} (methyl ester) and 1702 cm^{-1} (ketone)] and a hydroxyl group (3438 cm^{-1}) were detected in the IR spectrum of **2**. A comparison of the above data with the NMR spectra (Table 1) obtained for **1** and **2** indicated that these two meroterpenes have identical A, B and C rings. However, the presence of one methyl group at δ 2.24 was observed in the ^1H NMR spectrum of **2**. The correlation of these methyl hydrogens with a carbonyl carbon at δ 203.0 in the HMBC (J^2) suggested the presence of a methyl ketone in the molecule. In addition, the HMBC spectrum displayed a correlation for the hydrogens in the methyl ketone group with a quaternary carbon-bearing oxygen (δ 92.0, J^3), which is also correlated with a hydroxyl hydrogen (δ 4.89, J^2). The molecular formula ($C_{26}H_{36}O_6$) deduced from the mass spectrum, and the three-bound correlation (J^3) of this hydroxyl hydrogen with two carbonyl carbons (δ 207.0 and 203.0) suggested the presence of a C_4 unit (3-hydroxy-2,4-dioxo) as a partial structure of this meroterpene. Connectivity of these four carbons to the perhydrophenanthrene part of the molecule was made based on the HMBC correlation (J^3) detected for the methyl hydrogens at δ 1.04 (3H-9′) with the carbon at δ 92.0 (C-4′), whose chemical shift is consistent with the presence of a 2-hydroxycyclopentanone. Finally, the stereochemistry at C-4′ was established based on the NOE observed between the methyl hydrogens in the methyl ketone (δ 2.24) with the two vinyl hydrogens in the exocyclic methylene [δ 5.02 (H-1′a) and δ 5.24 (H-1′b)] and the methoxyl hydrogen in the methyl ester (δ 3.73) (Fig. 2). In addition, the hydroxyl proton (δ 4.89) showed strong NOE

Table 1
^1H NMR spectroscopic data (δ, J in Hz) and ^{13}C data (δ, multiplicity) for compounds **1** and **2** in chloroform-*d* (400 MHz)

	1		**2**	
Position	H	**C**[a]	H	**C**[a]
1α	α = 1.11 *m*	39.0 *t*	α = 1.65 *m*	37.8 *t*
1β	β = 1.78 *ddd* (13.0, 6.4, 6.4)		β = 1.75 *m*	
2α	2.38 *m*	33.5 *t*	2.44 *m*	33.5 *t*
2β	2.38 *m*		2.44 *m*	
3		217.0 *s*		217.0 *s*
4		47.0 *s*		47.0 *s*
5	1.23 *dd* (8.5, 4.0)	53.8 *d*	1.46 *m*	53.8 *d*
6α	1.52 *m*	19.6 *t*	1.58 *m*	20.0 *t*
6β	1.52 *m*		1.58 *m*	
7α	2.02 *m*	32.2 *t*	1.65 *m*	31.9 *t*
7β	2.26 *dt* (13.5, 3.3, 3.3)		2.05 *dt* (13.2, 3.3, 3.3)	
8		47.7 *s*		48.4 *s*
9	0.61 *dd* (13.0, 3.0)	51.9 *d*	1.59 *dd* (13.0, 3.0)	50.9 *d*
10		37.2 *s*		37.2 *s*
11α	1.93 *dd* (13.0, 3.0)	39.2 *t*	2.01 *dd* (12.6, 3.3)	35.9 *t*
11β	1.65 *dd* (13.0, 13.0)		1.40 *dd* (12.6, 12.6)	
12	1.26 *s*	16.8 *q*	1.27 *s*	16.9 *q*
13	0.85 *s*	16.0 *q*	0.86 *s*	16.7 *q*
14	1.0 *s*	20.9 *q*	1.01 *s*	20.6 *q*
15	1.05 *s*	26.9 *q*	1.05 *s*	26.9 *q*
1a′	4.89 *brs*	112.7 *t*	5.02 *brs*	107.7 *t*
1b′	5.40 *brs*		5.24 *brs*	
2′		145.0 *s*		151.0 *s*
3′		51.0 *s*		49.6 *s*
4′		207.8 *s*		92.0 *s*
5′		80.1 *s*		207.0 *s*[b]
6′		203.9 *s*		203.0 *s*[b]
7′		72.5 *s*		74.6 *s*
8′		168.5 *s*		169.0 *s*
9′	1.48 *s*	22.1 *q*	1.04 *s*	17.1 *q*
10′	1.38 *s*	15.1 *q*	2.24 *s*	28.2 *q*
OCH_3	3.73 *s*	52.5 *q*	3.72 *s*	52.2 *q*
OH	3.30 *s*		4.89 *s*	

[a] ^{13}C Multiplicity data were obtained through analysis of Pendant and HSQC spectra.
[b] These values may be exchangeable.

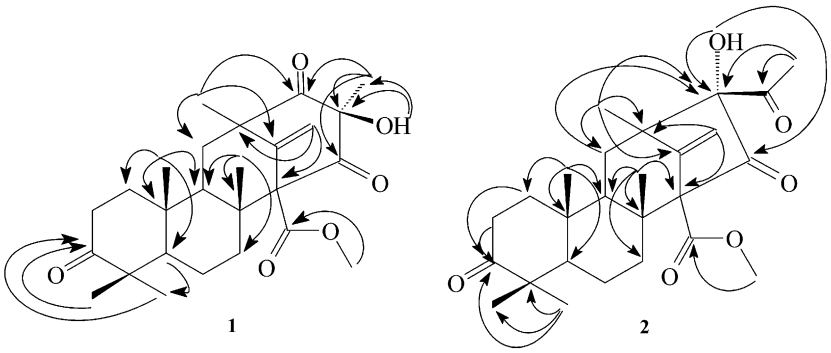
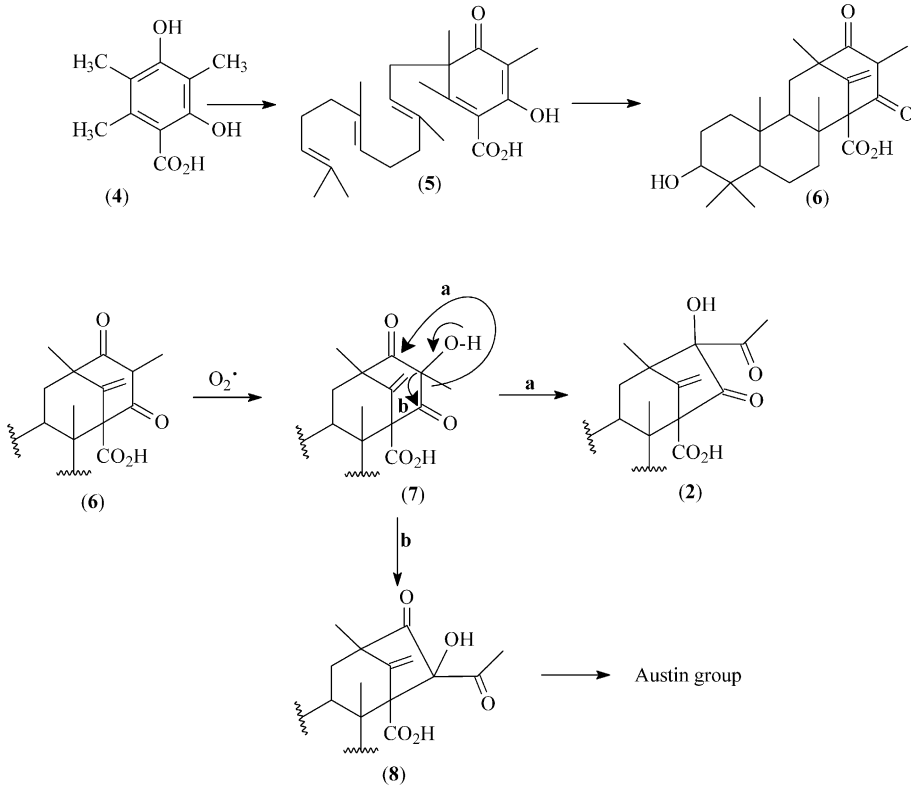


Fig. 1. HMBC correlations detected for **1** and **2**.



Scheme 1. Proposed biosynthesis of the meroterpenes produced by species of *Aspergillus* and *Penicillium*.

with H-9 (δ 1.59). Thus, the above data indicated that compound **2** has an *exo*-methyl ketone and an *endo*-hydroxyl in a [3:2:1]-bicyclo system forming a meroterpene, named preaustinoid B, which appears to be a new natural product.

Studies on the biosynthesis of meroterpenes produced by *Aspergillus* and *Penicillium* have suggested that this group of metabolites is formed by *C*-alkylation of 3,5-dimethyl-orsellinic acid (**4**) by farnesyl pyrophosphate (Scott et al., 1986; Ahmed et al., 1989; Simpson et al., 1997). Further alkylation and cyclization of the acyclic intermediate **5** would result in the formation of the 1,3-diketone (**6**), which has been postulated to be a precursor of the meroterpenes of the austin group and the meroterpenoids **1** and **2** (Scheme 1) (Ahmed et al., 1989). The diketone (**6**) had not been isolated from any fungal cultures prior to this report. The occurrence of these meroterpenes corroborates the earlier studies of this biosynthesis and apparently demonstrates that the α-ketol rearrangement in diketone **6** (paths a and b, Scheme 1) is not regioselective in *Penicillium* sp.

The antibacterial activity of the meroterpenes **1** and **2** and of the alkaloid verruculogen (**3**) was examined in the presence of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus* sp. Compounds **1** and **2** caused a bacteriostatic effect for all micro-organisms tested at a dosage of 250 μg/ml. Compounds **1** and **3** presented this effect at dosages of 125 and 250 μg/ml respectively to *E. coli*. The bactericidal effect was observed only with compound **1** on *E. coli*, *P. aerugi-*

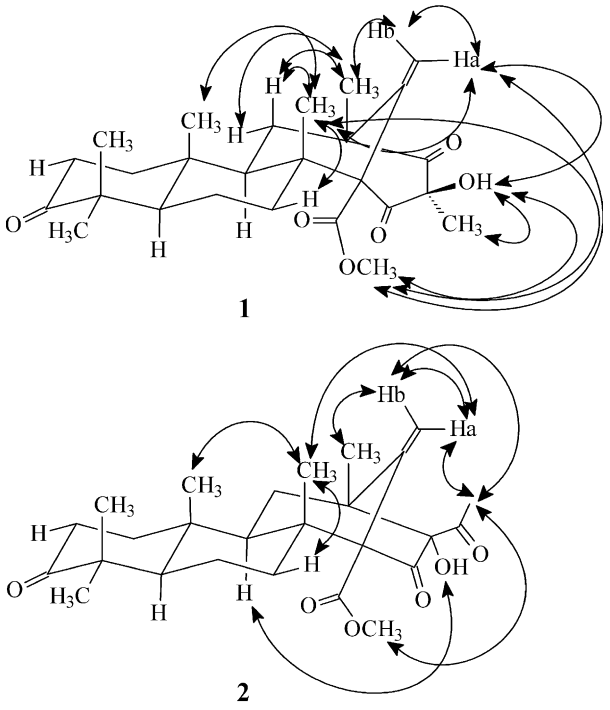


Fig. 2. Spatial correlations suggested for **1** and **2** based on NOE difference spectra.

nosa and *Bacillus* sp at dosages of 250 μg/ml. By comparison, penicillin, vancomycin and tetracycline were effective against all bacteria tested at a concentration of 25 μg/ml.

3. Experimental

3.1. General

Optical rotations were measured on a Perkin Elmer 241 polarimeter. UV spectra were obtained in CH_2Cl_2 solution on a Hewlett Packard 8452-A spectrophotometer, and IR spectra were measured with a Bomen MB-102 spectrophotometer in KBr pellets. HREIMS measurements were carried out on a VG-Autospec spectrometer, and electron impact was used as the ionization mode. Low-resolution APCIMS data were acquired in the positive ion mode, using a MICROMASS QUATTRO-LC instrument equipped with an ESI/APCI "Z-spray" ion source. ^1H and ^{13}C NMR spectroscopic experiments were recorded on a BRUKER DRX-400 spectrometer with deuterochloroform ($CDCl_3$) as solvent and TMS as internal standard.

3.2. Plant material

Roots of *Melia azedarach* were collected in Brazil in 1998. A voucher specimen was deposited in the Herbarium of the University's Department of Botany.

3.3. Isolation of the micro-organisms

The general procedures adopted followed the methodology described by Petrini et al. (1992). Immediately after collection, the root bark was separated mechanically from the xylem and washed with water followed by EtOH and then sterilized with 11% aq. sodium hypochlorite for 1 min. The material was then deposited on a Petri dish containing PDA medium (potato-dextrose-agar) and incubated in the dark at 25 °C for 1 week. *Penicillium* sp was isolated by replication and grew as a bluish colored culture. The fungus was identified and deposited (number 024) at the Laboratório de Bioquímica Micromolecular—LaBioMi—of the Departamento de Química at Universidade Federal de São Carlos, São Carlos, Brazil.

3.4. Rice culture of Penicillium sp and isolation of the meroterpenes

Fifty Erlenmeyer flasks (500 ml) containing rice (~90 g) ("*Uncle Ben's*"—parboiled) and distilled water (75 ml per flask) were autoclaved twice at 121 °C for 40 min. A small disc of the PDA medium from the Petri dish containing mycelium of *Penicillium* sp was transferred under sterile conditions to 47 of the 50 Erlenmeyer flasks containing sterilized rice. Three flasks were kept for control purposes. After 20 days of growth, the water that had accumulated in the flasks was removed by filtration from the fungal biomass. MeOH (~150 ml) was added to each flask and allowed to stand for 5 h, after which the insoluble residue was removed by gravity filtration. MeOH was removed in vacuo to give a yellowish residue (6 g), which was subjected to low-pressure Si gel CC eluted with a hexane–CH_2CL_2 (1:1) to MeOH gradient. The medium polarity fractions obtained with hexane–CH_2Cl_2–MeOH (25:25:2) were subjected to Si gel CC using CH_2Cl_2–MeOH (99:1); the meroterpene preaustinoids **1** (32 mg) and **2** (6 mg) were finally purified by preparative Si gel TLC [hexane–CH_2Cl_2–MeOH (25:25:3)] .

3.4.1. Meroterpene *1*

White amorphous powder; mp 244–245^0; $[\alpha]_D^{25}$ −4.97° (*c* 110.8, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} nm 238, 308(*s*), 344(*s*); IR (KBr) ν_{max} cm^{-1}3391, 1746, 1728, 1701, 1692, 1637; ^1H NMR (400 MHz, $CDCl_3$)—Table 1; ^{13}C data obtained by Pendant (Homer and Perry, 1995), HSQC and HMBC (400 MHz, $CDCl_3$)—Table 1; APCIMS (Daughter ions, 20 eV) *m*/*z* 445 (22) ($[M+H]^+$), 427(20), 413(100), 395(38), 385(19), 327(5); HREIMS *m*/*z* 444.2508 (calc. for $C_{26}H_{36}O_6$, 444.2512).

3.4.2. Meroterpene *2*

White amorphous powder; mp 198–200 °C; $[\alpha]_D^{25}$ −7 8.83° (*c* 6, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} nm 234, 312(*s*);

IR(KBr) ν_{max} cm^{-1}3438, 1738, 1702, 1656; ^1H NMR (400 MHz, $CDCl_3$)—Table 1; ^{13}C data obtained by Pendant (Homer and Perry, 1995), HSQC and HMBC (400 MHz, $CDCl_3$)—Table 1; APCIMS (Daughter ions, 20 eV) m/z 445(29) ($[M+H]^+$), 427(29), 413(100), 395(87), 385(30), 327(5); HREIMS m/z 444.2505 (calc. for $C_{26}H_{36}O_6$, 444.2512).

3.5. Bioassay

The minimal inhibitory concentrations (MICs) were determined by microbroth dilution assay as recommended by the Subcommittee on Antifungal Susceptibility Testing of the US National Committee for Clinical Laboratory Standards (NCCLS, 1997), which was performed on 96-well plates with 100 μl of Mueller Hinton Broth (MHB), 100 μl of test compound and 5 μl of test bacteria at 1.0×10^7 UFC/ml), followed by incubation at 37 °C (24 h). The test substances obtained from the fungus culture above were dissolved in DMSO at an initial concentration of 250 μg/ml. The test microorganisms were *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus* sp (obtained from Universidade de Maringá—PR, Brazil). Bioactivity was recorded as absence of red coloration in the wells and bacteriostatic or bactericidal effects were observed on MHB plates. Penicillin, vancomycin and tetracycline (25 μg/ml each) were used as positive controls; the cultivation medium (MHB) was used as a negative control.

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