Synergistic Antimicrobial Activity of Metabolites Produced by a Nonobligate Bacterial Predator*

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* Dedicated to the memory of Estevan B. Banegas, the founder of Dominion BioSciences, Inc.

Abstract:

A naturally occurring, gram-negative, nonobligate predator bacterial strain 679-2, exhibits broad-spectrum antimicrobial activity that is due, in part, to the production of three extracellular compounds. Antimicrobial-activity-directed fractionation of a culture of strain 679-2 against a panel of microorganisms has led to the isolation of three compounds: pyrrolnitrin, maculosin, and a new compound, which we have named banegasine. Although pyrrolnitrin is well known in the literature, it has not been found in cells with the herbicide maculosin. Further, this is the first report of production of maculosin by a prokaryote. Both maculosin and banegasine, which displayed no antimicrobial activities alone, were found to potentiate the antimicrobial activity of pyrrolnitrin. Based on 16S rRNA sequence, cellular fatty acid composition, and biochemical and cultural characteristics, strain 679-2 appears to represent a new genus and species of eubacteria, Aristabacter necator. The potent, broad-spectrum antimicrobial activity of predator strain 679-2 may be due to synergism between metabolites.

Article:

INTRODUCTION

Driven by the appearance of antibiotic-resistant bacterial pathogens, including Mycobacterium tuberculosis, Staphylococcus aureus, Neisseria gonorrohoeae, Haemophilus influenzae, and Pseudomonas aeruginosa (14, 17, 29), there is an ever-pressing need for new antibiotics. Similarly, few drugs are available for the treatment of infections caused by yeast (e.g., Candida albicans and Cryptococcus neoformans) and/or fungi (e.g., Histoplasma capsulatum and Aspergillus niger) (20). There are a multitude of modern technologies, such as the target-based design of novel compounds (27) and innovative treatment strategies (4), that can be applied to the discovery of new antibiotics. However, nature should not be overlooked as a valuable source of pharmaceuticals, especially with respect to antiinfective and anticancer agents. For example, over the time period from 1984 to 1995, >60% of new drugs for treatment of infections and cancer were derived either directly from natural sources, semisynthesized from a natural product parent, or completely synthesized but modeled after a natural product lead compound (15, 16, 22, 33). Predator bacteria, which are normal inhabitants of soil and present in high numbers (10), are an unexploited source of novel antibiotics (5, 10, 11).

Predator bacteria, such as strain 679-2 (7), are a group of nonobligate bacteria that prey on microorganisms, including other bacteria, fungi, yeast, and protozoa (10). Predators are capable of growing in laboratory media but can also grow on prey microorganisms as a sole source of nutrient (10). Although some predator bacteria kill via attachment (8), the majority do not require attachment for antimicrobial activity (10). In spite of their proven antimicrobial activities, none of the compounds responsible have been identified. The number of bacterial predators in soil can be higher than the number of total soil bacteria measured by usual bacteriological methods (9). Thus, they are important agents of soil ecology and microbial population dynamics (10).

Two predators with broad-spectrum, extracellular antimicrobial activity are Burkholderia ambifaria strain 2.2 N (5) and strain 679-2 (7). Due to its growth inhibitory properties against bacteria, fungi, and even other predator
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bacteria and yet with an ability to thrive in the absence of prey microorganisms, strain 679-2 was described as the top of a hierarchy of nonobligate bacterial predators in soil (7). A U.S. patent was issued for this strain stemming from its ability to inhibit the growth of a wide variety of microorganisms (11).

On the basis of a limited number of morphological, stain, culture, and biochemical characteristics, strain 679-2 appeared to be a representative of the genus Pseudomonas (7). Although members of that genus have not been identified previously as nonobligate bacterial predators, a number of pseudomonads display antimicrobial activities (6, 23, 24, 36). The most notable are members of the species Pseudomonas (now Burkholderia) cepacia (6, 24). Due to the novelty of nonobligate bacterial predators and their broad-spectrum antimicrobial activities, especially if they were to prove to be members of the genus Pseudomonas, we characterized strain 679-2 more fully by using a polyphasic approach (38). In addition, we demonstrate here that the activities of extracellular compounds are responsible for the broad-spectrum antimicrobial activities of this predator. Three compounds were isolated via antimicrobial-activity-directed fractionation of cultures of strain 679-2.

MATERIALS AND METHODS

Strain, media, and culture conditions

Strain 679-2 (ATCC 55089) is a nonobligate predator bacterial strain isolated from soil (**7**). It was grown in one-quarter strength tryptic soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 0.2% sucrose (TSB+S) or on TSB+S containing 1.5% agar at 30°C. Stock slant cultures were stored at 4°C for no longer than 1 month before transfer. Colony morphology was assessed on TSB+S agar. Its ability to grow at 25, 30, 37, and 45°C was determined in both TSB+S broth and TSB+S agar media. The biochemical and substrate utilization patterns of strain 679-2 were assessed by using the API-NFT, API-ZYM, and API-CH50 tests (bioMérieux Vitek, Inc., Hazelwood, Mo.), and MIDI, Inc. (Newark, Del.), generated a fatty acid profile.

General experimental procedures

UV and specific rotations ([ ]D) were recorded on a Varian Cary 3 UVVis spectrophotometer and a Rudolph Autopol IV polarimeter, respectively. All nuclear magnetic resonance (NMR) experiments were performed in either CDCl3 or CD3OD with tetramethylsilane as an internal standard; gs-COSY, gs-HSQC, gs-HMBC, and ^{1}H NMR spectra were run on a Bruker AMX-500 instrument by using a Bruker 5-mm broadband inverse probe with a z-gradient or a Nalorac 3-mm microinverse broadband probe with a z-gradient, whereas a Bruker DPX-300 instrument was utilized for the DEPT-135 and ^{13}C NMR spectra by using a Bruker 5-mm QNP probe. Low-resolution electrospray ionization mass spectra were determined on a Finnigan LCQ instrument (San Jose, Calif.) with an electrospray interface and via direct insertion probe on a HP5989A mass spectrometer (Hewlett-Packard; Palo Alto, Calif.); high-resolution mass spectra were obtained via direct insertion probe EI on a VG70S magnetic sector instrument (Micromass, Beverly, Mass.). Column chromatography was carried out on Si gel 60 (70-230 mesh; Merck, Darmstadt, Germany), and fractions were monitored via thin-layer chromatography (TLC; Si gel 60 $_{F254}$ plates, 0.25-mm thickness) visualized by spraying them with vanillin in H2SO4. Preparative high-pressure liquid chromatography (HPLC) was carried out on either a Waters Delta Prep 3000 or a Varian Pro Star System.

Extraction and isolation

Strain 679-2 was grown in TSB+S broth at 30°C in stages from 10-ml starter cultures to 20-liter fermentation cultures by 10-fold increases in volume. The 20-liter culture was freeze-dried after two successive freeze-thaw cycles to yield 167 g of solids. This material was divided into two equal aliquots and then, separately, each one was extracted with methanol (MeOH) in a Waring blender twice, and this extract was filtered through a fritted funnel in vacuo to remove insolubles. The volume of the eluant was reduced in vacuo and then partitioned between CHCl3-MeOH and H2O (4:1). The organic extracts were combined (21 g), mixed with Celite, and purified further on a flash Si gel column that was developed by using a gradient of 100% hexane to 100% CHCl3 to 20% MeOH. The fractions were combined into 12 pools based on TLC properties and examined against a panel of microorganisms. Pool 3 displayed the most potent activity, especially against *A. niger*, and it

was therefore purified further on a 5-μm diol HPLC column (250 by 20 mm; YMC) by using an isocratic solvent system of CHCl3-hexane (1:1) to yield compound 1 (Fig. 1; 20.5 mg, yield 0.012% [wt/wt]). The aqueous extracts mentioned above were combined and partitioned further by shaking them with *n*-butanol. The volume of the alcohol-soluble portion was reduced in vacuo, and the residue (5 g) was purified via size-exclusion chromatography over LH-20 (Amersham Pharmacia, Uppsula, Sweden) with 100% MeOH to yield nine pools. Pool 9, with a large amount of sample weight, was purified successively via preparative TLC with CHCl3-MeOH (10:1 3:1 3:1) to yield four fractions. Fraction four resulted in compound 2 (Fig. 1; 11.0 mg; yield, 0.0066% [wt/wt]) after preparative HPLC in the reversed phase on a 5-μm ODS-A column (250 by 20 mm; YMC) by using an isocratic solvent system of MeOH-H2O (15:85). The same HPLC column was used for fraction 2 with a MeOH-H2O solvent gradient of 2:98 up to 7:93 over 20 min and then kept isocratic to yield compound 3 (Fig. 1).

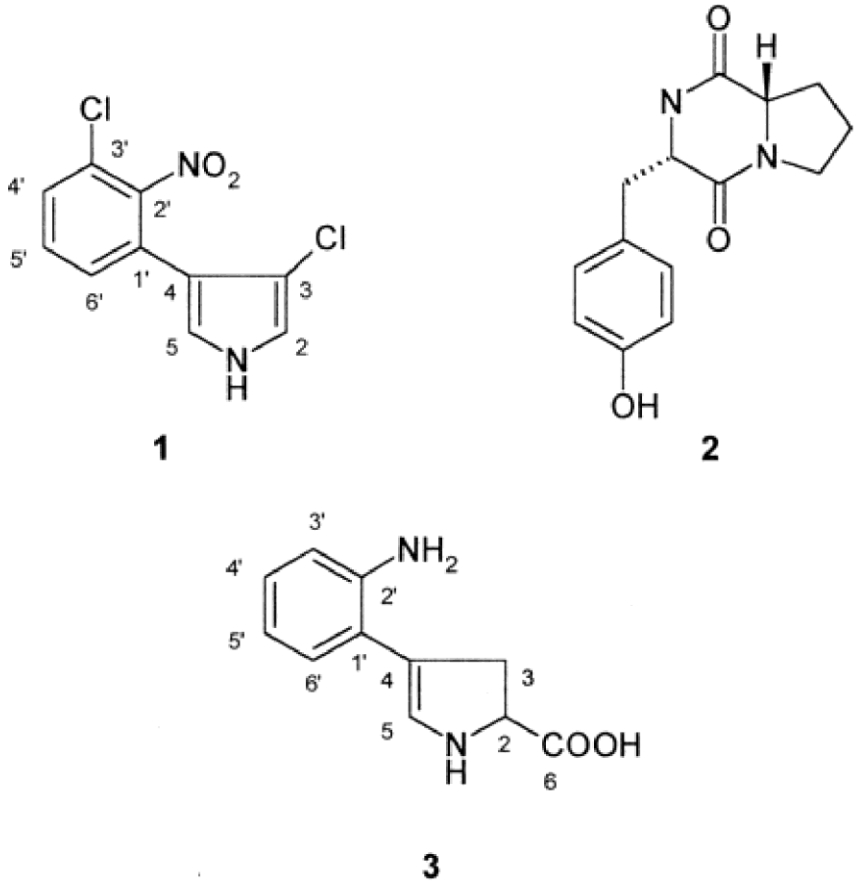

FIG. 1. Structures of pyrrolnitrin (compound 1), maculosin (compound 2), and banegasine (compound 3).

Measurement of antimicrobial activities

The antimicrobial activities of extracts, fractions, and pure compounds were measured by a zone-of-inhibition assay (5) against strains of Micrococcus luteus, Mycobacterium smegmatis, Saccharomyces cerevisiae, C. neoformans, C. albicans, and A. niger. The MIC was defined as the lowest concentration resulting in the inhibition of growth, as evidenced by clearing. For combinations of compounds, the MIC was expressed in terms of each individual compound.

Calculation of FIC of antibiotic combinations

To test for synergistic activity, the fractional inhibitory concentrations (FIC) of combinations of compounds 1, 2, and 3 were measured against the aforementioned six target microorganisms. FIC values were calculated by using the following formula: FIC = (MICDrug A in combination/MICDrug A alone) + (MICDrug B in combination/MICDrug B alone). Synergy was defined as an FIC value of 0.5, whereas FIC values between 0.5 to 1.0 were considered to be the result of additive antimicrobial effects (2, 12, 18).

DNA isolation and sequencing the 16S rRNA gene

DNA was isolated from cells of strain 679-2 as described by Marmur (28). The 16S rRNA gene was amplified by using PCR with a pair of universal 16S rRNA primers: 27f and 1522r (26). The DNA sequenced was a PCR product of genomic DNA generated by primers 27f and 1522r and, consequently, the sequence reported covered positions 27 through 1522 of the 16S rRNA gene (98%) according to the Escherichia coli 16S rRNA numbering system. Two sets of primers were used to sequence the PCR product (27f and 907r; 704f and 1522r), leading to sequence overlap in the center of the gene. The 16S gene was sequenced three times in both the forward and the reverse directions by using an automated DNA sequencer (UVA Biomolecular Research Facility, Charlottesville, Va.). In addition, the PCR product was cloned into pBluescript (Stratagene, La Jolla, Calif.) and sequenced with the T7 and T3 primers by both automated DNA sequencing and by Sequenase according to the manufacturer's directions (U.S. Biochemicals, Inc., Cleveland, Ohio). Sixfold coverage of the sequence was attained, and two different approaches were employed to reduce the frequency of sequence ambiguities; the sequence was deposited as GenBank accession number AF005994.

Banegasine (compound 3)

Our findings for banegasine were as follows: white powder (3.0 mg), yield 0.0018% (wt/wt); melting point, 213 to 215°C; [ ]D, -44.3°C (c 0.07, MeOH); UV (MeOH) max (log) 227 (3.86) and 280 (3.71) nm; 1H NMR (CD3OD, 500 MHz) 3.13 (1H, dd, J = 9.6 and 15.3 Hz, H-3), 3.50 (1H, dd, J = 3.9 and 15.3 Hz, H-3), 3.84 (1H, dd, J = 3.9 and 9.3 Hz, H-2), 7.03 (1H, dt, J = 1.2 and 7.8 Hz, H-5'), 7.11 (1H, dt, J = 1.5 and 8.4 Hz, H-4'), 7.18 (1H, s, H-5), 7.35 (1H, d, J = 8.1 Hz, H-3'), 7.68 (1H, d, J = 8.1 Hz, H-6'); 13C NMR (CD3OD, 125 MHz) 28.5 (C-3), 56.8 (C-2), 109.7 (C-4), 112.4 (C-3'), 119.3 (C-6'), 120.1 (C-5'), 122.7 (C-4'), 125.1 (C-5), 128.5 (C-1'), 138.4 (C-2'), 174.5 (C-6); HMBC correlations H-2 C-3, C-4, C-6; H-3 C-2, C-4, C-5, C-6, C-1'; H-5 C-3, C-4, C-1 '; H-3' C-1 '; H-6' C-4; EIMS m/z 204 (M+, 9), 130 (100), 103 (8); ESIMS m/z 205 [M + H]+; HREIMS m/z 204.0890, calculated for C1 1H12N2O2, 204.0899.

RESULTS

Characteristics and taxonomy of strain 679-2

Strain 679-2 formed four different types of colonies on TSB+S agar medium. The predominant (85%) type were 1- to 2-mm-diameter, amber-colored, beehive-shaped circular colonies seen after 48 h of incubation at 30°C. The colonial morphologies of the other three types (and the approximate frequencies of appearance) after 48 h of incubation of TSB+S agar medium at 30°C were as follows: 3- to 5-mm-diameter, irregularly shaped, watery mucoid colonies (6%); 0.2-mm-diameter translucent white circular colonies (5%); and 1-mm-diameter white colonies (3%). The amber-colored colony type gave rise to all of the other three types, as did the irregularly shaped, mucoid colony type. Only colonies or cultures of the amber-colored, beehive-shaped variant exhibited antimicrobial activity. For all experiments reported here, cultures were inoculated with cells from the amber-colored, beehive-shaped colony and the frequency of nonproducing variants in cultures was <20%. Most luxuriant growth in broth medium, and the largest-diameter colonies (1 to 2 mm) of the predominating type, occurred when cultures were incubated at 30 and 37°C. Less growth occurred when cultures were incubated at 25°C. The strain grew poorly at 45°C, yielding sparse turbidity in broth and only a few small colonies on agar. Strain 679-2 was oxidase and indol negative and was capable of reducing nitrates to nitrites, and it had the following enzymatic activities: acid and alkaline phosphatase, esterase (C4), lipase, gelatinase, *N*-acetyl-ß-glucosaminidase, naphthol-AS-BI-phosphohydrolase, ß-galactosidase, ß-glucosidase, and cysteine, leucine, and valine arylamidases. The strain lacked -glucosidase activity. The following substrates were utilized for growth or energy: adipic acid, D-arabinose, L-arabinose, capric acid, cellobiose, esculin, D-fucose, galactose, D-glucose, lactose, D-lyxose, malic acid, maltose, mannose, phenylacetic acid, sucrose, trehalose, and D-xylose. Although capable of utilizing glucose for growth, strain 679-2 failed to ferment glucose or other carbohydrates. Compared to members of the genus *Pseudomonas* or *Burkholderia* (particularly *B. cepacia*), strain 679-2 utilized a narrower range of substrates as carbon or energy sources (39). Especially noteworthy was the inability of strain 679-2 to utilize glycerol or sugar alcohols. Strain 679-2 had a unique cellular fatty acid composition. The pattern of low levels of $_{C16:0}$ (i.e., 15%), high levels of $_{C16:1}$ (i.e., 18%), and very high levels of C18:1 (i.e., 40%) cellular fatty acids distinguished strain 679-2 from members of the genus *Pseudomonas* or *Burkholderia* (i.e., high $_{C16:0,}$ ^{low} $_{C16:1,}$ and low $_{C18:1)}$ (39).

Based on the sequence of the 16S rRNA gene of strain 679-2, it is not a representative of *Pseudomonas*, *Burkholderia*, or *Ralstonia* spp. (i.e., <90% similarity). Further, no matches with similarity indices of >90% were found between the 16S rRNA gene sequence of strain 679-2, and any other sequence recorded in the databases. On the basis of the unique 16S rRNA sequence and cellular fatty acid profile of strain 679-2, it appears that this nonobligate predator with broad-spectrum antifungal activity represents a new genus of bacteria. Therefore, the scientific name *Aristabacter necator* (Aristaeus, protector of vines; Necator, slayer) is proposed.

Antimicrobial compounds from strain 679-2

Compounds responsible for the observed antimicrobial activities were pursued systematically via antimicrobial-activity-directed fractionation of a 20-liter culture of strain 679-2 grown in one-quarter strength TSB+S. After incubation to stationary phase (48 h at 30°C), the culture was freeze-dried. Crude extracts and partially purified fractions were tested against the panel of microorganisms by the zone of inhibition assay. Liquid-liquid partitioning of the freeze-dried culture residue led to an organic and an aqueous fraction. Successive purification procedures on the organic fraction led to the isolation and structure elucidation of pyrrolnitrin (compound 1) as the major antimicrobial compound. The structure of compound 1 was confirmed by comparisons of the ^{1}H and ^{13}C NMR data to those of the literature (13, 19, 32).

The aqueous fraction was extracted with *n*-butanol, and from this two major compounds were isolated by successive purification procedures. The structure of maculo sin (compound 2) was confirmed by comparison of the spectral data to those of the literature (3, 30, 35).

A new compound (compound 3) was obtained as an amorphous white powder, and its molecular formula was established as $_{C11H12N2O2}$ by HREIMS. The results obtained from the ^{1}H, ^{13}C, COSY, and HSQC NMR spectra indicated that compound 3 had a disubstituted benzene ring, a dihydropyrrole moiety, and a carboxyl substituent. From the ^{1}H NMR data, signals characteristic of an ortho disubstituted benzene ring were observed at H 7.35 (1H, doublet, *J* = 8.1 Hz, H-3'), H 7.11 (1H, triplet of doublets, *J* = 8.4 and 1.5 Hz, H-4'), $_{H}$ 7.03 (1H, triplet of doublets, *J* = 7.8 and 1.2 Hz, H-5'), and H 7.68 (1H, doublet, *J* = 8.1 Hz, H-6'); From the ^{13}C and DEPT-135 NMR data, substituents were attached to this ring at the quaternary $_{C}$ 128.5 (C-1') and C 138.4 (C2'). The dihydropyrrole moiety was assigned from the mutually coupled signals at H 3.84 (1H, doublet of doublets, *J* = 3.9 and 9.3 Hz, H-2), H 3.13 (1H, doublet of doublets, *J* = 9.6 and 15.3 Hz, H-3), and H 3.50 (1H, doublet of doublets, *J* = 3.9 and 15.3 Hz, H-3), and from the double bond between $_{C}$ 109.7 (C-4) and H/ C 7.18/125.1 (C-5). Long-range correlations in the HMBC NMR data supported these assignments. The carboxyl side chain was positioned at C-2 via HMBC correlations from both H-2 and H-3 to C-6, and the linkage between the pyrrole and benzene rings was determined from HMBC correlations from both H-3 and H-5 to C-1' and H-6' to C-4. Finally, the only unassigned functional group from the HREIMS was the amine moiety, which was attached by default to position C-2'. Thus, the structure of this new bacterial metabolite, banegasine (compound 3), was elucidated as 4-(2-amino-phenyl)-2,3 -dihydro- 1*H*-pyrrole-2-carboxylic acid (Fig. 1). The stereochemistry of position C-2 was not determined due to the limited amount of sample available.

Antimicrobial activities of compounds isolated from strain 679-2

The pure compounds isolated from 679-2 were evaluated against the panel of microorganisms by zone-of-inhibition assays, and pyrrolnitrin (compound 1) was the only compound with antimicrobial activity (Table 1). When tested alone, compounds 2 and 3 were inactive (Table 1). However, the antimicrobial potentiating effects of combinations of 2 and 3 with 1 were examined by FIC calculations. FIC values of 0.5 are considered synergistic, whereas values greater than 0.5 are considered additive (2, 12, 18). Based on MICs (Table 1) and FIC calculations (Table 2), the combination of compounds 1 and 2 was synergistic against *A. niger*, whereas the combination of compounds 1 and 3 was synergistic against *M. smegmatis*. Moreover, by combining all three compounds, synergistic activity was observed against *A. niger*, *M. smegmatis*, and *S. cerevisiae* (Tables 1 and 2). The results in Table 1 are from a representative experiment wherein duplicates were averaged and the FIC values (Table 2) were calculated as described in Materials and Methods.

TABLE 1. MICs of strain 679-2 metabolites

Metabolite(s)[a]	MIC (μg/μl)[b] against:					
	M. luteus	*M. smegmatis*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*A. niger*
1	0.22	0.30	0.054	0.108	0.108	0.29
2	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
3	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
1+2	0.185	0.30	0.027	0.054	0.054	0.108
	0.173	0.275	0.024	0.047	0.047	0.10
1+3	0.22	0.075	0.073	0.070	0.070	0.29
	0.188	0.063	0.062	0.060	0.060	0.25
2+3	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
1+2+3	0.20	0.10	0.025	ND	ND	0.10
	0.184	0.097	0.023	ND	ND	0.097
	0.167	0.084	0.021	ND	ND	0.084

[a] See Fig. 1 for definitions and structures of the metabolites.

[b] For the combination studies, the first value represents the MIC relative to the concentration of the first compound listed and the second (or third) value represents the MIC relative to the concentration of the second (or third) compound listed. ND, not determined.

TABLE 2. FIC of strain 679-2 metabolites

Metabolite combinations[a]	FIC[b] against:					
	M. luteus	*M. smegmatis*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*A. niger*
1+2	1.00	1.25	0.58	0.54	0.54	0.46
1+3	1.19	0.31	1.41	0.72	0.72	1.25
1+2+3	1.25	0.50	0.50	ND	ND	0.50

[a] See Table 1, footnote *a*.

[b] See the text for calculation of the FIC. ND, not determined.

DISCUSSION

Strain 679-2 is unique not only because it represents a novel bacterial genus but also because it produces concomitantly the antibiotic pyrrolnitrin (compound 1), the herbicide maculosin (compound 2), and the novel compound banegasine (compound 3). The antimicrobial (1, 37) and phytotoxic (35) activities, respectively, of pyrrolnitrin and maculosin have been described previously. Compound 1 is produced by a wide variety of bacteria, including the pseudomonads (1, 23), Enterobacter agglomerans (13), Burkholderia cepacia (19), and others (37). To the best of our knowledge, compound 2 has not been isolated from a prokaryote, nor has it been produced in combination with compound 1 by any organism.

Banegasine is a novel compound, and yet structural similarities can be drawn between it and pyrrolnitrin. Compound 3 has a 2,3-dihydropyrrole moiety, and an analogous compound, albeit with a fully unsaturated pyrrole ring, was postulated as an intermediate in the biosynthesis of compound 1 (21, 37). Thus, banegasine may be an intermediate in, or a by-product of, the biosynthesis of pyrrolnitrin by strain 679-2.

Maculosin and banegasine lacked antimicrobial activities when tested alone or in combination with each other (Table 1). However, the combination of either maculosin or banegasine with pyrrolnitrin resulted in increased antimicrobial activities as expressed by the FIC (Table 2). Synergistic activity suggests that the compounds

attack different targets. Cell-free culture filtrates of strain 679-2 (25) and pyrrolnitrin induce the rapid release of intracellular maltase from cells of S. cerevisiae (15 min at 30°C; data not shown), a finding consistent with the ability of pyrrolnitrin to effect ozomoregulation (31). Exposure of S. cerevisiae to the combination of compounds 1, 2, and 3 resulted in the release of twice as much maltase (data not shown), suggesting that synergism was due to enhanced lytic activity. Precedence for synergistic antimicrobial activity was noted recently between two unrelated, yet coproduced, compounds isolated from leaves of the barberry plant, Berberis frementii (34). That report suggests that occurrences of synergistic antimicrobial activity between structurally related (this report) or unrelated (34) compounds in nature may be more prevalent than realized currently.

Synergism between metabolites indicates that isolating and measuring the antimicrobial activity of single compounds may miss possible candidates for antibiotic development (34). For example, banegasine, which was inactive alone, may have potential as an antimicrobial-activity potentiator, and this would have been missed if the suite of compounds isolated from strain 679-2 had not been evaluated in combinations.

Evidence of synergistic antimicrobial activity may help to explain the "chemical warfare" used by Aristabacter necator strain 679-2 to kill other organisms. The spectrum of antimicrobial activities and the lytic ability of whole cells were reflected in the activities of the purified compounds in combination (Tables 1 and 2). Thus, there is no need to invoke some cell-associated activity (e.g., penetration) to explain the antimicrobial activities of this predator bacterium. Moreover, the unique chemical profile and biological activity observed with this microorganism points to the potential for predator bacteria to serve as valuable sources of novel antibiotics.

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C.C.C. and D.L. contributed equally to this study.

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