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Newly isolated marine *Bacillus pumilus* (SP21): A source of novel lipoamides and other antimicrobial agents*

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Abstract: A screening of marine bacteria for antimicrobial activity resulted in the isolation of *Bacillus pumilus* (SP21) from a sediment sample collected in the Bahamas. A bioassay-guided fractionation led to the isolation of five surfactin analogs, glycocholic acid, amicoumacins A and B in addition to three new compounds named lipoamides A–C. The chemical structure of all the bioactive compounds was elucidated using spectroscopic methods including 2D NMR and MS. The antimicrobial activity of each compound was evaluated against a panel of pathogens and is reported herein.

Keywords: *Bacillus pumillus*; antimicrobial; lipopeptides; amicoumacins; MRSA; lipoamides.

INTRODUCTION

The need for antibiotics and antimicrobials continues to be a major challenge as infectious diseases affect millions of people worldwide. Further, antimicrobial resistance is a growing concern as the number of resistant bacteria and the geographic distribution of these organisms are both rising. Due to such global health concerns, our laboratory has been engaged in a program to identify natural products with activity against microbes. Marine-derived microorganisms have proven to be a useful source of such natural products and progress has recently been reviewed [1,2].

Bacteria of the genus *Bacillus* are known to produce natural products which possess antagonistic activities against many bacterial and fungal pathogens and are often used as agents for the treatment and/or prevention of different plant and animal infections [3–6]. Their antimicrobial activities have mainly been attributed to the production of antibiotic peptide derivatives, lipopeptides [7–10], which have powerful surfactant like properties with numerous biotechnological applications including de-emulsification, health care, and food industry. Moreover, in contrast with synthetic homologs, microbial surfactants are often biodegradable and can be supplied by fermentation [2,11].

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RESULTS AND DISCUSSION

Bacterial isolation and screening for antimicrobial activity

During an expedition to the Bahamas in 2006, 445 bacterial strains from sediment samples were isolated. Extracts of all isolates were screened for antimicrobial activity against *Staphylococcus aureus* (ATCC 10832), *Pseudomonas aeruginosa* (ATCC 14210), and *Candida albicans* (ATCC 14053). From this screening, 41 showed antimicrobial activity against *S. aureus*, 4 (SP21, SPA3, VC2, and VC1) showed antimicrobial activity against *P. aeruginosa*, and 3 showed activity against *C. albicans*. All active strains were tested for antimicrobial activity against a second panel of microbes including *Enterococcus faecalis* (ATCC10741), *Proteus vulgaris* (ATCC 12454), and methicillin-resistant *S. aureus* (MRSA) (ATCC 33591). The activity in disk diffusion assays for the four isolates is summarized in Table 1. SPA3, VC2, and VC1 showed some selectivity while SP21 was active against all strains tested. SP21 was identified by 16S rRNA sequence analysis as *Bacillus pumilus* and selected for chemical analysis.

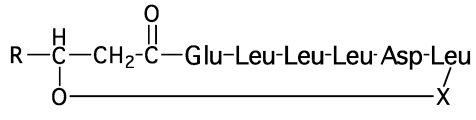
Table 1 Antimicrobial activity of isolates (inhibition zone in mm).

Isolate	*S. aureus*	*S. aureus* (MRSA)	*E. faecalis*	*P. aeruginosa*	*P. vulgaris*	*C. albicans*
SP21	14	12	11	13	19	14
SPA3	12	0	10	0	11	12
VC2	15	0	0	10	8	11
VC1	13	0	12	10	0	15

Lipopeptides

A 4-l culture of SP21 was harvested after 72 h, and the cells were separated from the supernatant. Both fractions were lyophilized and extracted with DCM:MeOH (1:1) to afford 133 mg of pellet extract and 12.6 g of supernatant extract. The pellet extract was further partitioned between H_2O and DCM, and the resulting organic fraction was evaporated to yield 79 mg of yellow oil. Analysis by ultra high performance liquid chromatography UPLC/MS with a standard gradient from 5 % ACN (0.1 % HCO_2H) in H_2O (0.1 % HCO_2H) to 100 % ACN (0.1 % HCO_2H) and an electrospray ionization (ESI) detector indicated that this fraction contained a mixture of known lipopeptides (Table 2) [2,12]. Indeed, the peaks related to the adducts $[M+H]^+$ 1036, 1050, 1064, and 1078, are characteristic of described surfactin analogs which are known to differ in the number of methylene groups (14 amu) in their lipid and/or peptide components.

Table 2 Lipopeptides isolated from *B. pumilus* SP21.



	R	X	$[M+H]^+$
1	$C_{11}H_{23}$	Val	1036.5
2	$C_{12}H_{25}$	Leu or Ileu	1050.5
3	$C_{13}H_{27}$	Leu or Ileu	1064.5
4	$C_{14}H_{29}$	Val	1064.5
5	$C_{14}H_{29}$	Leu or Ileu	1078.5

The surfactin analogs are composed of a heptapeptide fragment linked with an *n*-, *iso*-, or *anteiso*-hydroxy-fatty acid side chain with 13–16 carbons. In our case, tandem mass spectroscopy analysis allowed us to identify two different amino acid sequences among the various lipopeptides produced by *B. pumilus* (SP21). In order to evaluate the antimicrobial properties of these metabolites, the mixture was further purified by reverse-phase high-performance liquid chromatography (HPLC) with an isocratic mobile phase (75 % ACN in H_2O with 0.1 % TFA) to lead to compounds **1** (6.9 mg), **2** (17.8 mg), **3** (2.5 mg), **4** (2.9 mg), and **5** (4.9 mg). In addition to the MS evidence, the ^{1}H NMR spectra for each compound was consistent with the previously reported lipopeptide structures [11,12].

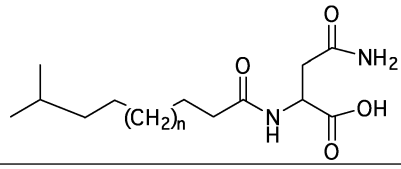
Lipoamides A–C, amicoumacin and glycocholic acid derivatives

The supernatant extract was fractionated by C_{18} flash chromatography, and three families of natural products were isolated by a series of HPLC purifications. Lipoamides A (**6**, 5.5 mg), B (**7**, 1.7 mg), and C (**8**, 1.8 mg) were isolated using a C_{18} column with isocratic conditions (75 % MeOH in H_2O with 0.1 % HCO_2H). Compound **6**, obtained as a colorless oil, was found to have the molecular formula $C_{15}H_{28}N_2O_4$ by HRESI (*m/z* 323.1933 $[M+Na]^+$, 323.1941 calcd for $C_{15}H_{28}N_2O_4Na$) which requires three degrees of unsaturation. The two methyl signals at δ_H 0.88 (d, 6.7 Hz) as well as a broad peak at 1.30 ppm indicated the presence of an *iso* fatty acid side chain, which was supported by evaluation of the correlation spectroscopy (COSY) data. The combined MS and NMR data clearly indicated the presence of a C11 acyl group with a methyl branch on the penultimate carbon. Based on the molecular formula, the remainder of the molecule was a $C_4H_7O_3N_2$ unit. The proton signals at δ_H 4.71 (bs), δ_H 2.77 (dd, 15.7; 6.9), and δ_H 2.72 (dd, 15.7; 4.8) as well as key heteronuclear multiple-bond correlation (HMBC) correlations indicated the existence of an asparagine unit. Therefore, the third unsaturation was explained by a carboxylic acid function in position 1' which was not detected through various NMR experiments including HMBC and H2BC. The proposed structure was further confirmed by a ^{1}H experiment in DMSO-d_6 which revealed three exchangeable protons at δ_H 6.84, δ_H 7.56, and δ_H 7.84 ppm.

Compound **7** (*m/z* 337.2095 $[M+Na]^+$, 337.2103 calcd for $C_{16}H_{30}N_2O_4Na$) and **8** (*m/z* 351.2240 $[M+Na]^+$, 351.2260 calcd for $C_{17}H_{32}N_2O_4Na$) had very similar ^{1}H and ^{13}C spectra to compound **6** and the only significant differences were molecular weight differences of 14 and 28 amu, respectively. Thus, the structures of lipoamides B (**7**) and C (**8**) are as shown in Table 3.

Glycocholic acid (**9**, 7.7 mg) was readily isolated from the supernatant extracts by HPLC, and its structure was confirmed by comparison of 1D and 2D NMR data with that in the literature [13]. Compound **9** may be related to sterol derivatives present in the marine broth media and thus are believed to be produced through metabolism by *B. pumilus* SP21 during the fermentation process. A mixture of known amicoumacins was purified by HPLC using an isocratic system of 48 % MeOH in H_2O with 0.1 % HCO_2H leading to amicoumacin A (**10**, 5.4 mg) and amicoumacin B (**11**, 7.6 mg). Metabolites **10** and **11** were identified based on comparison of our spectroscopic data with that previously reported [14,15].

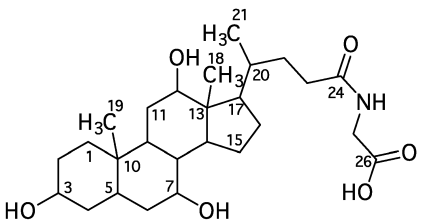
Table 3 ^{1}H and ^{13}C NMR (500 and 125 MHz, respectively) data of lipoamide A (**6**) in CD_3OD.



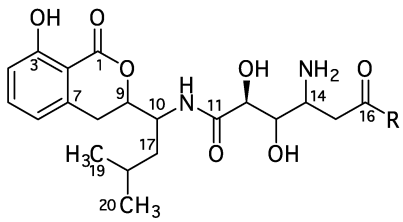
6 $n = 3$
7 $n = 4$
8 $n = 5$

Carbon	δ_C	δ_H (m, J in Hz)	COSY	HMBC (C → H)
1	175.3[b]			H2
2	36.6	2.23 (t, 7.4)	H3	H3
3	26.9	1.61 (m)	H2, H4	H2
4	30.2[a]	1.31 (m)	H4	H3, H2
5	30.5[a]	1.31 (m)		
6	30.9[a]	1.31 (m)		H8
7	28.5	1.31 (m)		H8, H9
8	40.2	1.18 (m)	H7, H9	H9, H10, H11
9	29.1	1.52 (sept, 6.7)	H8, H10, H11	H10, H11
10	23.0	0.88 (d, 6.7)	H9	H8, H9, H11
11	23.0	0.88 (d, 6.7)	H9	H8, H9, H10
1'	–			
2'	49.8	4.71 (bs)	H3'a, H3'b	H3'a, H3'b
3'	37.9	2.77 (dd, 15.7; 6.9)	H2'	H2'
		2.72 (dd, 15.7; 4.8)	H2'	H2'
4'	174.3			H3'a, H3'b

[a]Chemical shift can be interchanged.
[b]Chemical shift determined by the HMBC correlations.



9



10 R=NH_2
11 R= OH

Minimal inhibitory concentrations (MICs)

The MICs were evaluated for all isolated compounds and are summarized in Table 4 [16]. The lipopeptides, the most abundant secondary metabolites of the SP21 fermentation extract, showed the lowest MIC value with no significant difference between compounds **1–4**. There is interesting selectivity with good activity against *S. areus*, *P. vulgaris*, and *E. faecalis* yet no activity against *P. aeruginosa*. Amicoumacin A (**10**) exhibited better antimicrobial activities than amicoumacin B (**11**), which may be due to the presence of an amide function at C-16. This report includes the first description of the activity of amicoumacin A against the "superbug" methicillin-resistant *S. aureus* (MRSA). Lipoamides showed no antimicrobial activity against *S. aureus* and *P. aeruginosa* with MIC values above 100 μg/ml.

Table 4 MIC values (μg/ml) of metabolites isolated from *B. pumilus* SP21.

Compounds	*S. aureus*	*P. aeruginosa*	*P. vulgaris*	*E. faecalis*	*S. aureus* (MRSA)	*C. albicans*
Amicoumacin A (**10**)	6.5	>100	50	50	50	50
Amicoumacin B (**11**)	50	>100	>100	50	n/a	>100
Lipoamide A (**6**)	>100	>100	n/a	n/a	n/a	n/a
Lipopeptide **1**	6.5	>100	25	12.5	n/a	50
Lipopeptide **2**	12.5	>100	6.25	12.5	n/a	25
Lipopeptide **3**	12.5	>100	12.5	25	n/a	25
Lipopeptide **4**	25	>100	12.5	25	n/a	25

n/a data not available

In summary, new lipoamides isolated from *B. pumilus* have been reported for the first time. These were not found to exhibit significant antibacterial activity, however, the known lipopeptides and amicoumacins were found to exhibit selective antibacterial activity.

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