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Bacilosarcins A and B, novel bioactive isocoumarins with unusual heterocyclic cores from the marine-derived bacterium *Bacillus subtilis*☆

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ABSTRACT

Two novel isocoumarins, bacilosarcins A (**1**) and B (**2**) were isolated from a culture broth of the marine-derived bacterium *Bacillus subtilis* TP-B0611. The structures and absolute configurations of **1** and **2** were determined on the basis of spectroscopic analyses and chemical conversions. Compound **1** possesses an unprecedented 3-oxa-6,9-diazabicyclo[3.3.1]nonane ring system while **2** has a 2-hydroxymorpholine moiety that is rare in nature. These compounds showed growth inhibition against barnyard millet.

1. Introduction

There is substantial interest in discovering new lead molecules from natural products for agricultural application including plant growth regulators. We previously isolated an α-pyrone derivative that inhibits spore germination of plant pathogenic fungi,[2] a spirocyclic polyketide with auxin-like plant growth promoting activity,[3] and an indole derivative with antifungal activity[4] from actinomycetes. As part of our continuing studies in this field, the marine-derived bacterium *Bacillus subtilis* TP-B0611 was found to produce two new isocoumarins, bacilosarcins A (**1**) and B (**2**) as well as three previously reported isocoumarins, amicoumacins A, B, and C.

Isocoumarin-type metabolites from microorganisms are characterized by the amino-containing substituent, which is presumably derived from leucine, at 3-position in the dihydrocoumarin core. There have been several such isocoumarin compounds as baciphelacin,[5] amicoumacins,[6] xenocoumacins,[7] Y-05460 M-A,[8] PM-94128,[9] and Sg17-1-4[10] reported to date. These compounds possess the common chromophore, 3,4-dihydro-8-hydroxyisocoumarin in their structures and many of them are produced by the genus *Bacillus*. These isocoumarins have been reported to exhibit antibacterial,[5,6b] cytotoxic,[9,10] and antiulcer[6a,7] activities. The structures of **1** and **2** were assigned on the basis of NMR analysis and chemical conversions. Compound **1** possesses an unprecedented 3-oxa-6,9-diazabicyclo[3.3.1]nonane ring system whereas **2** has a 2-hydroxymorpholine substructure that is rare in nature. The new compounds showed plant growth inhibitory activity against barnyard millet. We herein describe the isolation, structural elucidation, absolute stereochemical assignment, and biological activities of **1** and **2** (Fig. 1).

2. Results and discussion

During the course of screening marine-derived bacteria for bioactive compounds, we assessed the strain *B. subtilis* TP-B0611 from intestine content of a sardine (*Sardinops melanosticta*) collected in Toyama Bay, Japan. Upon large-scale liquid fermentation, compounds **1** and **2** were isolated from the culture extract using plant growth inhibition bioassay to detect bioactivity and to direct the fractionation.

Compound **1** was obtained as a colorless powder. High-resolution EIMS revealed an $[M]^+$ at *m/z* 491.2272, corresponding to the molecular formula $C_{24}H_{33}N_3O_8$ (calcd 491.2267). ^1H and ^{13}C NMR spectral data (Table 1) were in full agreement with the assigned molecular formula. The ^{13}C NMR and DEPT spectra of **1** displayed signals for 24 carbons including 8 aliphatic carbons, 7 carbons bonded to nitrogen and/or oxygen, 6 carbons in the olefinic region,

☆ See Ref. 1.

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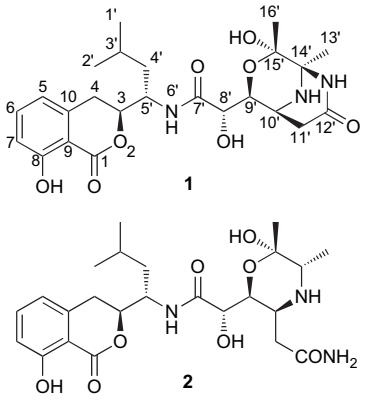

Figure 1. Structures of bacilosarcins A (**1**) and B (**2**).

and 3 carbonyl carbons. These data also indicated that **1** contained 10 double bond equivalents, which on the basis of the carbon chemical shifts were assigned to 3 carbonyls, 3 C=C double bonds, and 4 rings. The IR spectrum was consistent with the presence of hydroxyl (3265 cm^{-1}) and carbonyl functional groups (1655 cm^{-1}). The UV spectrum was nearly identical to that reported for amicoumacins (λ_{max} 206, 247, 314 nm),[6] suggesting that **1** possessed a chromophore similar to dihydroisocoumarin.

Several substructures were assigned by analyses of the ^{1}H, ^{13}C, ^{1}H–^{1}H COSY, TOCSY, NOESY, HMQC, and HMBC NMR spectral data recorded in $CDCl_3$ (Fig. 2). The first part was the isocoumarin moiety, which contained two isolated spin systems. One spin system consisting of the three aromatic protons H-5, H-6, and H-7 displayed the coupling patterns for 1,2,3-trisubstituted benzenoid ring system. The HMBC correlations from H-5 and H-7 to C-9 and from H-6 to C-8 and C-10, and from 8-OH to C-7 and C-8 established the hydroxyl group at C-8 and substitutions at C-9 and C-10. The second spin system was elucidated starting from the two secondary methyl groups (H-1′ and H-2′) that showed HMBC correlations to one another, to C-3′ and to C-4′. The proton signal attached to C-4′ showed a COSY correlation to a methine proton H-5′, which in turn showed COSY correlations to H-3 and an XH proton (δ_H 6.98). The methine proton H-3 showed COSY correlation to H-4. This XH proton was considered an NH proton on the basis of the carbon chemical shift of C-5′ (δ_C 49.6) and an HMBC correlation from the XH proton to C-7′. The HMBC correlations from H-4 to the aromatic carbons C-5, C-9, and C-10 established the connectivity between C-4 and C-10. The deshielding characters of H-3 and C-3 nuclei were indicative of the ester linkage between C-1 and C-3 to complete the isocoumarin-type substructure **A**.

Substructure **B** was constructed starting from the methylene proton (H-11′) that showed a COSY correlation to H-10′ and HMBC correlations to C-9′ and C-12′. This fragment was expanded by COSY correlations from H-10′ to H-9′ and from H-9′ to H-8′, and a TOCSY correlation from H-8′ to 8′-OH. The carbon chemical shifts of C-9′ (δ_C 72.5) and C-10′ (δ_C 47.9) were indicative of bonding of oxygen and nitrogen to these carbons, respectively. Finally, HMBC correlations from H-8′ and H-9′ to C-7′ and 12′-NH to C-11′ established this as a functionalized adipate **B**. Substructures **A** and **B** were

Table 1
^{1}H and ^{13}C NMR data for bacilosarcins A (**1**) and B (**2**)

Position	Bacilosarcin A (**1**)			Bacilosarcin B (**2**)		
	δ_H (mult, *J* in Hz)[a]	δ_C[b]	HMBC	δ_H (mult, *J* in Hz)[a]	δ_C[b]	HMBC[c]
1		169.7			170.0	
3	4.59 (ddd, 13.2, 2.7, 2.5)	81.2		4.54 (ddd, 12.9, 2.5, 2.4)	81.3	5′
4	3.09 (dd, 16.4, 13.2)	30.5	3, 10	3.21 (dd, 16.4, 13.2)	29.4	3, 10
	2.84 (dd, 16.4, 2.7)		5, 9, 10	2.85 (dd, 16.4, 2.4)		5, 9, 10
5	6.71 (d, 7.3)	118.2	4, 7, 9	6.50 (d, 7.3)	118.6	4, 7, 9
6	7.42 (dd, 8.3, 7.3)	136.6	8, 10	7.37 (dd, 8.6, 7.3)	136.5	8, 10
7	6.89 (d, 8.3)	116.3	5, 8, 9	6.97 (d, 8.6)	116.0	5, 8, 9
8		162.1			162.3	
9		108.1			108.9	
10		139.4			140.6	
1′	0.96 (d, 6.4)	21.8	2′ 3′ 4′	0.83 (d, 6.6)	21.6	2′, 3′, 4′
2′	0.97 (d, 6.3)	23.1	1′, 3′, 4′	0.80 (d, 6.6)	23.3	1′, 3′, 4′
3′	1.66 (m)	24.8		1.72 (m)	24.8	
4′	1.76 (ddd, 13.6, 10.2, 5.1)	41.0	5′	1.85 (ddd, 13.7, 11.5, 4.4)	39.0	1′, 3′, 5′
	1.44 (ddd, 13.6, 9.0, 4.6)			1.38 (ddd, 13.7, 9.8, 3.9)		3′
5′	4.39 (dddd, 10.2, 10.0, 4.6, 2.5)	49.6		4.64 (m)	49.1	4′, 7′
7′		172.3			172.9	
8′	3.94 (d, 9.8)	68.4	7′, 9′, 10′	4.90 (d, 8.6)	71.5	7′, 9′, 10′
9′	4.10 (dd, 9.8, 2.4)	72.5	7′, 8′, 11′	5.47 (dd, 8.6, 2.4)	69.5	7′, 8′, 11′, 15′
10′	3.50 (dd, 6.6, 2.4)	47.9	12′, 14′	4.74 (m)	51.3	11′, 12′, 14′
11′	2.62 (d, 18.3)	30.3	9′, 12′	3.57 (dd, 16.1, 8.0)	29.4	9′, 12′
	2.45 (dd, 18.3, 6.6)		9′, 10′, 12′	3.44 (dd, 16.1, 5.4)		9′, 10′, 12′
12′		171.2			174.8	
13′	1.33 (s)	21.3	14′, 15′	1.43 (d, 6.8)	14.5	14′, 15′
14′		69.7		3.72 (q, 6.8)	52.2	13′, 15′
15′		98.0			95.7	
16′	1.31 (s)	23.8	14′, 15′	1.45 (s)	25.0	14′, 15′
8-OH	10.69 (s)		7, 8	ND[d]		
6′-NH	6.98 (d, 10.0)		7′	9.15 (d, 9.0)		4′, 5′, 7′
8′-OH	3.80 (br s)			ND		
12′-NH	6.46 (br s)		11′, 12′	9.19 (br s)[e]		12′
				8.43 (br s)[e]		11′, 12′
15′-OH	3.80 (br s)		14′, 15′, 16′	ND		

[a] Recorded at 400 MHz.
[b] Recorded at 100 MHz.
[c] Recorded at 600 MHz.
[d] ND=not detected.
[e] 12′-NH_2.

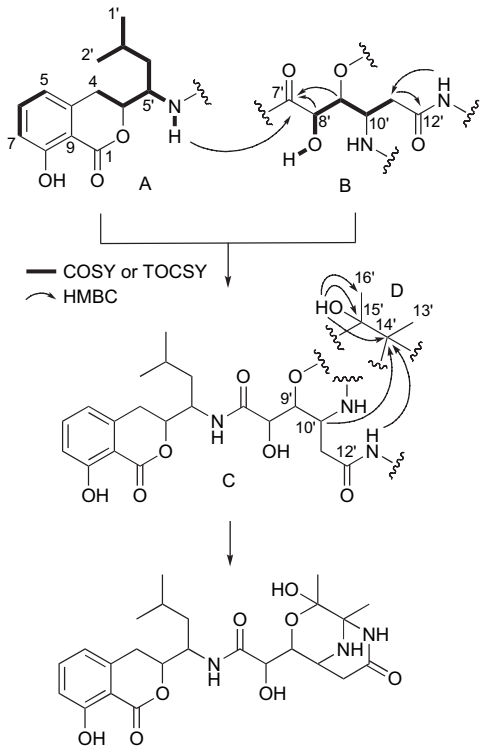

Figure 2. Partial structures and COSY, TOCSY, and key HMBC correlations of **1**.

connected on the basis of an HMBC correlation from 6′-NH to C-7′ and a NOESY correlation between 6′-NH and H-8′, providing substructure **C**.

Substructure **D** consisted the remaining four carbons including two singlet methyls and two quaternary sp^3 carbons. Connectivities of these carbons were established by the HMBC correlations from the two methyls (H-13′ and H-16′) to C-14′ and C-15′. Attachment of a hydroxyl group to C-15′ was indicated by the HMBC correlations from 15′-OH to C-14′, C-15′, and C-16′. Substructures **C** and **D** were linked through two nitrogen atoms at C-14′ on the basis of HMBC correlations from H-10′ to C-14′ and from 12′-NH to C-14′. Finally, an ether linkage between C-9′ and C-15′ that was consistent with the deshielded character of the hemiacetal carbon C-15′ (δ_C 98.0) completed the planar structure of **1**.

The relative configuration of the bicyclic ring system was elucidated by analysis of ^1H–^1H coupling constants and interpretation of the NOESY spectrum (Fig. 3). A large diaxial coupling constant (9.8 Hz) between H-8′ and H-9′ and a small coupling constant (2.4 Hz) between H-9′ and H-10′ indicated the axial and equatorial orientations for H-9′ and H-10′, respectively. The axial orientation of C-11′ methylene was supported by an NOESY correlation between H-8′ and H-11′ (δ_H 2.45). The H_3-16′ methyl was assigned to be placed in the equatorial orientation since NOE was not observed between the methyl and H-9′. On the basis of biogenesis consideration, it seemed reasonable that **1** was derived from amicoumacin A (**3**) and a biacetyl equivalent C_4 unit along with formation of two water molecules. Upon treatment in trifluoroacetic acid (60 °C, 3 h), **1** underwent a transformation to amicoumacins B (**4**) and C (**5**) (Fig. 4). The identity of amicoumacins was confirmed by HPLC-DAD-ESIMS comparisons with the compounds isolated from the

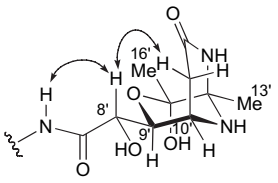

Figure 3. Relative configuration and key NOESY correlations of **1**.

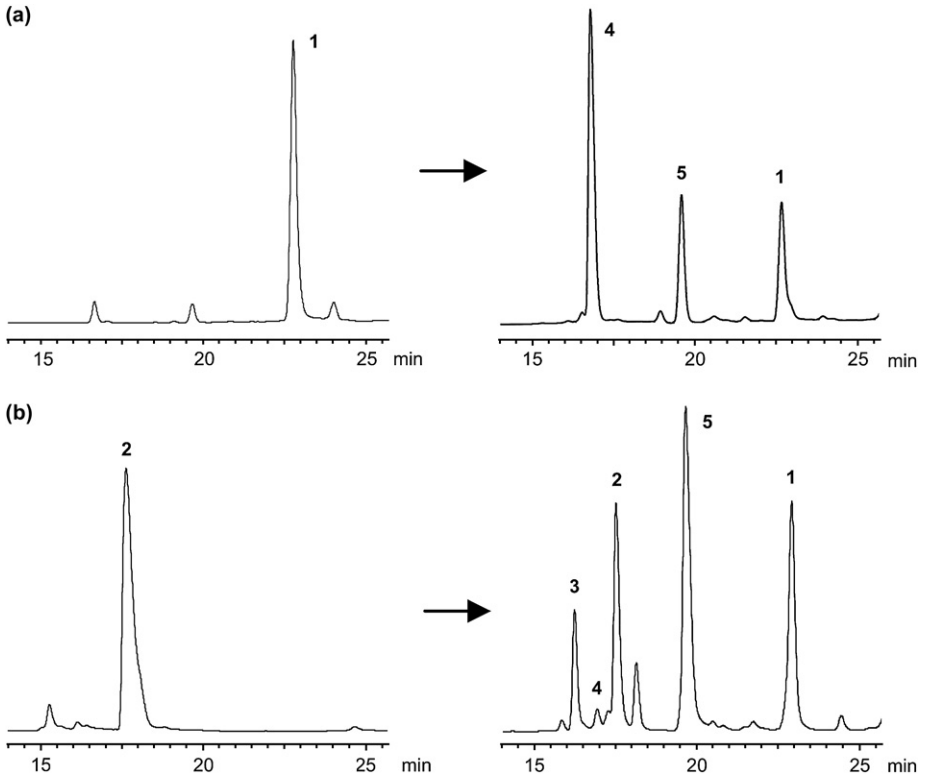

Figure 4. HPLC analysis of transformation of bacilosarcins to amicoumacins. (a) Treatment of **1** with TFA at 60 °C for 3 h. (b) Treatment of **2** with TFA at rt for 0.5 h. Compound **1**: bacilosarcin A, compound **2**: bacilosarcin B, compound **3**: amicoumacin A, compound **4**: amicoumacin B, compound **5**: amicoumacin C.

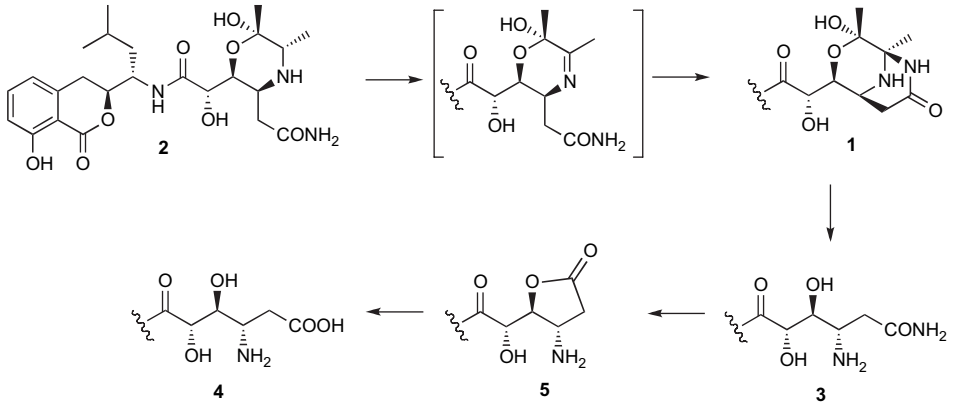

Figure 5. Chemical correlations of bacilosarcins (**1** and **2**) and amicoumacins (**3**–**5**).

fermentation mixture. To further confirm the absolute configuration, CD spectra of **4** derived from **1** and the authentic **4** were compared. The CD spectrum of **4** obtained from **1** by acid-catalyzed degradation showed positive Cotton effects at 221 nm ($\Delta\varepsilon$ +0.437) and 240 nm ($\Delta\varepsilon$ +1.643) and negative Cotton effects at 259 nm ($\Delta\varepsilon$ −2.183) and 315 nm ($\Delta\varepsilon$ −0.555), that was in good agreement with those for the authentic **4** ($\Delta\varepsilon_{219}$ +1.112, $\Delta\varepsilon_{240}$ +1.779 $\Delta\varepsilon_{260}$ −2.311, $\Delta\varepsilon_{314}$ −0.809). The absolute configuration of **1** was thus determined as illustrated in Figure 1. A plausible mechanism for the conversion is presented in Figure 5. After hydrolysis of **1** into biacetyl and **3**, the latter compound was transformed into **5** by acid-catalyzed lactonization, followed by hydrolysis of lactone leading to **4**.

Compound **2** was isolated as a colorless powder. FT-ICRMS analysis of **2** showed a molecular formula of $C_{24}H_{35}N_3O_8$ (obsd $[M+H]^+$ at m/z 494.24966; calcd $[M+H]^+$ 494.24969), which differed from **1** by an extra molecule of hydrogen. Because **2** was unstable in $CDCl_3$, NMR measurements were performed in pyridine-d_5. The NMR data of **2** were similar to those of **1**, with differences attributable to a reductive cleavage of the C–N bond between C-14′ and N-12′. This was revealed by the absence of the singlet methyl signal for H-13′ and the appearance of signals for a doublet methyl (H-13′, δ_H 1.43) and a quartet methine (H-14′, δ_H 3.72), and was supported by the COSY correlation between these protons and the HMBC correlations from H-9′ to C-15′, H-10′ to C-14′, H-13′ to C-14′ and C-15′, H-16′ to C14′ and C-15′. The relative and absolute configurations of **2** were determined by an NOESY experiment and chemical correlation with **1** and amicoumacins. The coupling values for $J_{H\text{-}8',H\text{-}9'}$ of 8.6 Hz and $J_{H\text{-}9',H\text{-}10'}$ of 2.4 Hz indicated that the morpholine ring had a chair conformation, which was supported by a NOESY correlation between H-11′ and the axial H-14′ (Fig. 6). Treatment of **2** in trifluoroacetic acid (rt, 0.5 h) gave a mixture of **1**, **3**, **4**, and **5**. The CD spectrum of **4** derived from **2** displayed the Cotton effect pattern ($\Delta\varepsilon_{219}$ +1.384, $\Delta\varepsilon_{241}$ +1.324 $\Delta\varepsilon_{258}$ −2.675, $\Delta\varepsilon_{313}$ −0.708) similar to that for the authentic **4**, thereby confirming the absolute configuration of **2**. Oxidative formation of the imine intermediate is plausibly involved in the conversion of **2** to **1**.

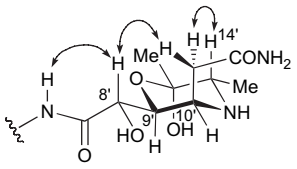

Figure 6. Relative configuration and key NOESY correlations of **2**.

Bacilosarcin A (**1**) showed 82% inhibition at 50 μM against growth of barnyard millet sprouts while bacilosarcin B (**2**) showed very weak activity at the same concentration (Table 2). Of particular interest, amicoumacin A (**3**) showed more potent activity than **1**. This may imply that **1** behaves as a prodrug of **3** in plant cells. The activity levels shown by **1** and **3** are higher than that of herbimycin A, a potent herbicidal compound from *Streptomyces*,[11] suggesting that they may be lead molecules for plant growth regulators.

Table 2
Plant growth inhibition activity of bacilosarcins A (**1**) and B (**2**) and related compounds[a]

Compound	Concentration (μM)		
	2	10	50
Bacilosarcin A (**1**)	17	40	82
Bacilosarcin B (**2**)	NA	NA	7
Amicoumacin A	35	95	98
Amicoumacin B	NA	14	13
Herbimycin A	NA	NA	58

[a] Growth inhibition of barnyard millet is indicated in % of control. NA=not active.

3. Conclusions

Two new isocoumarins, bacilosarcins A (**1**) and B (**2**) were isolated from the culture extract of *B. subtilis*. The structures of bacilosarcins are unique for their nitrogen-containing heterocyclic cores. The 3-oxa-6,9-diazabicyclo[3.3.1]nonane ring system in **1** is unprecedented both in natural and synthetic compounds. The 2-hydroxymorpholine substructure in **2** is rare in natural products although it is more popular in synthetic community.[12] There have been a few compounds containing 2-hydroxymorpholine such as convolutamine E[13] and akashin C[14] reported to date.

4. Experimental

4.1. General

Optical rotations were measured using a JASCO DIP-3000 polarimeter. CD spectra were measured in MeOH on a JASCO J-720 spectropolarimeter. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. IR spectra were recorded on a Perkin Elmer Spectrum 100 spectrophotometer. NMR spectra were obtained on a JEOL JNM-LA400 or a Bruker AVANCE 600 spectrometer. The ^1H and ^{13}C chemical shifts were referenced to the solvent signals (δ_H=7.26 and δ_C=77.0 in $CDCl_3$ or δ_H=7.19 and δ_C=123.5 in pyridine-d_5). FT-ICRMS was measured on a Bruker Daltonics Apex-Q94e spectrometer. HR-ESITOFMS were recorded on a Bruker microTOF focus spectrometer. HR-EIMS was recorded on a JEOL GC-mate2 spectrometer. LC–ESIMS spectra were measured on an Agilent 1100 MSD spectrometer with a diode-array detector. HPLC analyses were carried out on an Agilent HP1100 system using a Cosmosil 5C18-AR-

II column (Nacalai Tesque Inc., 4.6×250 mm). Silica Gel 60-C18 (Nakalai Tesque 250–350 mesh) was used for ODS column chromatography. HPLC separation was performed using a Cosmosil 5C18-AR-II column (Nacalai Tesque Inc., 20×250 mm).

4.2. Microorganism

B. subtilis TP-B0611 was isolated from the intestine of a sardine (*S. melanosticta*) collected in Toyama Bay, Japan. The intestine content was streaked on the isolation medium consisting of glucose 0.5%, soluble starch 0.5%, meat extract 0.1%, yeast extract (Difco laboratories) 0.1%, NZ-case (Humco Scheffield Chemical Co.) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1%, agar 1.5%, 0.005% amphotericin B and 0.02% benomyl. After cultivation at 32 °C for 1 week, the strain was isolated and preserved. The isolated strain was identified as *B. subtilis* on the basis of 16S rRNA analysis, which indicated 99.87% identity to *B. subtilis* subsp. *subtilis*. The strain was kept at −78 °C as a stock culture in 20% glycerol.

4.3. Fermentation

Strain TP-B0611 grown on a slant culture was inoculated into 500-mL K-1 flasks each containing 100 mL of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, triptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, and $CaCO_3$ 0.3% (pH 7.0). The flasks were cultivated on a rotary shaker (200 rpm) at 30 °C for 3 days. Seed culture (3 mL) was transferred into 500-mL K-1 flasks each containing 100 mL of the production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein) 1.5%, yeast extract 0.3%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were cultured on a rotary shaker (200 rpm) at 30 °C for 5 days.

4.4. Extraction and isolation

A 5-day old fermentation broth of strain TP-B0611 (100 mL×50 flasks) was extracted with 1-butanol (50 mL per flask) on a rotary shaker (200 rpm) for additional 1 h. The mixture was centrifuged at 6000 rpm for 10 min and the organic layer was separated from the aqueous layer. Evaporation of the solvent provided approximately 4.3 g of extract from 5 L of culture. The crude extract was defatted by partitioning with MeOH and *n*-hexane several times and the MeOH layer was concentrated in vacuo to give 3.8 g of brown viscous oil. The oily material was repeatedly subjected to reversed-phase ODS column chromatography with a step gradient of MeCN–0.15% KH_2PO_4 solution (pH 3.5) (20:80, 25:75, 30:70, 35:65, 40:60, 45:55 and 50:50 v/v). Fractions 2–4 showed the potent seed germination inhibitory activity and by HPLC contained bacilosarcins and amicoumacins. Evaporation of combined fractions 2–4, followed by extraction with 1-butanol, provided 307 mg of brown powders. Final purification was achieved by preparative reversed-phase HPLC with a linear gradient of MeCN–0.15% KH_2PO_4 (pH 3.5) (50:50–75:25 over 30 min) to give **1** (9.3 mg) and **2** (6.3 mg), along with amicoumacins A (**3**, 10.1 mg), B (**4**, 1.5 mg), and C (**5**, 4.8 mg).

*4.4.1. Bacilosarcin A (**1**)*

Colorless powder; $[\alpha]_D^{25}$ −76.9 (*c* 0.48, $CHCl_3$); IR (film) 3265, 1655 cm^{-1}; UV (MeOH) λ_{max} (log ε) 206 (4.61), 247 (3.90), 314 (3.76); ^1H and ^{13}C NMR (Table 1); HR-EIMS molecular ion calcd for $C_{24}H_{33}N_3O_8$ *m*/*z* 491.2267 $[M]^+$, found 491.2272 $[M]^+$.

*4.4.2. Bacilosarcin B (**2**)*

Colorless powder; $[\alpha]_D^{25}$ −22.9 (*c* 0.78, MeOH); IR (film) 3188, 1653 cm^{-1}; UV (MeOH) λ_{max} (log ε) 206 (4.07), 247 (3.34), 315 (3.23); ^1H and ^{13}C NMR (Table 1); FT-ICRMS molecular ion calcd for $C_{24}H_{36}N_3O_8$ *m*/*z* 494.24969 $[M+H]^+$, found 494.24966 $[M+H]^+$.

4.5. Acid-catalyzed degradation of bacilosarcins A (1) and B (2)

A solution of **1** (50 μg) in trifluoroacetic acid (400 μL) was heated at 60 °C for 3 h. The solution was concentrated in vacuo, diluted with MeOH, and subjected to the HPLC analysis [Cosmosil 5C18-AR-II (4.6×250 mm), a linear gradient of 25–75% MeCN in 0.15% KH_2PO_4 solution (pH 3.5) over 32 min, 0.7 mL/min, 254 nm detection]. The retention times of **1**, **2**, amicoumacins A (**3**), B (**4**), and C (**5**) were 20.6, 15.5, 15.2, 15.4, 15.9 min, respectively. Additionally, LC–MS analysis was conducted for the identification of the degradation products [Cadenza CD-C18 (4.6×75 mm, Imtact), a linear gradient of 2.5–97.5% MeCN in 0.1% HCOOH over 30 min, 0.8 mL/min]. Compound **2** (50 μg) was treated with trifluoroacetic acid (20 μL) in MeOH (400 μL) at room temperature for 0.5 h. The reaction mixture was analyzed in the same manner as described for **1**.

4.6. Determination of absolute configuration of bacilosarcins A (1) and B (2)

As described above, bacilosarcin A (**1**, 1 mg) was treated in trifluoroacetic acid to yield the mixture of amicoumacins. The mixture was purified by preparative C-18 reversed-phase HPLC [Waters XTerra RP18 (4.6×250 mm), a linear gradient of 25–50% MeCN in 0.15% KH_2PO_4 solution (pH 3.5) over 30 min, 0.7 mL/min, 254 nm detection]. The fraction containing amicoumacin B (**4**) was lyophilized and the remaining solid was extracted with MeOH to give **4** (116 μg). Similarly, degradation of bacilosarcin B (**2**, 1 mg) and HPLC purification yielded **4** (13 μg). The yields were calculated on the basis of the optical density at 315 nm. The CD spectra of **4** derived from **1** and **2** were similar to that of **4** isolated from the fermentation mixture.

*4.6.1. Compound **4** isolated from the fermentation mixture*

$[\alpha]_D^{23}$ −80.8 (*c* 0.36, MeOH) {lit.[6] $[\alpha]_D^{22}$ −78.2 (*c* 0.08, MeOH)}. The ^1H and ^{13}C NMR data were identical to those reported in the literature.[15] CD (MeOH) $\Delta\varepsilon_{219}$ +1.112, $\Delta\varepsilon_{240}$ +1.779 $\Delta\varepsilon_{260}$ −2.311, $\Delta\varepsilon_{314}$ −0.809; HR-ESITOFMS molecular ion calcd for $C_{20}H_{27}N_2O_8$ *m*/*z* 423.1773 $[M-H]^-$, found 423.1780 $[M-H]^-$.

*4.6.2. Compound **4** obtained from bacilosarcin A (**1**)*

CD (MeOH) $\Delta\varepsilon_{221}$ +0.437, $\Delta\varepsilon_{240}$ +1.643, $\Delta\varepsilon_{259}$ −2.183, $\Delta\varepsilon_{315}$ −0.555; HR-ESITOFMS molecular ion calcd for $C_{20}H_{27}N_2O_8$ *m*/*z* 423.1773 $[M-H]^-$, found 423.1765 $[M-H]^-$.

*4.6.3. Compound **4** obtained from bacilosarcin A (**2**)*

CD (MeOH) $\Delta\varepsilon_{219}$ +1.384, $\Delta\varepsilon_{241}$ +1.324, $\Delta\varepsilon_{258}$ −2.675, $\Delta\varepsilon_{313}$ −0.708; HR-ESITOFMS molecular ion calcd for $C_{20}H_{27}N_2O_8$ *m*/*z* 423.1773 $[M-H]^-$, found 423.1772 $[M-H]^-$.

4.7. Bioassay

Barnyard millet seeds (*Echinochloa frumentacea* L.) were incubated for 18 h on a wet paper towel at 25 °C in the dark. Germinated seeds were selected and incubated in a solution (2 mL) of test compounds in 0.5% aqueous MeOH containing 0.1% Tween 20 in glass containers (50 mm cube) for 5 days at 25 °C in the dark. Each experiment was carried out triplicate. The sprouts were measured and the inhibition was indicated by percent (%) to the control (0.5% aqueous MeOH with 0.1% Tween 20). The data were statistically analyzed.

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