دانشگاه علوم پزشکی و خدمات بهداشتی،درمانی کرمانشاه
Kermanshah University Of Medical Sciences

SCIENTIFIC REPORTS

Streptomyces sp. VN1, a producer of diverse metabolites including non-natural furan-type anticancer compound

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12:30 p.m.
دو شنبه، ۱۱ بهمن ۱۴۰۰ ، ساعت ۱۲:۳۰

SCIENTIFIC REPORTS
nature research

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Streptomyces sp. VN1, a producer of diverse metabolites including non-natural furan-type anticancer compound

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Introduction

Natural products (NPs) have been starting points of drug discovery for several decades. Major antimicrobials and chemotherapeutics entering clinical trials are often based on NPs. Drugs with a natural origin can be produced as primary or secondary metabolites from versatile living organisms. Different microorganisms such as *Streptomyces*, myxobacteria and uncultured bacteria are major sources of such beneficial NPs.

Streptomyces

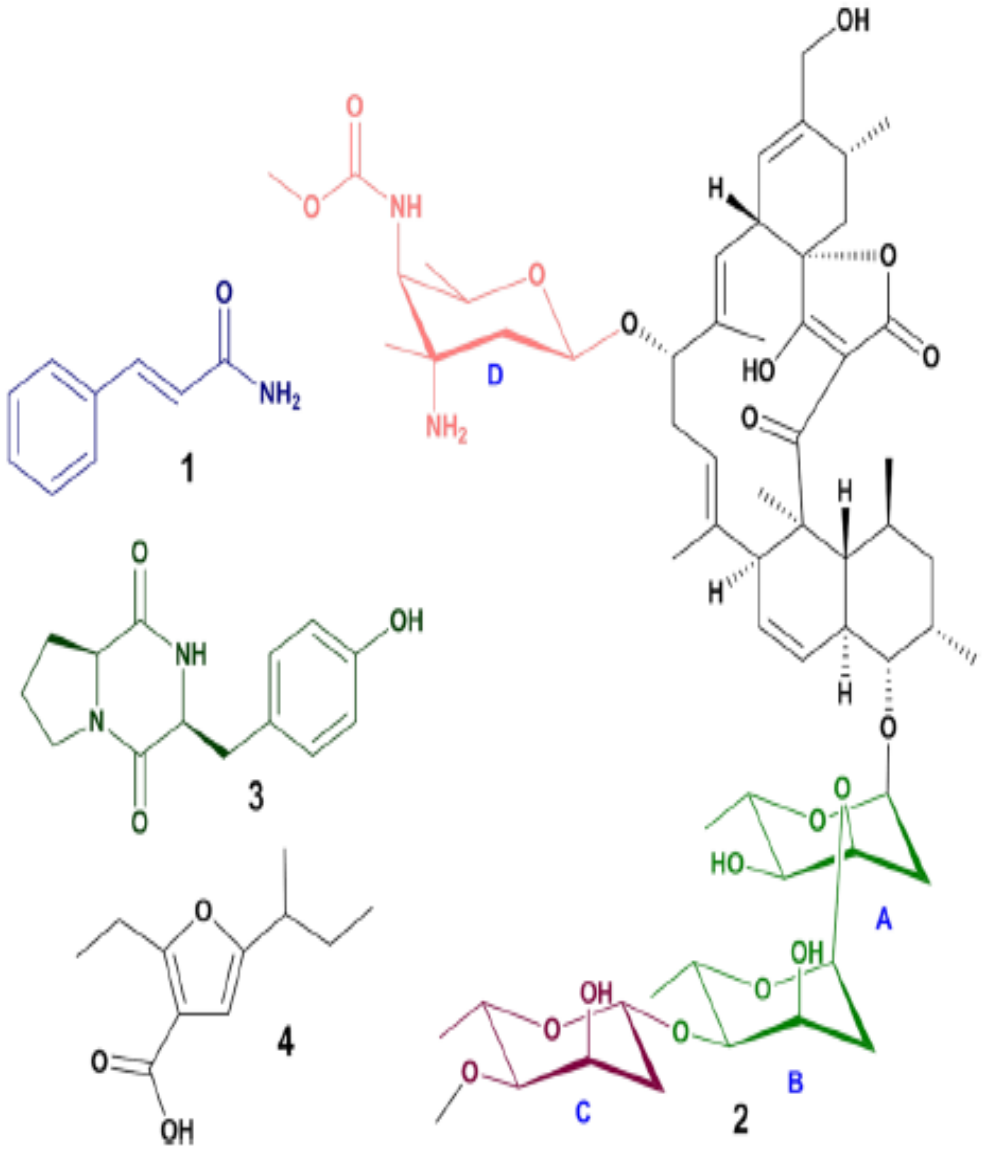
- *Streptomyces* are Gram-positive, aerobic bacteria in the order of *Actinomycetales* within the class of *Actinobacteria*.
- Previous studies have shown that more than 74% of current antibiotics are derived from the genus *Streptomyces*.
- Multiple approaches such as ribosome engineering and genome mining have been used to find new secondary metabolites in old *Streptomyces* strains.
- Besides the effort to work on old strains to explore novel biosynthetic gene clusters (BGCs), isolating new bioactive strains from the environment is also a great way to find novel BGCs.

Marine Products

- Recently, marine bacteria have been known as sources for many novel compounds.
- In this study, we characterized a *Streptomyces* sp. VN1 isolated from the coastal region of Phu Yen Province, Da Nang, central Viet Nam.
- Based on phylogenetic, chemotaxonomic, and morphological characteristics, this strain belongs to genus *Streptomyces*.
- After a large-scale fermentation and bioassay-guided isolation, cinnamamide (**1**) lobophorin A (**2**) diketopiperazines cyclo-L-proline-L-tyrosine (**3**) and a furan-type compound (**4**) were characterized from fermentation broth of *Streptomyces* sp. VN1.

Structures of compounds 1, 2, 3, and 4. Letters “A-D” indicate four sugar units in lobophorin A.

Interestingly, the furan-type compound (**4**) exhibited anticancer activity against five types of tumor cell lines. Compound **4** effectively suppressed both migration and invasion of AGS cells. The ability of the strain to produce cinnamamide, antibacterial compounds **2** and **3,** anticancer compound **4**, and diverse secondary metabolites make *Streptomyces* sp. VN1 an attractive target for bioactive compound screening.



Materials and Methods

4

Phylogenetic analysis:

- 16S rRNA sequencing

3

Chemotaxonomic analyses:

- Sherlock Microbial Identification software package

2

Morphological and phenotypic analyses:

- FE-SEM JEOL JSM-6700

1

Collection and isolation of strain

8

Cytotoxicity assay:

- MTT

7

Isolation of compounds from *Streptomyces* sp. VN1 and elucidation of their structures:

- HPLC
- NMR
- Infrared Spectroscopy

6

Mass spectrometric analysis of crude extraction sample:

- UPLC-ESI-Q-TOF-HRMS

5

Genome sequencing, assembly, and annotation:

- PacBio
- EggNOG
- antiSMASH

11

Statistical analysis:

- Student's t-test

10

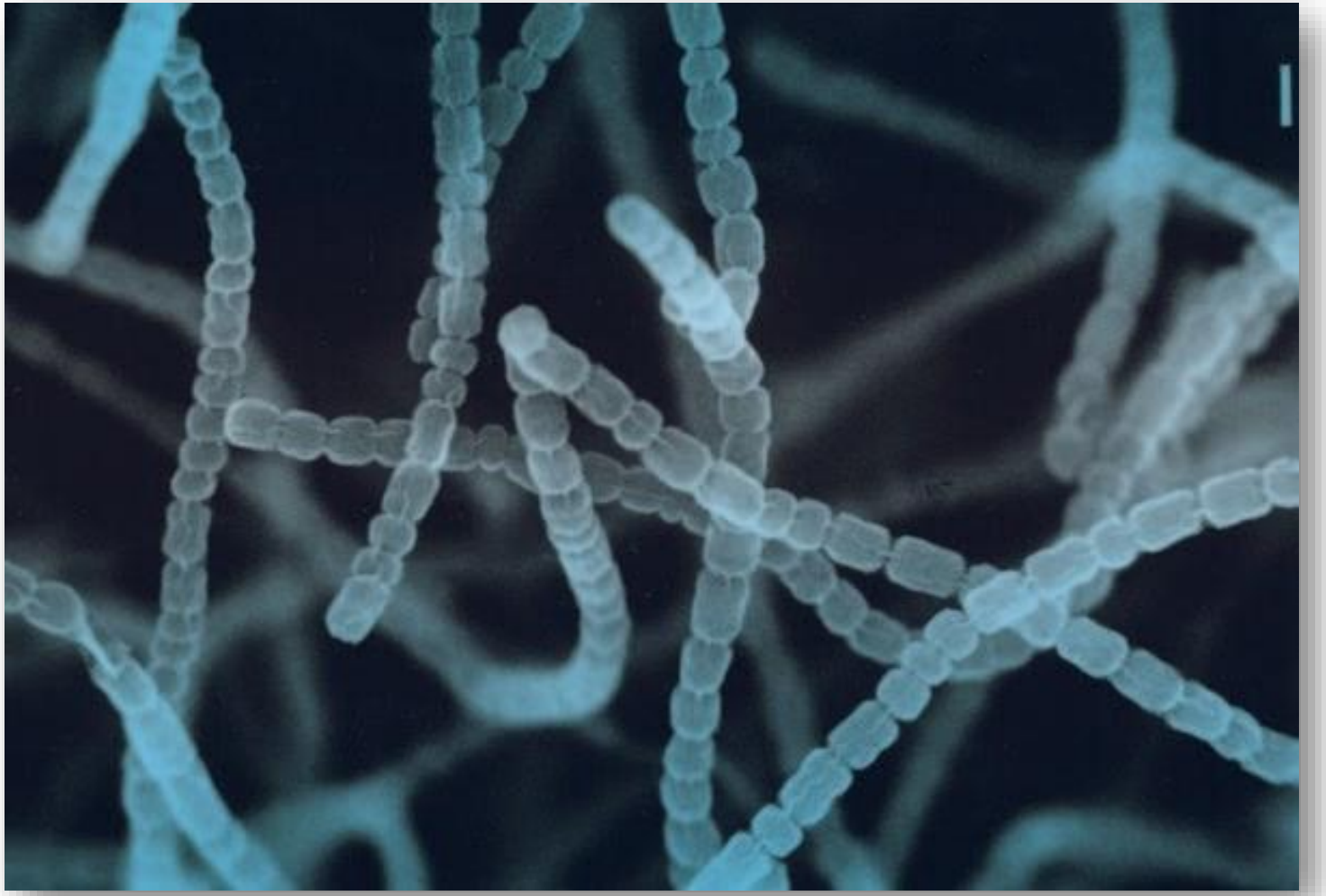
Cell invasion assay:

- Transwell chamber
- hematoxylin /eosin

9

Wound-healing assay

Results

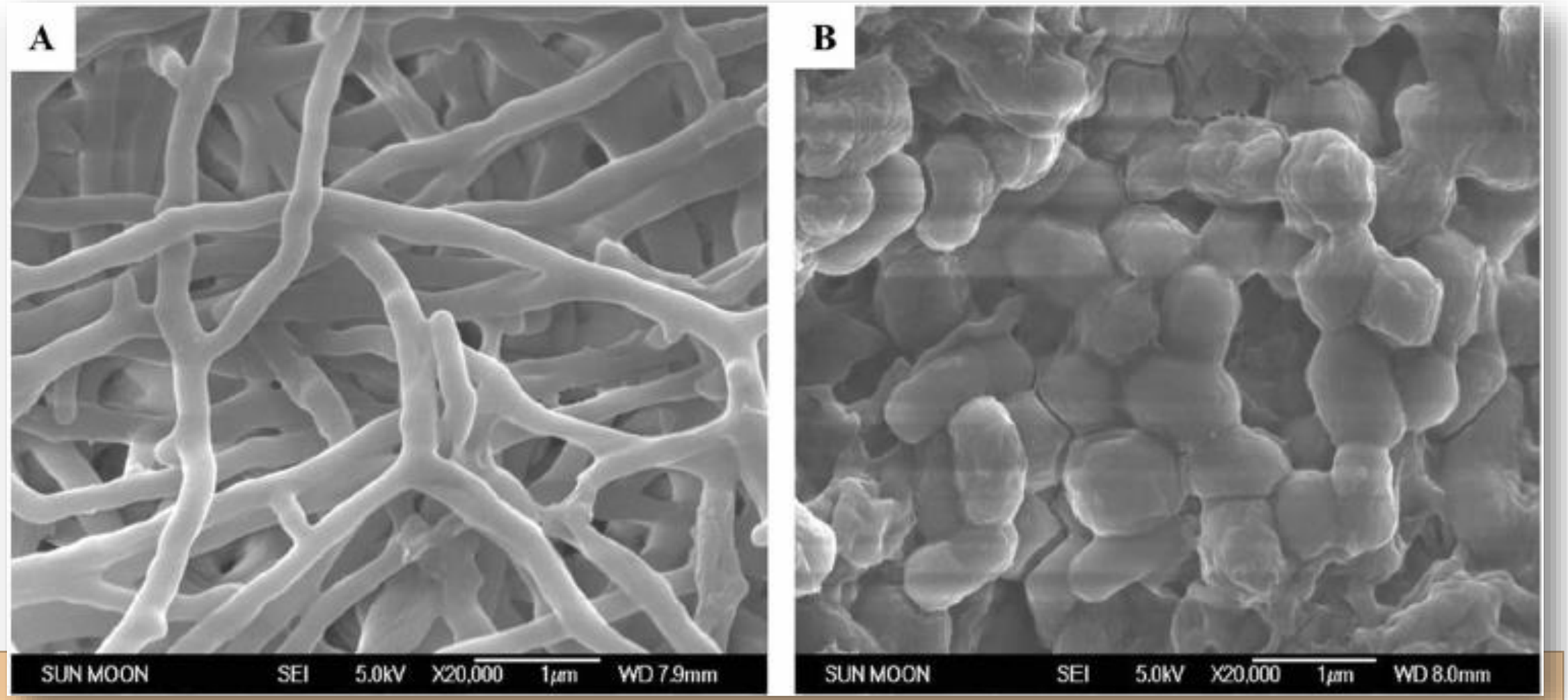


Morphological and phenotypic characteristics

- Scanning electron microscope observations revealed that the substrate mycelium of *Streptomyces* sp. VN1 was rectiflexible. When the culture of *Streptomyces* sp. VN1 reached maturity and adequate aerial mycelium was produced, aerial hyphae differentiated into short, straight to flexuous chains with smooth surfaces. Its spores were found to be regular and smooth.

- *Streptomyces* sp. VN1 displayed survival on sole carbon sources of **L-arabinose, fructose, mannitol, sucrose, xylose, lactose, and starch**. Starch and yeast extract were preferred carbon source and nitrogen source, respectively.

- *Streptomyces* sp. VN1 produced brownish to grayish mycelium with good sporulation on the media (i.e., Marine broth-malt extract medium) used for metabolite isolation. It showed growth at temperatures of 24 °C to 40 °C with pH between 4 and 9. Various salts had different effects on the growth of *Streptomyces* sp. VN1.



Scanning electron microscopic image showing the morphology of *Streptomyces* sp. VN1. (**A**) Rectiflexible mycelium; bar, 1 μm. (**B**) Smooth and oval-shaped spores; bar, 1 μm.

Chemotaxonomic characteristics

- Predominant fatty acids in *Streptomyces* sp. VN1 were C16:0 iso (23%), C15:0 antesio (19%), C15:0 iso (10%), and C16:0 (11%).
- Fatty acids in genus *Streptomyces* are known to contain straight chains as well as iso- and anteiso-branched chains.
- Cell-wall peptidoglycans of *Streptomyces* sp. VN1 contained L-diaminopimelic acid, typical of cell-wall type I. Galactose, arabinose, and xylose were detected as major carbohydrates in its whole-cell hydrolysate.

Phylogenetic analysis and genome annotation

- Whole genome sequencing of *Streptomyces* sp. VN1 produced a total of 179,193 sequence reads, yielding a total consensus of 8,341,703 bp with GC content of 72.5%.
- Predicted proteins were annotated by blasting the eggNOG database. In eggNOG functional classification, 6,987 (97.36%) of 7,176 proteins were assigned. The following four top categories were classified: transcription, carbohydrate metabolism, amino-acid metabolism, and energy production.
- Genome analysis with autoMLST showed that this new isolate had the highest sequence similarities with *Streptomyces* sp. FXJ7.023 (GCF_000404005), *Streptomyces pactum* (GCF_001767375) and *Streptomyces olivaceus* (GCF_000721235).

Characteristics	*Streptomyces* sp. VN1
Spore morphology	Short, flexuous chains
Spore surface	Smooth
Production of diffusible pigments	–
Growth at 24 °C	+
Growth at pH 5	+
Growth at pH 11	–
Growth on carbon sources	
L-Arabinose	+
Fructose	+
Mannitol	+
Sucrose	+
Xylose	+
Lactose	+
Starch	+
Growth on nitrogen sources	
Cystine	+
L-Proline	+
Glycine	+
L-Asparagine	+

Morphological and physiological characteristics and carbon usage of *Streptomyces* sp. VN1.

Sample	*Streptomyces* sp. VN1
Length (bp)	8,341,703
No. of reads	179,193
Coding density (%)	87.57
Average CDS length (bp)	1,018
No. of protein-coding genes	7,716
No. of tRNA genes	86
No. of rRNA	18
GC content	72.5

General characteristics of the genome of isolated *Streptomyces* sp. VN1.

In silico analysis of secondary metabolite biosynthesis pathways

Thirty-four secondary metabolite biosynthetic gene clusters were identified in the *Streptomyces* sp. VN1, including:

- type I polyketide synthases (T1PKS),
- type II polyketide synthases (T2PKS),
- type III polyketide synthases (T3PKS),
- non-ribosomal peptide synthetases (NRPS),
- terpenes,

lassopeptides,

thiopeptides,

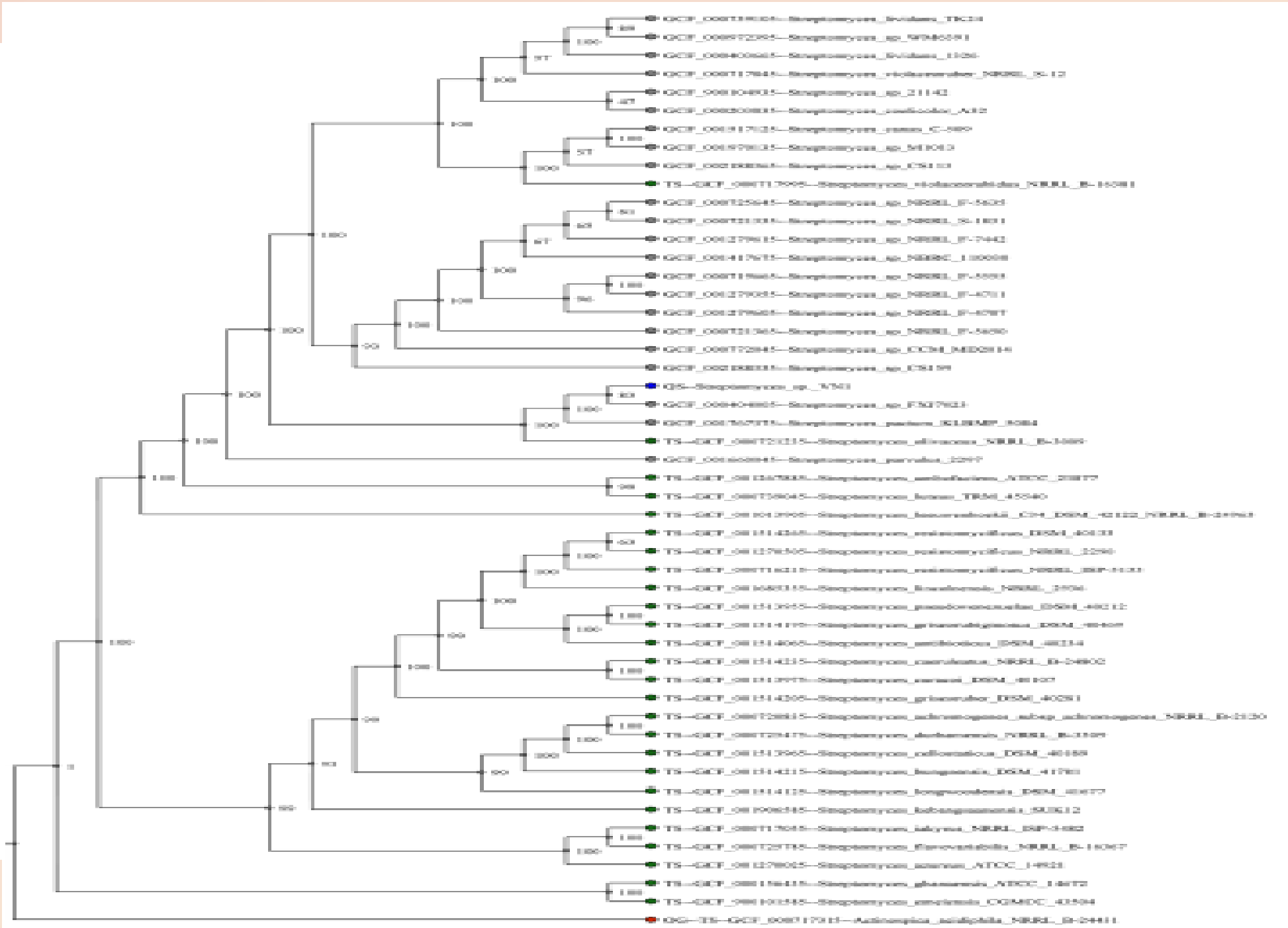
lanthipeptides,

indoles,

siderophores,

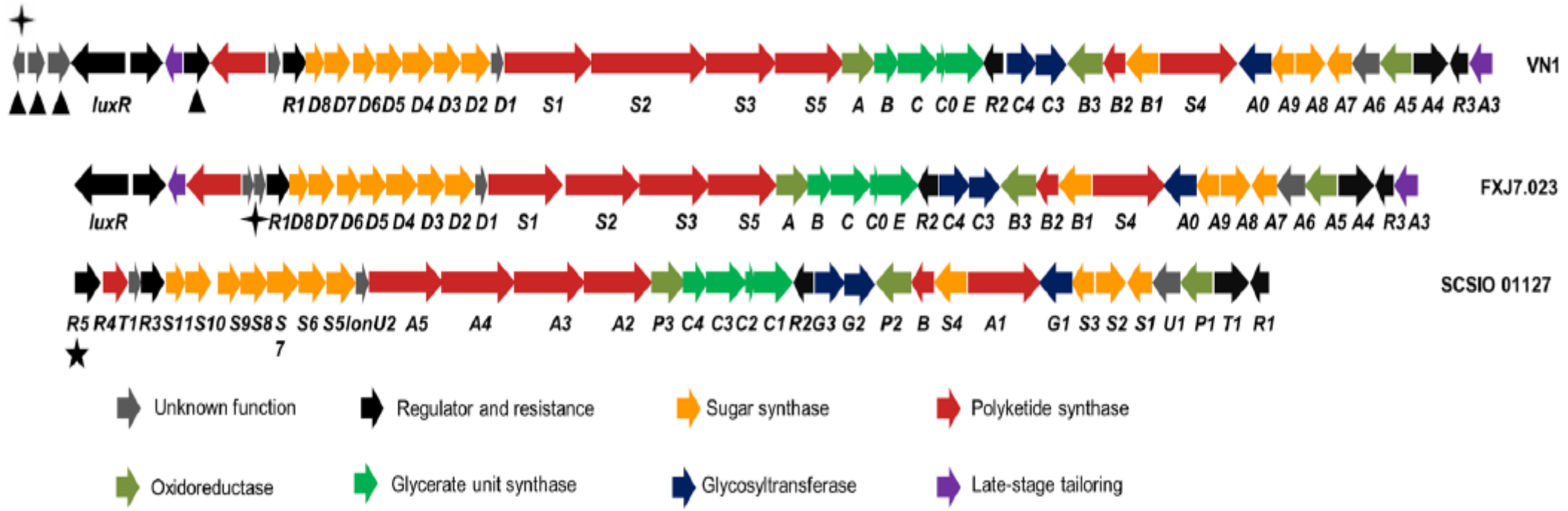
bacteriocins.

Molecular phylogenetic analysis of using default parameters (>100 core genes) by autoMLST. Bootstrap confidence levels are indicated at internodes whereas scale bar indicates nucleotide substitutions per nucleotide position.



No	Cluster	Type	From	To	Most similar known biosynthetic gene cluster (percent of similarity)	Reference strain	Accession number
1	1	Oligosaccharide-T1PKS-T3PKS-NRPS	10491	225803	Lobophorin A (96%)	*Streptomyces* sp. FXJ7.023	JX306680
2	2	Terpene	237497	258555	2-Methylisoborneol (100%)	*Streptomyces griseus*	AP009493
3	5	Terpene	539486	563714	Carotenoid (54%)	*Streptomyces avermitilis*	AB070934
4	9	Ectoine	1684875	1695273	Ectoine (100%)	*Streptomyces anulatus*	AY524544
5	10	Melanin	2650272	2660898	Melanin (100%)	*Streptomyces coelicolor* A3(2)	AL645882
6	11	Lassopeptide	2720839	2743360	SSV-2083 (50%)	*Streptomyces sviceus*	NZ_CM000951
7	12	Siderophore	2756614	2768407	Desferrioxamine_B (83%)	*Streptomyces coelicolor A3(2)*	AL645882
8	15	Lantipeptide	4240796	4272172	SBI-06990 alpha/SBI-06989 beta (50%)	*Streptomyces bingchenggensis*	CP002047
9	17	Terpene	5321623	5342708	Albaflavenone B (100%)	*Streptomyces coelicolor* A3(2)	AL645882
10	18	T2PKS	5408372	5450926	Spore_pigment (66%)	*Streptomyces avermitilis*	AB070937
11	20	T1PKS-NRPS	6048796	6212376	Friulimicin (75%)	*Actinoplanes friuliensis*	AJ488769
12	21	T1PKS-NRPS	6260144	6309491	Xiamycin (77%)	*Streptomyces* sp. SCSIO 02999	JQ812811
13	26	T2PKS	6650019	6692435	Enterocin (95%)	*Streptomyces maritimus*	AF254925
14	28	Terpene-NRPS	7137719	7221298	Hopene (92%)	*Streptomyces coelicolor* A3(2)	AL645882
15	29	T1PKS	7275849	7358369	Divergolide (100%)	*Streptomyces* sp. HKI0576	HF563079
16	30	Bacteriocin	7687326	7697541	Informatipeptin (42%)	*Streptomyces viridochromogenes* DSM 40736	GG657757
17	32	NRPS	7898917	7949836	Coelichelin (100%)	*Streptomyces coelicolor* A3(2)	AL645882

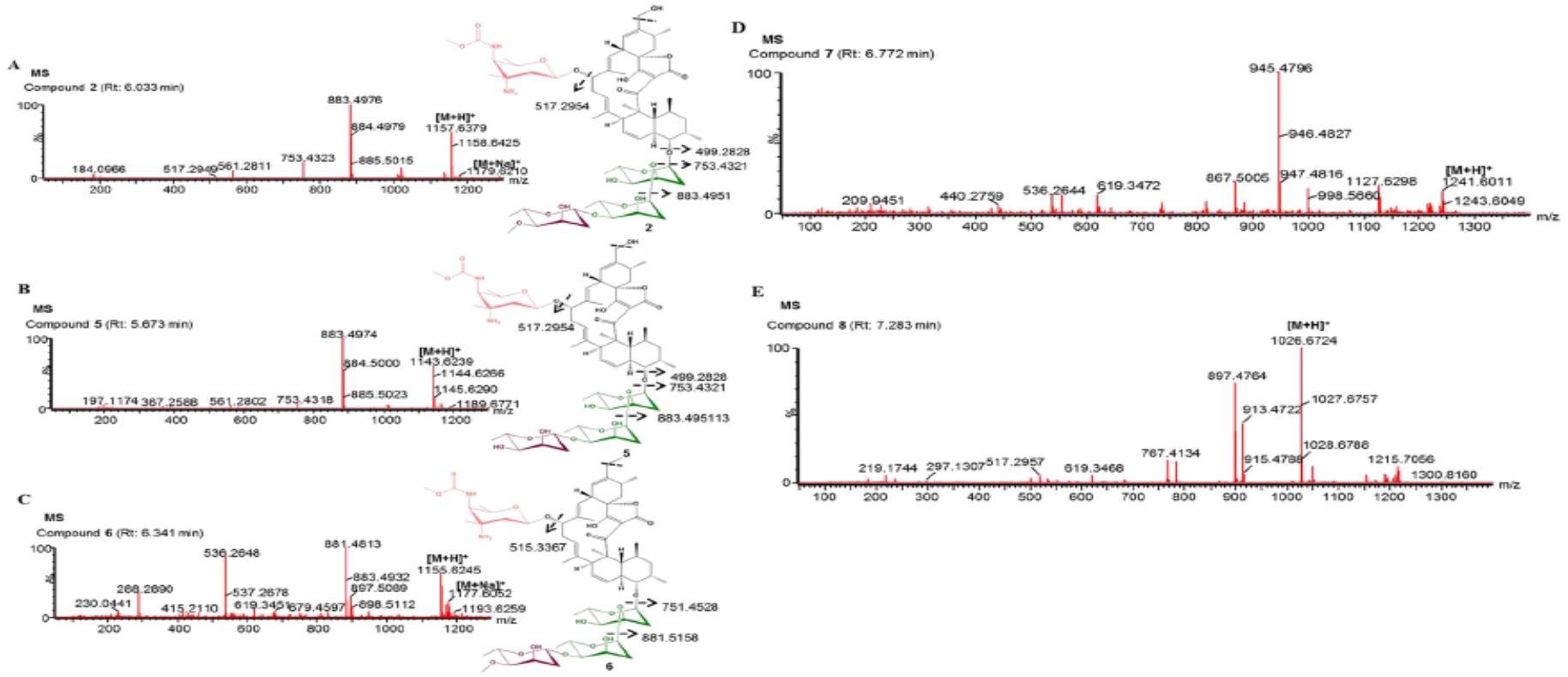
Overview of *Streptomyces* sp. VN1 genome analysis by antiSMASH of 17 secondary metabolites of biosynthetic gene clusters and seven PKS and NRPS gene clusters sharing similarity of more than 40%. The number of BGCs is determined with antiSMASH and ClusterFinder OFF.



Genetic organization of the lobophorin biosynthetic gene cluster of *Streptomyces* sp. VN1 (GenBank: SUB5241063), lobophorin gene cluster of *Streptomyces olivaceus* FXJ7.023 (GenBank: JX306680), and lobophorin gene cluster of *Streptomyces* sp. SCSIO 01127 (GenBank: KC013978). "▲"; " "; "★" denote unique genes in *Streptomyces* sp. VN1, *Streptomyces olivaceus* FXJ7.023, and *Streptomyces* sp. SCSIO 01127, respectively.

Identification of lobophorin A analogs by mass spectrometry.

Mass spectra data were obtained in positive mode. From HR MS/MS profiles, metabolic substances in crude extract samples were found to be correlated with the mass-to-charge ratio (*m/z*) of molecular ions. Mass profiles of secondary metabolites existing in isolated strains were compared with genome mining data. Herein, we identified lobophorin A and its analogs in the crude extract through this technique.

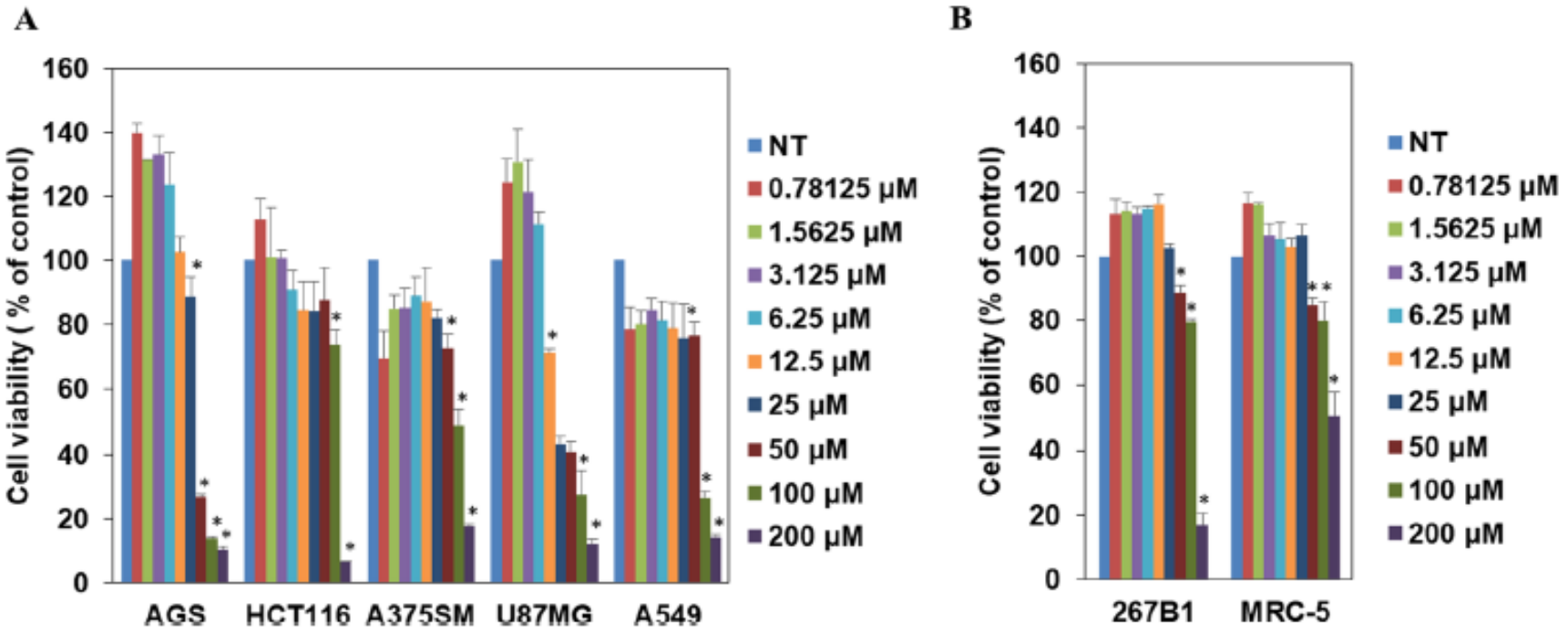


HR-MS and MS/MS analyses of lobophorin analogs. (**A**) The component of lobophorin A generates a [M + H]+ ion at *m/z* 1,157.6379. (**B**) The component of compound **5** (demethylation of lobophorin A) generates a [M + H]+ ion at *m/z* 1,143.6239. (**C**) The component of compound **6** (dehydroxylation of lobophorin A) generates a [M + H]+ ion at *m/z* 1,155.6245. (**D**) The component of compound **7** generates a [M + H]+ ion at *m/z* 1,2041.6011. (**E**) The component of compound **8** generates a [M + H]+ ion at *m/z* 1,026.6724.

Anticancer activity

- To assess whether compound **4** might have anticancer activity, we evaluated the inhibitory effect of compound **4** on the growth of different tumor cell lines. Compound **4** showed anti-proliferative activities against five types of cancer cell lines . This compound exhibited more sensitive growth-inhibitory activities for gastric adenocarcinoma (AGS), glioblastoma (U87MG), and lung cancer (A549) than for melanoma (A375SM) and colon cancer cells (HCT116).

- These data suggest that compound **4** possesses an anticancer activity by inhibiting the growth and metastasis abilities of cancer cells.



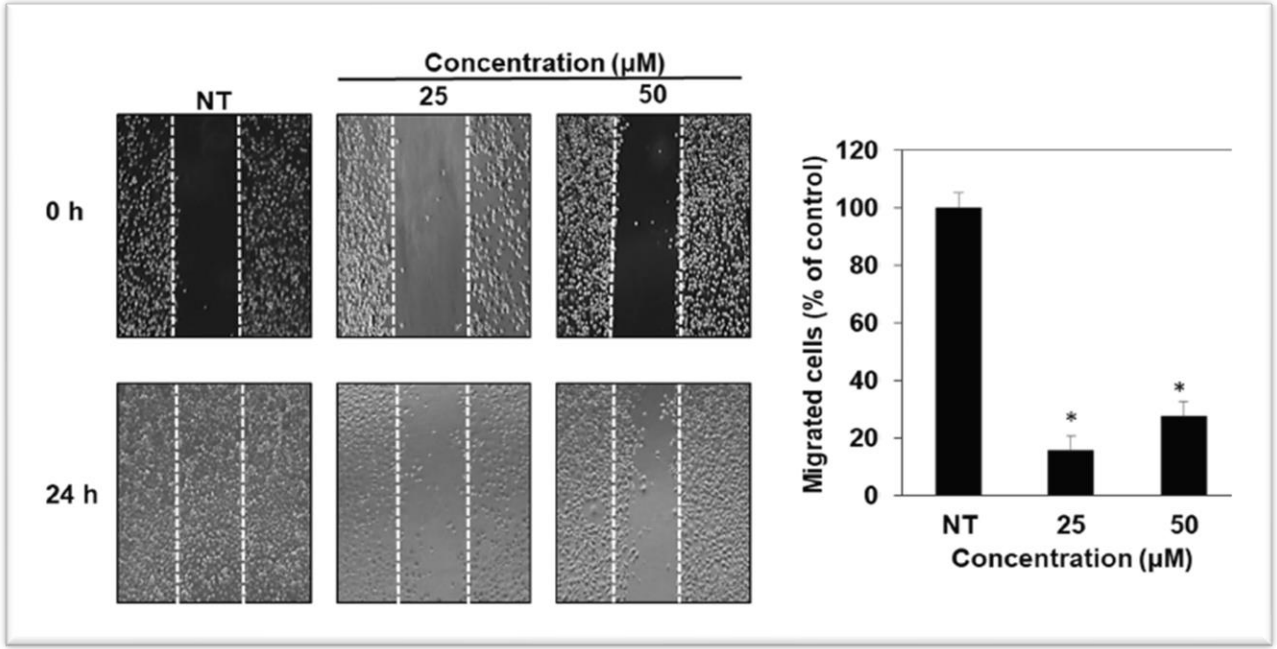
In vitro growth inhibitory activities of compound **4** against different cell lines. (**A**) cancer cell lines, (**B**) normal cell lines. NT: no treatment. *p < 0.05 vs. control.

Compound 4	AGS (Gastric cancer)	HCT116 (Colon cancer)	A375SM (Melanoma)	U87MG (Glioblastoma)	A549 (Lung cancer)	267B1 (prostate epithelial)	MRC-5 (lung fibroblast)
IC_{50} (µM)	40.5	123.7	84.67	50	58.64	157.2	>200

IC50 value of compound **4**.

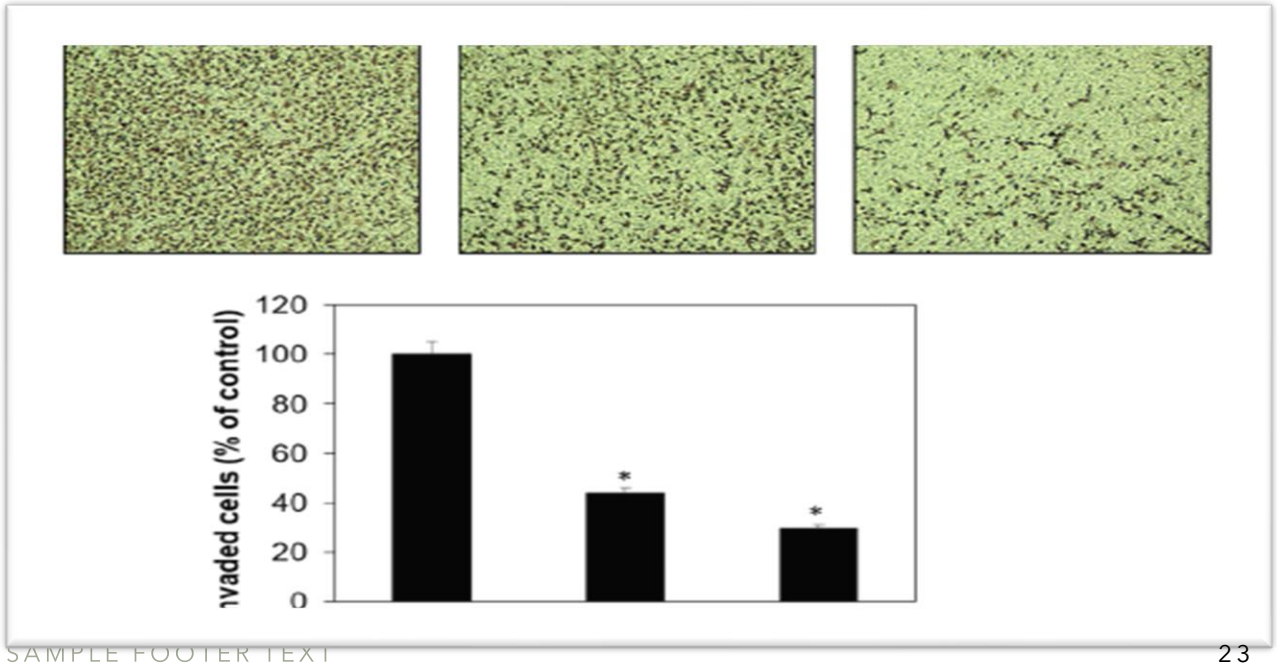
Cell migration inhibitory activity of furan-type compound against AGS cancer cells. NT: no treatment.

*p < 0.05 vs. control



Cell invasion inhibitory activity of furan-type compound against AGS cancer cells. NT: no treatment.

*p < 0.05 vs. control.



Discussion

Marine *Streptomyces* are valuable sources of various secondary metabolites for drug discovery. In this study, we found that *Streptomyces* sp. VN1 as a new strain produced many active compounds, including cinamamide, lobophorin A, cyclo-L-proline-L-tyrosine, and a furan-type compound based on HR-MS/MS and 2D-NMR studies. Besides these compounds, possible analogs of lobophorin A were found based on HR-MS/MS analysis. We found that fragments of those compounds were similar to a fragment of lobophorin A. However, in mass profiles they appeared in trace amounts. Thus, we were unable to elucidate structures of these compounds.

Cinamamide is the product of phenylalanine ammonia lyase. Cinamamide is a nonlethal chemical repellent. Recently, cinamamide derivatives have been synthesized. They show potential activities in both peripheral and central nervous systems, including antiepileptic, antidepressant, neuroprotective, analgesic, anti-inflammatory, muscle-relaxant and sedative/hypnotic properties. Our results indicate that *Streptomyces* sp. VN1 is a promising producer of cinamamide compounds for drug discovery.

Lobophorin A and related analogs are members of the spirotetronate family. Their anti-inflammatory and antibacterial properties have been reported. Genome sequence analysis results revealed that the putative lobophorin biosynthetic gene cluster in *Streptomyces* sp. VN1 showed very high similarity with *Streptomyces* sp. FXJ7.023 and *Streptomyces* sp. SCSIO 01127

They examined the biological activity of compound **4** against five different types of cancer cell lines. Compound **4** showed growth inhibitory activity most effectively against an AGS cancer cell line with an IC_{50} value of 40.5 μM. Although concentration ranges of compound **4** for growth inhibition of cancer cells were much higher than those of known anticancer drugs such as taxol with effective concentrations below 10 μM, compound **4** inhibited the growth of cancer cell lines more effectively as compared to normal cell lines at tested concentrations. Interestingly, compound **4** also showed anti-migration and anti-invasion activities against AGS cancer cell line after 24 hours of exposure. These results confirm compound **4** possesses anticancer activity.

Results of *in sillico* genome analysis showed that *Streptomyces* sp. VN1 encodes the putative biosynthetic gene clusters of diverse interesting secondary metabolites, such as **carotenoid**, **friulimicin**, **xiamycin**, divergolide, and **informatipeptin**. However, biosynthetic pathways for these metabolites in *Streptomyces* sp. VN1 were **inactive under normal culture conditions**. This study displays the biosynthetic potential of *Streptomyces* sp. VN1 through isolation and characterization of four compounds. Due to its ability to grow fast and produce various secondary metabolites, it is suitable for further metabolic exploration to produce useful metabolites. We present *Streptomyces* sp. VN1 as a good candidate for strain optimization to produce therapeutically and industrially relevant compounds. Our results reinforce the need of further exploring marine *Streptomycetes* as a rich source of novel metabolites relevant for biotechnological applications.

Thank you

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