ORIGINAL ARTICLE

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Rec. Nat. Prod. 9:4 (2015) 509-517

records of natural products

A New Siderophore from Sponge Associated *Pseudomonas fluorescens* 4.9.3

F. Can Özkaya[1,2], Erdal Bedir[2*] and E. Esin Hameş[2*]

[1]*İzmir Katip Çelebi University, Faculty of Fishers, Aquaculture Section, 35620 İzmir, Türkiye*

[2]*Ege University, Faculty of Engineering, Department of Bioengineering, Bornova, 35100 İzmir, Türkiye*

(Received July 30, 2014; Revised February 26, 2015; Accepted March 28, 2015)

Abstract: In this study, *Pseudomonas fluorescens* 4.9.3 isolated from a sponge sample was investigated for its secondary metabolites. Two metabolites were isolated, and their structures were elucidated by 1D-, 2D NMR and LC-MS/MS experiments. The new compound (**1**) was established as (5*R*)-N-(2-(1H-imidazol-4-yl)ethyl)-2-(2-hydroxyphenyl)-5-methyl-4,5-dihydrooxazole-4-carboxamide, while the known metabolite was determined as pre-pseudomonine.

Keywords: *Pseudomonas fluorescens;* siderophore; pseudomonine.

1. Introduction

Since iron plays key role as a cofactor in biological processes such as amino acid synthesis, oxygen transport, respiration, nitrogen fixation, methanogenesis, the citric acid cycle, photosynthesis and DNA biosynthesis, it is a universally essential element for microorganisms [1,2]. However, iron is naturally found in the form of Fe (III) oxide that is not freely available for aerobic microorganisms in physiological conditions, and its levels in the oceans are very low [3,4]. Besides, ferrous iron (Fe^{+2}) is unstable under aerobic conditions, and it is transformed to ferric ion (Fe^{+3}) by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$) [5]. Thus, to acquire iron from the environment, many aerobic bacteria, including marine species, synthesize siderophores, low molecular weight iron chelating molecules to solubilize, capture and deliver Fe (III) into the cells. These molecules are very specific for the producer organism to form ferric-siderophore complexes [2,6,7,8,9]. Many siderophores are produced by non-ribosomal peptide (NRP) biosynthesis machinery as peptic structures and combine with high affinity to the Fe (III) form to reduce it to the soluble form Fe (II) in physiological conditions [9-12]. Alpha-hydroxycarboxylic acid, catechol or hydroxamic acid moieties can be found in the iron binding sites, and they can be classified as hydroxycarboxylate, catecholate or hydroxamate type siderophores [13].

Varies strains of *Pseudomonas fluorescens* produce pyoverdine derived siderophores, composed of the basic fluorescent dihydroxyquinoline chromophore, variable peptide chains (6-14 amino acids), and an acyl side chain bound to chromophore and amino group of the peptide chain [14-16]. Pyoverdine is the primary iron uptake system of fluorescent pseudomonads and its catecholate and hydroxamate groups are known as a high affinity-binding site [17]. However, many fluorescent

* Corresponding author: E-Mail: erdalbedir@gmail.com; esinhames@gmail.com; Phone:+90-232-3884955 *Fax:* :+90-232-3884955

The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 06/01/2015 EISSN:1307-6167

pseudomonad species can synthesize low iron affinity siderophores with different structural forms such as pyochelin or quinolobactin [14-16]. Besides, *P. fluorescens* strains that are found in the rhizosphere of plants can be beneficial for the production of antifungal compounds [18-19].

As a part of our ongoing studies to obtain bioactive agents from natural sources, we made an attempt to isolate and identify endophytic bacteria from the sponges collected from different areas of Mediterranean and Aegean coasts of Turkey to screen their antimicrobial activities and to investigate secondary metabolites of the selected strains. After surface sterilization, homogenated inner tissues of the sponges were used for inoculation to R2A, M1 and M6 media and incubated at room temperature for 2 weeks, resulting in isolation of 195 bacterial species [20-22]. The pure isolates were cultured in seawater medium and Muller Hinton broth that was prepared with 50% seawater at 150 rpm and 28 ^{o}C for 2 days in a rotary shaker. Cell-free fermentation broths were extracted with ethyl acetate. The extracts were screened for their antimicrobial activities, and based on the activity data and thin layer chromatography (TLC) profiles, the strain 4.9.3 was characterized and taken into further studies to purify its secondary metabolites. Here we report two metabolites isolated from the selected strain, a sponge derived *P. fluorescens*.

2. Materials and Methods

2.1. Isolation and Cultivation of Bacteria

A sponge sample was collected by scuba diving at a depth of 20 m from Ayvalik-Agean Sea coast of Turkey (39°19'N 26°42'E) in June 2010. The sponge was identified as *Axinella damicornis* by M. Baki Yokes (PhD) from Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Haliç University, Istanbul, Turkey. Sample was transferred to sterile 500 mL polypropylene bottles containing seawater and kept in ice until processing.

The sponge sample was rinsed with sterile seawater for five times to remove surface bacteria and cut into small pieces (1 cm^3) using sterile scalpel aseptically. The pieces were homogenized in 10 volumes of sterile seawater using a blender. Prior to inoculation, homogenate was diluted tenfold series with sterile seawater and dilutions were spread onto agar plates containing **R2A** [yeast extract 0.5 g, glucose 0.5 g, peptone 0.5 g, casein hydrolysate 0.5 g, soluble starch 0.5 g, sodium pyruvate 0.3 g, K_2HPO_4 0.3 g, magnesium sulphate 0.05 g, agar 20 g and 500 mL natural seawater and 500 mL distilled water (50%), pH 7.2] [20], **M1** (soluble starch 10 g, yeast extract 4 g, peptone 2 g, agar 20 g and 1 L natural seawater, pH 7.5) [22] and **M6** (beef extract 4 g, peptone 4 g, yeast extract 1 g, glucose 10 g, NaCl 20 g, agar 20 g and 1 L of distilled water, pH 7.5) [21]. All media were supplemented with cycloheximide (100µg/mL) for inhibition of fungal growth and nalidixic acid (50 µg/mL) for inhibition of fast growing Gram-negative bacteria [23]. At least two plates for each medium and each dilution were used for inoculation and incubated 2 weeks at room temperature. Representative colonies were transferred on the fresh agar plates containing Muller Hinton agar for purification of the isolates. The pure isolates were stored at –20 ^{o}C in 50% (v/v) sterile glycerol.

2.2. Fermentation

For preliminary fermentation, pure bacterial strains were inoculated in 250 mL Erlenmeyer flask, containing 50 mL Sea Water Medium (SWM) [peptone 5 g, yeast extract 2.5 g, glucose 1 g, K_2HPO_4 0.2 g, $MgSO_4.7H_2O$ 0.1 g [24] and 500 mL natural seawater and 500 mL distilled water, pH 7.5] and Muller Hinton Broth that was prepared with 50% seawater. Incubation was performed at 28 ^{o}C and 150 rpm for 48 h. For large-scale fermentation, selected bacterial strain was inoculated in 1L Erlenmeyer flask containing 250 mL SWM.

2.3. Extraction

The biomass and fermentation broth were separated by centrifugation at 7000 rpm for 5 min after incubation, and the cell free supernatant was used for the extraction with ethyl acetate (EtOAc) (1:1). The EtOAc extracts were evaporated under reduced pressure at 37 ^{o}C.

2.4. DNA Extraction and 16S rDNA Sequence Analysis

Total genomic DNA was isolated using combined methods as described by Cheng and Lee [25,26]. Briefly, the selected strain was cultivated in MHB at 28 ^{o}C and 150 rpm for 16 h. The cell pellet was obtained using centrifuge at 4000 rpm for 5 min at +4 ^{o}C and then chilled by liquid nitrogen and crushed with glass rod. Lysis buffer (500 µl) [Tris–HCl 0.05 M, pH 7.6, NaCl 0.1 M, ethylenediaminetetraacetic acid (EDTA) 0.05 M, sodium dodesyl sulphate (SDS) 2%, polyvinylpyrolidone (PVP) (to remove phenolic compounds) 0.2%, β-mercaptoethanol 0.1%, and tris-saturated phenol] was added on the pellet and incubated for 30 min at room temperature. The cell debris was removed by centrifuge at 13000 rpm for 5 min and then supernatant was mixed gently by tris-saturated phenol (500 µl) for 2 min. In the next step, 500 µl of chloroform was also added gently to the DNA extract to remove phenol residues, and the separation of the two phases was achieved by centrifugation at 13000 rpm for 5 minutes. The phenol and chloroform extraction step was repeated to obtain good phase dispersion. Total genomic DNA in the aqueous phase was precipitated by adding 60 % (v/v) isopropanol, and centrifuged at 8000 rpm, +4^{o}C for 10 min. Finally, the upper phase was removed, and DNA pellet was washed with 70% ethanol (v/v) and dried at room temperature. The success of the genomic DNA extraction was confirmed by gel electrophoresis (1%) for integrity and quantity.

27F (5'-AGAGTTTGATCCTGGCTCAG-3') and R1492 (5'- TACGGTACCTTGTTACGACTT-3') were used as amplification primers of polymerase chain reaction (PCR). PCR program was initiated with denaturation cycle at 94 °C for 5 min, and 30 amplification cycle (denaturing; 94 °C for 45 s, primer annealing; 50 °C for 45 s, primer extending; 71 °C for 45 s). A BLAST search of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) was performed to identify the nearest neighbor to the amplified sequence.

2.5. Extraction and Isolation

The fermentation broth (60 L) was centrifuged at 10000 rpm by industrial centrifuge (Westfalia Seperator), and cell-free fermentation broth was extracted with EtOAc (20 L). The EtOAc was evaporated under reduced pressure at 37 ^{o}C to yield a dark brown solid (2.3 g). Then the crude extract was subjected to vacuum liquid chromatography, using silica gel (Merck 7734, 115 g). Elution was performed with EtOAc:*n*-hexane (50:50, 75:25), EtOAc:MeOH (95:5, 90:10, 80:20, 70:30, 60:40, 50:50) and MeOH to afford 20 main fractions (Fr 1-20). Fr 10 (285.6 mg), exhibiting antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus feacium* (VREF), was applied to silica gel (Merck 7734, 50 g) column chromatography. Elution was carried out under isocratic condition with *n*-hexane:EtOAc:MeOH:NH_3 (10:10:2: 1% – 10:10:3:1% – 10:10:4: 1% – 10:10:5:1% – 10:10:7: 1% – 10:10:10:1%) to afford 4 fractions (Fr 10.1-10.4). Fr 10.2 (10 mg) was applied to silica gel preparative TLC (prep-TLC). Elution was performed by EtOAc:MeOH:H_2O:NH_3 (30:5:4:3%) to give **1** (3 mg). Fr 10.3 (201 mg) was applied to silica gel (Merck 7734, 40 g) column chromatography. Elution was carried out under isocratic condition $CHCl_3$:MeOH:NH_3 (95:5:1% – 90:10: 1% – 80:20: 1% – 70:30: 1% – 50:50: 1%) to afford 8 fractions (Fr 10.3.1-10.3.8). Fr 10.3.7 (10 mg) was chromatographed on RP (C-18) column (13 g CHROBAND® C18 column) by using MeOH:H_2O (6:4) to yield **2** (6 mg).

All chromatographic experiments were performed using EtOAc/MeOH/H_2O/NH_3 (30:5:4:3%) as a mobile phase and 30% H_2SO_4 as a visualization agent on TLC (SiO_2) plates.

3. Results and Discussion

3.1. Structure elucidation

A bacterium was isolated from the sponge *Axinella damicornis*, collected from Ayvalık cost line in 2010 June. The isolate was identified by 16S rDNA sequence analysis as *Pseudomonas fluorescens* 4.9.3 with 99% similarity and was deposited to GenBank under accession number JX127246 (Figure

1). The metabolites were produced in SWM during 48 hours of incubation, and further extraction and isolation studies resulted in purification of two compounds (**1** and **2**).

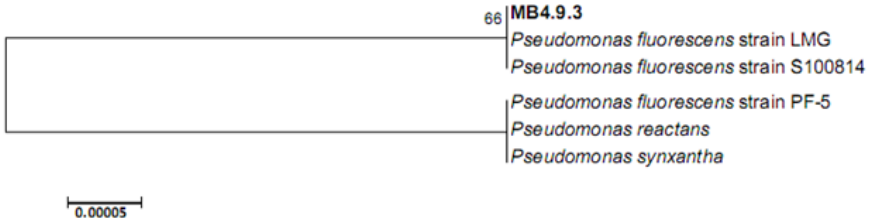

Figure 1. Phylogenetic tree of *P. fluorescens* 4.9.3. based on 16S rDNA sequences.

The HR-Q-TOF mass spectrum of yellow-brown amorphous compound **1** [*m/z* 315.1456 $[M+H]^+$ calcd. for $C_{16}H_{19}N_4O_3$ (+ mode)] supported a molecular formula of $C_{16}H_{18}N_4O_3$, indicating 10 degrees of unsaturation (Figure 2). Chemical shift values from ^1H and ^{13}C spectra of **1** are summarized in Table 1 (Supplementary information for 1D and 2D spectra).

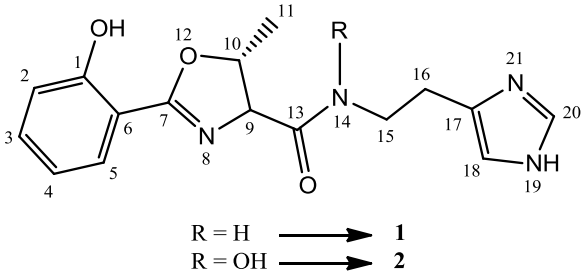

Fig. 2. Structures of **1** and **2**

The ^1H NMR spectrum displayed 1,2 disubstituted (ortho) benzene moiety [δ 7.00, dd, J=1.6 and 8.4 Hz, H-2; δ 7.45, dt, J=1.6 and 8.0 Hz, H-3; δ 6.94, dt, J=1.2 and 8.0 Hz, H-4; δ 7.62, d, J=1.6 and 8.0, H-5), which was confirmed with proton−proton correlations of H-2/H-3, H-3/H-4 and H-4/H-5 in the COSY spectrum. The HMQC and HMBC experiments helped us to assign the carbon resonances of the aromatic ring suggesting a 2-hydroxy phenyl unit. The downfield shift of H-5 implied an electron-withdrawing group at ortho position. As a matter of fact, a carbon signal at δ 165.8 (C-7) showed a correlation with H-5 in the HMBC spectrum. The same quaternary carbon exhibited another long-range correlation with a proton signal at δ 4.44 (d, J=7.2 Hz, H-9), which was part of a spin system CH(O/N)/(H-9)→CH(O/N)→CH_3 deduced from COSY experiment (δ 4.85, p, J=7.2 and 6.4 Hz, H-10; δ 1.46, d, J=6.4 Hz, H-11). After assigning carbons of the second spin system by HMQC, the data was consistent with presence of an oxazole ring (C_4H_5NO) [27]. Thus interconnectivity of oxazole and 2-hydroxy phenyl rings based on the HMBC correlations from C-7 to H-5 and H-9 was apparent (Figure 3). This substructure coupled with a histamine residue is frequently encountered in *Pseudomonas* genus as siderophore-type molecules [28-30].

Detailed inspection of the COSY spectrum exhibited an additional spin system; a proton resonance at δ 8.38, (t, J=4.8), an N(H) group as part of an amide system, showed a correlation with a CH_2 signal (δ 3.32, m, H_2-14), while the latter proton coupled with another methylene signal at δ 2.67 (t, J=7.2 Hz, H_2-15). The remaining part of **1** had $C_2H_3N_2$ molecular formula suggesting presence of an imidazole ring. As rapid conversion of charge distribution occured between tautomeric forms in the NMR timescale for the imidazole ring [31-33], its proton signals were barely observed at δ 6.81 (s, H-18) and 7.49 (s, H-20), and their vicinal proton-proton coupling constants were very small (J~1 Hz). Besides, the carbon NMR signals appeared as weak signals. Additionally, a correlation between the amide carbonyl (δ 170.0, C-12) and H-9 (δ 4.44) proved connectivity of oxazole ring and histamine moiety through C-12. Finally, structure of **1** was elucidated as (5*R*)-2-(2-hydroxyphenyl)-N-[2-(1H-imidazol-4-yl)ethyl]-5-methyl-4,5-dihydro-1,3-oxozole-4-carboxamide.

Table 1. ^{1}H and ^{13}C NMR data of **1***

C. No	^{1}H (δ)	^{13}C (δ)	Multiplicity
1**		159.2	C
2	7.00, dd, *J* = 1.6, 8.0 Hz	116.6	CH
3	7.45, dt, *J* = 2.0, 8.0 Hz	134.0	CH
4	6.94, dt, *J* = 1.6, 8.0 Hz	119.0	CH
5	7.62, d, *J* = 1.6, 8.0 Hz	128.0	CH
6		110.1	C
7		165.1	C
9	4.44, d, *J* = 7.2 Hz	73.7	CH
10	4.85, p, *J* = 6.4, 7.2 Hz	78.9	CH
11	1.46, d, *J* = 6.4 Hz	20.6	CH_3
13		169.2	C
14 (NH)	8.38, t, *J* = 4.8 Hz	-	NH
15	3.32, m	39.5	CH_2
16	2.67, t, *J* = 7.2 Hz	25.5	CH_2
17		129.6	C
18	6.81, br.s	115.1	CH
19 (NH)	***	-	NH
20	7.49 (br.s, 1H)	134.6	CH

*Spectra were measured in DMSO-d_6 at 400 MHz for ^{1}H and at 100 MHz for ^{13}C.
**C-1 (OH) = δ 11.77 (s, 1H)
*** Not observed

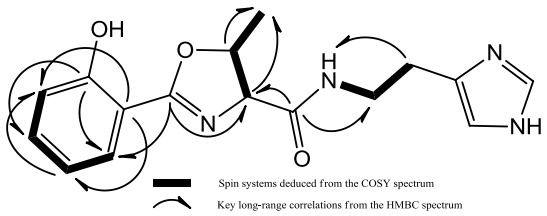

Figure 3. Spin systems and selected long-range correlations of **1**

The HR-MALDI-TOF mass spectrum of **2** (*m/z* 331.1386 $[M+H]^{+}$ calcd. for $C_{16}H_{20}N_4O_4$ and 329.1185 $[M-H]^{-}$ calcd. for $C_{16}H_{18}N_4O4$) supported a molecular formula of $C_{16}H_{19}N_4O_4$. Both the ^{1}H and ^{13}C NMR data of compounds **1** and **2** were very similar. The only difference was consistent with the presence of an N(13)-hydroxyl group in **2**. Compound **2**, namely pre-pseudomonine, was previously prepared chemically and enzymatically [34].

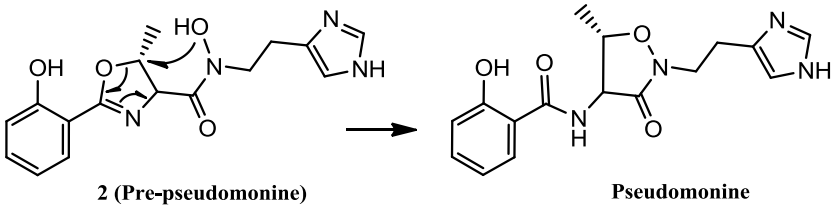

Figure 4. Proposed rearrangment mechanism for the formation of pseudomonine [34]

3.2. Discussion

Pseudomonads biosynthesize and secrete iron-chelating compounds due to iron limitation in the

environment. These secreted compounds, called siderophores, bind ferric iron and transport back to the bacterial cells [2,6,7,8,9]. Iron deficient conditions prompt microorganisms to up-regulate siderophore biosynthesis, including those experienced during infection of a host. Sometimes the iron competition between a host and pathogenic microbes is one of the most important features influencing the course of a microbial infection. In many infections, microbial siderophores are even able to get iron from the host's storage proteins such as lactoferrin or transferrin. To insure a competitive growth advantage, some microorganisms not only express specific outer membrane proteins to recognize the iron complexes of their siderophores, but also evolve receptors of siderophore-iron complexes made by other microbes [40-42].

As a part of our attempts to obtain antimicrobial agents from marine derived endophytic bacteria, an active strain, which was identified as *P. fluorescens* 4.9.3, was selected for further studies. Two siderophores (**1** and **2**) were isolated and identified as major constituents. Acquirement of two siderophores with no antimicrobial activity was surprising. However, when one takes into consideration the environmental factors, the results make sense. Although iron is one of the most abundant elements in the Earth's crust, its level in the ocean is surprisingly low, ranging from 20 pM to 1 nM. Thus, iron is perhaps the most important of all the bioactive metals in the marine environment [43]. To imitate the microorganism's original environment, the seawater medium (SWM) was used instead of a complex medium during the course of our research. Most probably, utilization of this medium during large-scale fermentation led to the up-regulation of siderophore biosynthesis genes compared to those regulating antibiotic production.

From chemical perspective, compound **1** has similar structural framework with vibriobactin (*Vibrio cholerae*) [35], whereas pseudomonine (**2**) (*Pseudomonas fluorescens*) [36] and acinetobactin (*Acinetobacter baumaii* ATCC 19606) [37] are nearly constitutional isomers only differing in the presence of second hydroxyl group of aromatic acid. These siderophores' unique scaffold is generated via coupling of L-histidine, threonine and salicylic acid units, and their functionalizations in non-ribosomal peptide synthetase (NPRS) cluster explain the structural diversity.

It needs to be pointed out that pre-pseudomonine is confirmed to be precursor of pseudomonine (Figure 4). This conversion is spontaneous under the physiologic buffered conditions at pH 7, and it goes through S_N2 mechanism, where the oxygen of hydroxamate acts as an intermediate nucleophile in the isoxazolidinone biosynthesis [34]. We speculate that compared to the previous studies, the short incubation period (48 h versus 96 h) and use of SWM instead of complex media might be the overriding basis for not obtaining pseudomonine. Additionally, pre-pseudomonine (**2**) was prepared in the laboratory by chemical or enzymatic methods; therefore, its isolation and identification as a natural product was being reported for the first time.

In this study, it was aimed to isolate antimicrobial compounds from the marine bacteria, and *P. fluorescens* 4.9.3 was chosen due to its antimicrobial activity against VREF and MRSA based on our preliminary results. However, MIC test revealed that both purified compounds were inactive toward the resistant pathogens. Based on this finding, it is hypothesized that the unstable nature or insufficient amount could be the reason for lacking to isolate and identify the active metabolites.

Moreover, to the best of our knowledge, there has been only one study on marine derived *P. fluorescens* (91QQ48), affording potent antimicrobial compounds. This marine isolate was obtained from tissues of an unidentified tunicate collected from Alaska, and its extract inhibited MRSA potently. Preliminary experiments showed that liquid shake cultures of *P. fluorescens* 91QQ48 failed to produce extracts with any antibacterial activity but that cultures grown on solid media gave extremely active extracts. Therefore, the strain was grown in moderate scale as lawns on solid media. Bioassay guided fractionation of the ethyl acetate extract (12.4 g) resulted in the isolation of a known metabolite (andrimid) and three new compounds, moiramides A-C. Andrimid and moiramide B exhibited potent in vitro antibacterial activity. Especially, the amount of purified andrimid was noteworthy (1.0 g: 8% yield). When compared to our study, Needham and co-workers used a different culturing protocol, solid media instead of liquid media. Moreover, their solid media comprised tryptic soy broth medium, 1% agar, and 1% sodium chloride; whereas our liquid media composed of peptone, yeast extract, glucose, K_2HPO_4, $MgSO_4.7H_2O$, 500 mL natural seawater and 500 mL distilled water. The differences in regards to fermentation methodology and medium composition might be another reason for failing to obtain the active metabolites in our study [44].

On the other hand, it should also be considered that several isoxazolidinone-type metabolites are

well known as antimicrobial agents (*i.e.* antituberculosis D-cycloserine, *β*-lactamase inhibitor lactivicin) [38,39], the precursors or degradation products of these siderophores with the same ring system (*i.e*. **1** and **2**) might be of interest for further exploration. In addition, siderophores have recently become important for drug delivery. Albomycin was actively transferred into Gram (+) and Gram (-) bacteria by iron transport processes and, the conjugated thio-nucleoside-type drug was enzymatically released to exert its toxic effect [45]. Miller and his group studied on the intracellular release of the amino glycoside-like antibiotic from salmycins. The siderophore-drug conjugate exhibited exceptional microbial selectivity and activity against Staphylococcus and Streptococcus (MIC=0.01 μg/mL), including multidrug resistant strains [46, 47]. These are only a few examples of siderophore-linker-drug combinations, and many studies are in progress in this field.

Consequently, yet the siderophores are not effective antibiotics, they hold a great potential for exploitation in the development of microbe-selective drug delivery, as well as the transport of selective anticancer agents.

Acknowledgments

This work is supported by TUBITAK (Project No: 109S361).

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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