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MICROBIOLOGY

Isolation and Partial Characterization of Bacteria with Potential Antimicrobial Activity from the Caspian Sea[1]

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Abstract—Due to the constant increasing of bacterial resistance against known antibiotics, it is now necessary to find new sources of antimicrobials including the marine environment. The aim of this study was to evaluate antimicrobial activity of bacterial strains isolated from different coastal regions of the Caspian Sea and to provide phylogenetic analyses of antibiotic producing strains. Water samples collected from the Caspian Sea were serially diluted and plated on selective media. Isolates were tested against a panel of reference strains (*Streptococcus pyogenes, Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) by microbial antagonism and disc diffusion assay. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method was also employed to produce a phylogenetic tree based on 16S rDNA sequences. Amongst 162 isolates, 8 strains (4.93%) showed antibacterial activity. Isolated bacteria displayed more activity against gram-positive bacteria than gram-negative bacteria. Moreover, the 16S sequences obtained for the 8 selected strains were compared using a BLAST algorithm and allowed us to determine the strains genus as followed: *Bacillus* (RS28, RS54, RS56, RS82, RS116, and NS53), *Brevundimonas* (RS32), and *Arthrobacter* (NS25). The findings of the present study recommend that culturally marine bacteria collected from the Caspian Sea might be a potent source of novel bioactive compounds such as antibiotics.

Keywords: Marine bacteria, Antimicrobial agents, the Caspian Sea, 16S rDNA

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INTRODUCTION

In spite of the tremendous progress in human medicine, infectious diseases caused by bacteria are still a major problem in public health and global economies [1, 7]. Moreover, multidrug-resistant bacteria have increased dramatically over the past few years and have now reached a level that places future patients in real danger [6]. As a result, there is an urgent need for the development of novel and effective antimicrobial compounds with different mode of action and chemical structures. Although progress has been made in the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature still remains the richest and the most versatile source for new antibiotics [4, 15]. Indeed, products from nature are unsurpassed in their ability to provide novelty and complexity. Compared to the terrestrial environment, which was the focus of the pharmaceutical industry for more than 50 years, marine environment has remained virtually unexplored for producing pharmacological compounds [1, 9, 17].

The ocean, which occupies almost 70% of earth's surface, harbors most of the planet's biodiversity. However, the microbial diversity of ocean remains relatively unexplored. Such microbial diversity can constitutes an infinite pool of novel chemistry, making up a valuable source for innovative biotechnology [2, 8, 17]. Moreover, the recent development of procedures for cultivation and identifying microorganisms, through the isolation and sequencing of ribosomal RNA or DNA, has aided microbiologists in their assessment of microbial diversity [7].

Historically, the first antibiotic isolated from marine bacterium was identified and characterized in 1966 [5]. In the last decades, the number of reported secondary metabolites isolated from marine bacteria has steadily increased, reflecting the growing attention by groups from academia and industry [14].

The Caspian Sea, known as the largest land-locked saltwater sea in the world, represents a great biodiversity which makes it one of the most valuable ecosystems in the world [11]. Until now, very few investigations have been conducted to study the antibiotic properties of marine bacteria isolated from the Cas-

[1] The text was submitted by the authors in English.

pian Sea. For instance, recently, it has been reported that marine actinomycetes of the Caspian Sea sediments were potent sources of novel antibiotics and bioactive compounds [13]. Considering the substantial biodiversity of the Caspian Sea, it should be a promising source for exploring microorganisms and novel antibacterial molecules. The present study followed two main objectives. The first was to screen the bacterial strains for their antimicrobial activity and the other objective was to identify the antibiotic producing strains by 16S rDNA PCR sequencing.

MATERIALS AND METHODS

Sampling and Isolating of Marine Bacteria

Water samples were collected at 150 m distance from the coastal regions of the Caspian Sea (Mazandaran province, Iran) at the depths of 0.5 m. Two sampling stations were located along of the Caspian Sea with the following latitudes and longitudes: Ramsar (36°55′ N, 50°41′ E) and nashtarud (36°45′ N, 51°00′ E) (Fig. 1). In the field, the water samples were collected in 500 mL sterile bottles. The samples were brought to the laboratory in aseptic condition. In order to keep the temperature low, all bottles were placed in the full-of-ice flask [12]. The serial dilution (10^{-1}–10^{-8}) were prepared immediately and 150 μL of each diluent was cultured on plates containing marine-agar medium (1 : 10), 0.5 g Peptone, 0.1 g yeast extract, 0.1 $FePO_4$, and 15 g agar-agar dissolved in one liter of sea water (Merck, Germany) adjusted to pH 7.2–7.6, as described by Zheng et al. (2005) [19]. The plates were incubated at 25°C for 48, 72 h, and 20 days. The appeared colonies were separated based on morphology and sub-cultured for colony purification. Single colonies were stored at 4–8°C for further analysis.

Preparation of Bacterial Cultures and Crude Extracts

According to the method described by Zheng et al. (2005) [19], each isolated bacterium was cultured into 300 mL marine broth medium containing 5 g peptone, 1 g yeast extract, 0.1 g $FePO_4$ dissolved in one liter sea water and then adjusted to pH 7.2–7.6. The cultures were incubated at 25°C with stirring for 7 days and then centrifuged at 5000 g for 30 min. The supernatants were treated with ethyl acetate three times. After the solvent removed under reduced pressure at 37°C, the extracts were used as the crude samples for bioactivity assay in disc diffusion method.

Antibacterial Assay

Streptococcus pyogenes PTCC 1447 (ATCC 8668), *Escherichia coli* PTCC1399 (ATCC 25922), *Staphylococcus aureus* PTCC 1431 (ATCC 25923), and *Pseudomonas aeruginosa* PTCC 1430 (ATCC 27853) were obtained from Iran Research Organization for Science and technology, Tehran, Iran. These strains were used as reference for antimicrobial assays. The antimicrobial activity test was performed by microbial antagonism (cross streak assay) and disc diffusion assay. Cross streak method was assessed using the method described by Mohseni et al., (2013) with modifications [13]. In the cross culture method, each isolated marine bacteria were cultured in a half of Petri dish massively at 30°C for 48 h on Mueller-Hinton agar medium (Merck, Germany) in order to let antimicrobial diffusion (sea water was used to make the Mueller-Hinton agar to provide fundamental minerals for marine bacterial growth). Afterward, each reference strain (0.5 Mc Farland dilutions) was cultured perpendicular to the marine bacteria culture in the other half of the plate with the same condition. The absence or presence of growth was monitored for 48 h at 30°C as triplicate. *Streptomyces erythraeus* PTCC 1120 (ATCC 11635) was used as reference strain and positive control. In the disc diffusion assay, antimicrobial activity was analyzed using standard paper discs, as described by Zheng et al. (2005) with modifications [19]. The dried crude extracts were dissolved in ethyl acetate (EtOAc) to a concentration of 100 mg/mL and 20 μL of each sample was used to saturate discs. The saturated discs were placed onto the agar surface containing the test microorganism and incubated at 37°C for 24 h after a diffusion process for 10 h at 8°C. EtOAc disc was used as negative control. The diameters of inhibition zones were measured.

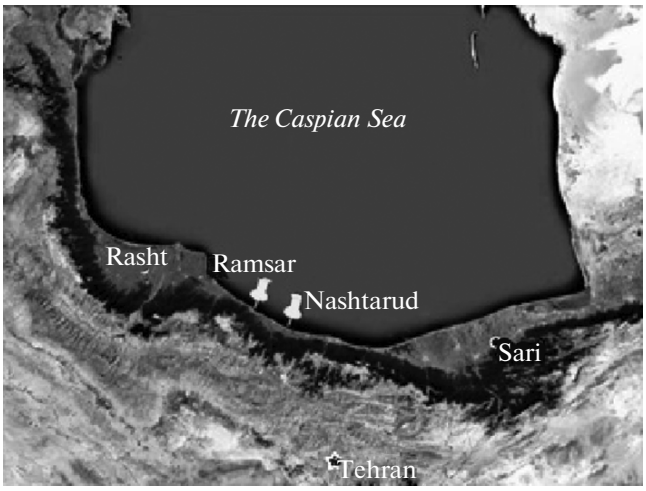

Fig. 1. Sample sites along the Caspian Sea (Ramsar and Nashtarud).

Characterization of Bacterial Strains Producing Antibiotic Agents

The DNA of antimicrobial producing strains was extracted using DNA extraction kit (CinnaGen, Iran) and then 16S rRNA genomic regions were analyzed using 5'-AACTGGAGGAAGGTGGGGAT-3' and

Table 1. Antimicrobial activity of isolated strains by cross streak assay

Strain name	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Streptococcus pyogenes*
RS28	+	–	–	–
RS54	+	–	–	+
RS56	+	–	–	+
RS82	+	–	–	+
RS116	+	–	+	+
NS53	+	–	–	+
NS25	+	–	–	–
RS32	–	–	–	+
Control	+	–	–	+

No inhibition of growth and inhibition of growth are marked with – and +, respectively.

Table 2. Antimicrobial activity measurements of isolated strains by disc diffusion assay

Strain name	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Streptococcus pyogenes*
RS28	++	+	–	+
RS54	+	–	–	+
RS56	+++	–	–	+++
RS82	++	+	–	+
RS116	+	–	–	+
NS53	+++	–	–	++
NS25	+	–	–	+
RS32	+	+	–	++
Control	–	–	–	–

Growth inhibitory calculations were carried out as follows; <5 mm (–), 6–9 mm (+), 10–13 mm (++), >14 mm (+++).

5'-AGGAGGTGATCCAACCGCA-3' as forward and reverse primers, respectively [10]. Total volume for each reaction was adjusted to 50 μL and Taq DNA polymerase (Fermentas, Lithuania) was used for all amplification. PCR amplification conditions on thermocycler (eppendrof, Germany) were as follows: 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1% agarose gel and extracted by gel extraction kit (CinnaGen, Iran). Purified products were subjected to 16S rDNA sequencing (Macrogen Inc., South Korea). The sequences were analyzed by nBLAST tool at Center for Biotechnology Information (NCBI). The isolates were identified based on homologous sequences, similarity search and new recognized strains were submitted to NCBI. Furthermore, these sequences were aligned by using MAFFT software (http://mafft.cbrc.jp/alignment/software). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method was also employed to produce a phylogenetic tree based on 16S rDNA sequences.

RESULTS

The antimicrobial activity of isolated bacteria was evaluated by two assays: cross streak assay (Table 1) and disc diffusion assay (Table 2). According to the Table 1, isolated strains from Ramsar and Nashtarud cities were indicated as RS and NS, respectively. Of 162 isolates, eight (4.93%) displayed antibacterial activity mainly against *Staphylococcus aureus* and *Streptococcus pyogenes*, suggesting that several compounds may be produced by these marine isolates. Moreover, none of the strains was active against *P. aeruginosa*. Among the eight isolates selected, antibacterial activities of the crude extract of two isolates are of interest: NS53 and RS56 showed highest activity against *S. aureus* with an inhibition zone >14 mm. Examples of exhibiting the antibacterial activity of RS56 are shown in Fig. 2.

Phylogenetic tree of the eight isolates with antimicrobial activity was constructed using the UPGMA method (Fig. 3). The 16S rDNA sequences were submitted to the NCBI. The Accession numbers of 16S rDNA sequences as follows: RS28 (accession no. JN084040), RS54 (accession no. JN084042), RS56 (accession no. JN084043), RS82 (accession no. JN084046), RS116 (accession no. JN084048), NS53 (accession no. JN084049), NS25 (accession no. JN084039), and RS32 (accession no. JN084041). In addition, the isolates were classified into 3 main clusters. Of 8 isolates, 6 strains were belonged to *Bacillus* genus. The second cluster comprised *Arthrobacter* genus. Also, one isolate belonged to *Brevundimonas* genus.

DISCUSSION

During the screening of the novel secondary metabolites, isolated bacteria displayed more activity against gram-positive bacteria (*Streptococcus pyogenes* and *Staphylococcus aureus*) than gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). The findings of the current study are consistent with those of Cetina et al. (2010) who found that the gram-negative human bacterial pathogens were not as susceptible to marine antagonists as were the gram-positive human bacterial pathogens [7]. The difference observed among the antibacterial activity of isolated bacteria suggests that the antibiotic substances produced by them may not be identical compounds. Moreover, Mohseni et al. (2013) isolated 44 strains of actinomycetes from the Caspian Sea sediments and

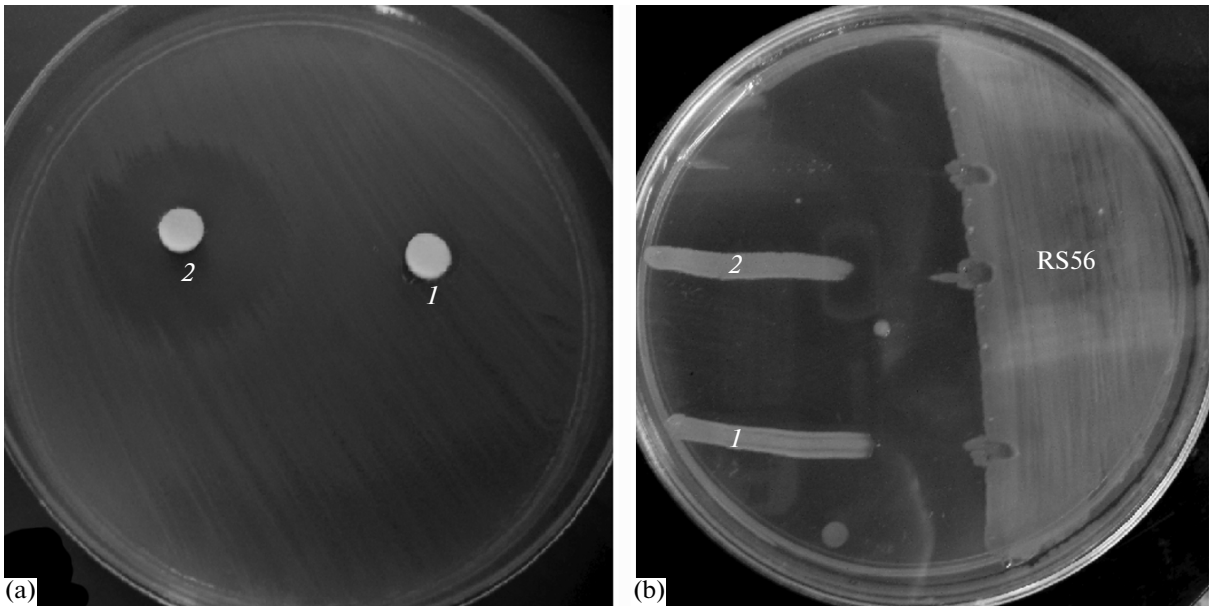

Fig. 2. Antibacterial activity of RS56. (a) The disc diffusion assay: *1*, negative control; *2*, inhibition of *Staphylococcus aureus* by RS56 isolate. (b) The cross streak assay: *1*, *Staphylococcus aureus*; *2*, *Streptococcus pyogenes.*

evaluated their antibacterial activities against two gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and four gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Klebsiella pneumonia*) bacteria. Their results demonstrated that among the tested bacteria, crude extracts exhibited highest antibacterial activity against *B. subtilis* and *S. aureus* [13].

In this study, highest zone of inhibition was observed against *S. aureus* (Table 2). *S. aureus* is one of the major human pathogens which can cause various infections like dermal, pulmonary and blood infections. These infections are highly prevalent in pediatric patients and healing options are limited [7, 16]. Therefore, antimicrobial agents isolated from marine bacteria could be an important therapeutic alternative particularly those directed against *S. aureus*.

With the steady decreasing costs of genome sequencing, genome mining of microbial genera and species with high potential for biosynthesis of biologically active compounds such as antibiotics represents a great potential and opportunity for drug discovery [17]. In the present study, the 16S sequences obtained for the 8 selected strains were compared using a BLAST algorithm (http://blast.ncbi.nlm.nih.gov) and allowed us to determine the strains genus as followed: *Bacillus* (RS28, RS54, RS56, RS82, RS116, and NS53), *Brevundimonas* (RS32), and *Arthrobacter* (NS25). Based on our results, a phylogenetic dendrogram was constructed with the 300 bp of the 16S rDNA sequence obtained. The tree showed that the closest neighbours of strains RS54, RS56, RS82, and RS116 were *Bacillus pumilus*. The closest species of NS53 was *Bacillus subtilis*. The UPGMA analysis showed that the strain RS28 is closed to *Bacillus cereus*. Furthermore, strain NS25 was closely related to *Arthrobacter* genus. RS32 is closely related to *Brevundimonas subvibrioides.* In a study on antibiotic production in marine bacteria, isolated from coastal areas of China, Zheng et al. (2005) have reported that the active marine bacteria were assigned to the genera *Alteromonas*, *Pseudomonas*, *Bacillus*, and *Flavobacterium* [19]. In another study conducted by Dufourcq et al. (2013), isolates with potential antibacterial activities, which recovered from marine costal environment from New Caledonia, belonged to the genera *Pseudoalteromonas*, *Photobacterium*, and *Salinivibrio* [8].

Considering the 16S phylogenetic analyses and the antibacterial activity observed, *Bacillus* presents an interesting antibacterial profile and might be further investigated. Among *Bacillus* species, the main antibiotic producer of this genus is *B. subtilis*. The findings of the present study led to conclude that the antibiotic producing *Bacillus* strains could easily be isolated from the Caspian Sea. Many gram-positive bacteria such as *Bacillus* are known to generate spores under adverse conditions, such as those encountered in marine ecosystems [2]. Being capable of producing a large number of antimicrobial peptides, *Bacillus* is an interesting genus to search for inhibitory substance and *B. subtilis* is one of the major producers of these substances, including several bacteriocins [18]. In addition to producing antimicrobial compounds, *B. subtilis* is considered a benign organism as it does not possess traits that cause disease. Since *B. subtilis* is not considered pathogenic or toxigenic to humans, it can safely be used for fermentation and genetic manipulation [3].

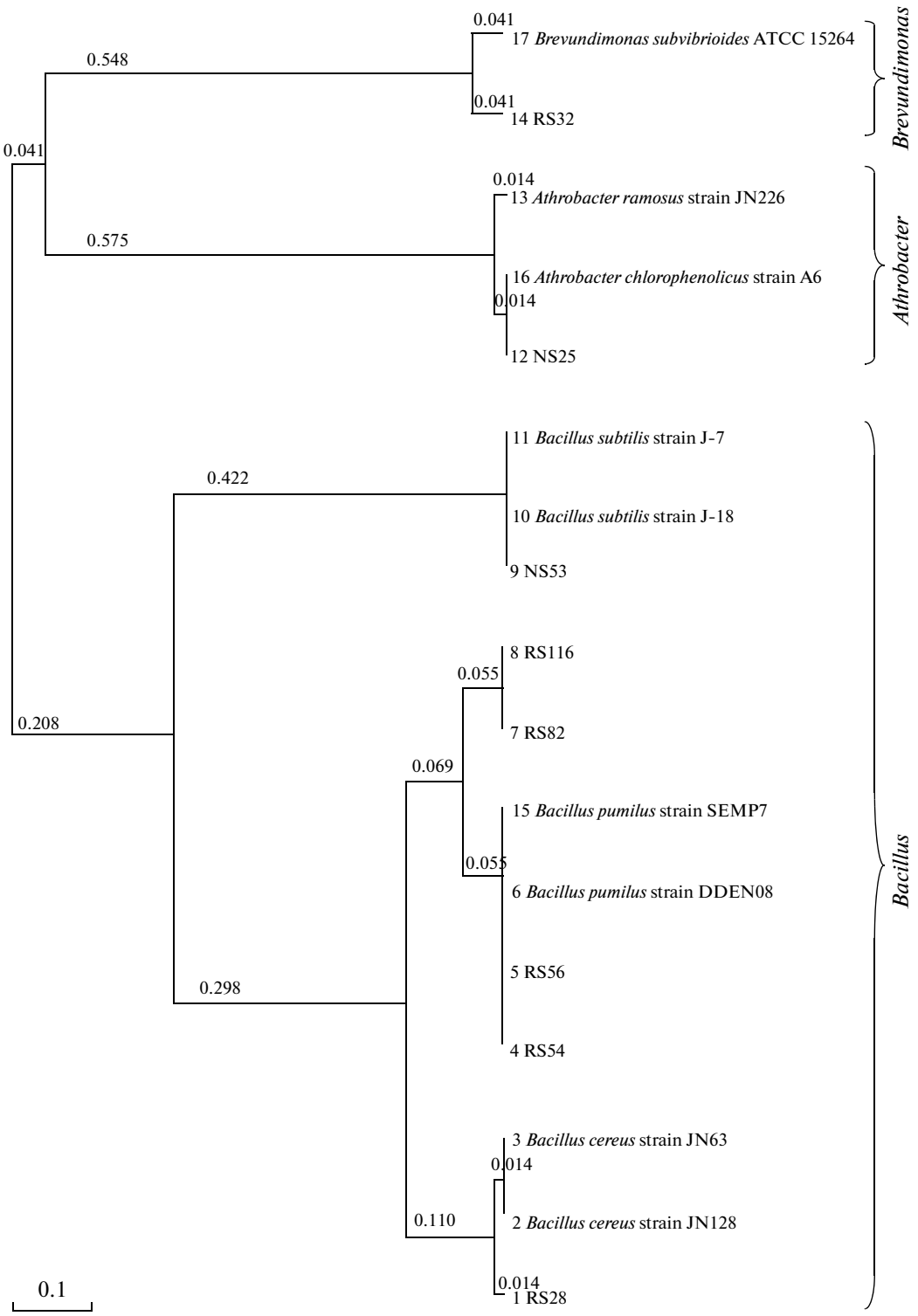

Fig. 3. Phylogenetic tree of partial 16S rDNA gene sequences of *Bacillus*, *Arthrobacter*, and *Brevundimonas*. A tree has been build using the UPGMA method (similarity clustering).

The Caspian Sea, known as the largest enclosed sea in the world, is located in an inland depression on the border of Europe and Asia. One of the most important features of the Caspian Sea is its changing water level, which has a significant effect on biodiversity in the extensive shallow areas [11]. The biodiversity of the Caspian Sea offers enormous scope for the discovery of novel metabolites. However, due to the lack of stud-

ies in this field, many of active metabolites are still unknown and need to be explored. The results of present investigation revealed that the marine bacteria collected from the Caspian Sea might be a potent source of novel antibiotics. Complementary investigations will be undertaken to better characterize the bioactive compounds.

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