Protatranes, effective growth biostimulants of hydrocarbon-oxidizing bacteria from Lake Baikal, Russia

Olga N. Pavlova\textsuperscript{a,c,*}, Sergey N. Adamovich\textsuperscript{b,c}, Angelina S. Novikova\textsuperscript{a}, Alexander G. Gorskhov\textsuperscript{d}, Oksana N. Izosimova\textsuperscript{a}, Igor A. Ushakov\textsuperscript{b}, Elizaveta N. Oborina\textsuperscript{b}, Anna N. Mirskova\textsuperscript{b}, Tamara I. Zemskaya\textsuperscript{a}

\textsuperscript{a} Limnological Institute, Siberian Branch, Russian Academy of Sciences, 3, Ulan-Batorskaya, 664033, Irkutsk, Russia
\textsuperscript{b} A.E. Favorsky Irkutsk Institute of Chemistry, Siberian Branch, Russian Academy of Sciences, 1, Favorsky st., 664013, Irkutsk, Russia
\textsuperscript{c} Irkutsk Scientific Center of the Siberian Branch, Russian Academy of Sciences, 134, Lermontov st., 664033, Irkutsk, Russia

**ABSTRACT**

Under natural conditions, biodegradation processes proceed slowly, especially in regions with low temperature. To activate vital processes in hydrocarbon-oxidizing microorganisms at low temperatures, biologically active compounds can be employed as growth stimulants. A low-temperature (10 °C) study has shown that tris-(2 hydroxyethyl) ammonium arylchalcogenylacetates, “protatranes” exert an effect on the growth of hydrocarbon-oxidizing strains of Rhodococcus erythropolis and Pseudomonas fluorescens, isolated from natural oil seepage on Lake Baikal. It has been found that “protatranes”, at microconcentrations, increase the growth rate of R. erythropolis bacteria by 2–16 times. It has been established that compounds slightly effect the growth of P. fluorescens. The positive effect of “protatranes” compounds on the growth rate of hydrocarbon-oxidizing microorganisms at low positive temperatures can be used for the development of environmentally benign methods for the restoration of natural objects after their contamination with oil.

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**1. Introduction**

Among the numerous substances of anthropogenic origin, that affect the environment, oil and its products are the most widespread. Almost all elements of economic infrastructure, i.e., industry, transport, and defence enterprises, face a problem of environmental pollution with oil products during production and in the case of emergencies [1]. Estimations have shown that up to 50% of crude oil gets to marine ecosystems in a natural fashion, whereas the second half is due to oil spills during production, transportation, and storage [2,3].

There are a large number of methods to remediate environmental pollution with oil and oil products [4,5]. Biological methods are considered the most promising. They are mainly based on the application of biological products containing hydrocarbon-oxidizing microorganisms as well as the activation of the microbial community of the polluted object [1,6–11].

Apart from biological components, the above products usually contain adsorbents, conservation agents, stabilizers (succrose, boric acid, benzoic acid, malachite green, and polyethylene glycol), growth stimulants (yeast extract and biotin), cryoprotectants (sucrose, gelatin, and glycerol) and nitrogen-phosphorus-potassium compounds, which are necessary for development of microorganisms [12–15]. The bioavailability of poorly water soluble and adsorbed organic pollutants can be increased with the introduction of surfactants, which are either added to the pollution sites or are produced by microorganism in situ [16]. At the same time, currently developed and patented compositions intended for remediation do not contain synthetic chemical compounds possessing physiological activity that affects the growth rate of hydrocarbon-oxidizing microorganisms.

Under natural conditions, biodegradation processes proceed slowly, especially in regions with low temperature. To activate vital processes in hydrocarbon-oxidizing microorganisms at low temperatures, biologically active compounds can be employed as growth stimulants. In light of this, the chemical compounds synthesized at the Irkutsk institute of chemistry SB RAS can be considered as potential such materials. In particular, numerous tris-(2 hydroxyethyl) ammonium arylchalcogenylacetates, so-called “protatranes”, attract special interest [17–20].
Biogenic ethanolamines (in particular, triethanolamine) and biologically active arylchalcogenylacetic acids have been employed as the starting materials for the synthesis of protatranes. Ethanolamines participate in the processes of intracellular metabolism. Their structural motif is seen in phospholipids, choline, and acetylcholine, etc. [21]. Arylchalcogenylacetic acids possess a plethora of biological activity and have found applications in medicine and agriculture. For instance, arylxyxacetic acids (Y = O) are used as regulators of plants growth [22]. Sulfur-containing acids (Y = S, SO, SO2) also show various high pharmaceutical activities [23].

Protatranes (A) have been synthesized by the reaction of arylchalcogenylacetic acids with triethanolamine (Scheme):

\[
\text{ArYCH₂CO₂H} + \text{N(CH₂CH₂OH)₃} \rightarrow \text{ArYCH₂CO₂} \cdot \text{HN}^+ (\text{CH₂CH₂OH})₃ \quad \text{(A)}
\]

where Ar=het(aryl); Y=O, S, SO, SO₂, Se.

A Compounds have an “atrace” structure (Fig. 1), usually found as colourless low-melting powders or viscous protonic alkanno-lammonium ionic liquids (PAIL) [24–29]. They are stable upon storage and highly soluble in water, alcohols, and other organic solvents [17,20,30].

Some A protatranes are non-toxic compounds (LD₃₀ = 1300–6000 mg/kg), which can be used in agriculture, medicine, clinical microbiology, and biotechnology due to their antioxidant, immunotropic, anti-allergic, antitumor, anti-metastatic, protective, growth-, and enzyme-stimulating properties. Effective growth biostimulants of microorganisms in genera such as Staphylococcus, Streptococcus, Neisseria meningitidis, Salmonella typhi surnephol, and Escherichia coli have been synthesised from protatranes and their analogs, which allows for the diagnosis time of dangerous diseases to be shortened [17,18,31].

It has been suggested that synthesised biostimulants can influence the growth rate of hydrocarbon-oxidizing microorganisms and their activity, not only at room temperature, but also at low temperatures, characteristic of soils of the northern regions and waters of the Arctic seas and Lake Baikal.

Among the most promising places for the search of psychrophilic hydrocarbon-oxidizing microorganisms are areas of natural oil seepage on Lake Baikal, which have been known since the 18th century [32], where the temperature of the surface waters in open parts of the lake seldom exceeds 9°C, even in the summer [33]. Owing to geological processes, oil, and products of its transformation, are constant components of the ecosystem of the eastern shore of the central hollow of the lake [34]. As shown earlier, in zones of oil seepage on Lake Baikal, there is a dynamic microbial community, which can effectively clean the water of pollution with oil, as evidenced by the stability of its area [35]. Aerobic oil destructors that show hydrocarbon-oxidizing activity at the lower temperatures (4°C), and biological surfactants, have been isolated from samples of water and bottom sediments of these areas [36].

The ability to degrade hydrocarbons is inherent in the genomes of the Baikal microorganisms, and this is dependent on the existence of alkB genes that are responsible for the synthesis of alkane-1-monooxygenases, which allows for the aerobic degradation of a wide range of normal alkanes [37,38].

The presence of hydrocarbon-oxidizing activity genes in the genomes of microorganisms isolated from areas of natural oil seepage on Lake Baikal enables their use as test-objects for the development of new technologies for biodrugs design.

The present work is aimed at examining compounds from a number of A protatranes:

\[
\begin{align*}
2\text{-CH₃-C₆H₄OCH₂CO₂} \cdot \text{HN}^+ (\text{CH₂CH₂OH})₃ & \\
4\text{-Cl-C₆H₄SCH₂CO₂} \cdot \text{HN}^+ (\text{CH₂CH₂OH})₃ & \\
3\text{-Cl-C₆H₄SO₂CH₂CO₂} \cdot \text{HN}^+ (\text{CH₂CH₂OH})₃ &
\end{align*}
\]

as growth biostimulants of hydrocarbon-oxidizing bacteria Rhodococcus erythropolis (No 4–08) and Pseudomonas fluorescens (No 5–05) isolated from Lake Baikal.

2. Materials and methods

2.1. General procedure for synthesis of compounds 1–3

A solution of tris-(2-hydroxyethyl)amine (triethanolamine) and the corresponding arylchalcogenylacetic acid (in 1:1 M ratio) in ethanol was heated at 65°C for 15–30 min, and then allowed to stand at 20–22°C for 1 h. The mixture was poured into diethyl ether (absolute), which was also allowed to stand for 12 h at 5–10°C. The residue was filtered off, washed with ether, and dried in a vacuum to give colourless powders, which are highly soluble in water and alcohol [17–19].

2.2. Objects of investigation

The microorganisms Rhodococcus erythropolis (No 4–08) and Pseudomonas fluorescens (No 5–05) were isolated as pure cultures from the area of natural oil seepage near Cape Gorevoy Utes (Central Baikal).

The strain No 4–08 (R. erythropolis) was isolated from bitumen structures, which are formed during oil seepage at the bottom / water boundary [34]. The strain is capable of growing at wide range of temperatures (±4–±37°C) and various levels of salinity (3–5%). It possesses hydrocarbon-oxidizing activity: it oxidises n-alkanes C₁₅–C₂₉ by 60–100% over 92 h at 22°C, while C₂₃–C₂₉ hydrocarbons are utilised completely under the action of this strain. The consumption of hydrophobic hydrocarbon substrates by R. erythropolis is accompanied by the synthesis of surface-active glycolipid compounds. It has been found that its genome contains alk-genes (alkanehydroxylases) responsible for the degradation of numerous n-alkanes [39].

The strain No 5–05 (Pseudomonas fluorescens) was isolated from waters near the oil seepage. The experiments have shown that the strain P. fluorescens (No. 5–05) oxidises up to 35% of n-alkanes C₁₀–C₂₄ over 36 days at 4°C. At the same time, short-chain alkanes are not accumulated in the medium, which indicates that this strain oxidizes these alkanes to simpler compounds [35].

2.3. Experimental growth conditions for the hydrocarbon-oxidizing strains of R. erythropolis and P. fluorescens

To evaluate the effect of compounds 1–3 on the growth of the microorganisms, the cultivation was carried out in mineral medium of the following composition [g/L]: KH₂PO₄: 1.0, K₂HPO₄: 1.0; CaCl₂·2H₂O: 0.02, MgSO₄·7H₂O: 0.2, FeCl₃: 0.05, NH₄NO₃: 1, pH 7.2. The mineral medium (100 ml) was added to experimental flasks (250 ml), along with oil (50 µL), a suspension of a strain (1 mL),
The bacteria), concentration, medium and dehydrated solution 0.0001% were prepared directly in the experiment. After hydrocarbon-oxidizing oil was prepared, it was used as the internal standard, added to an experimental mixture. The mixture was vigorously shaken and allowed to stand until complete phase disengagement. Then an aliquot was collected from the lower phase (chloroform) and transferred to an autosampler flask of the chromatograph with a division of flow into the injector at a ratio of 1:10. The analysis was carried out under the following conditions: capillary column OPTIMA-17 ms, 30 m, internal diameter of 0.32 mm, the temperature of the injector was 280 °C; temperature of the column was 50 °C, 5 min, then a gradient of temperature of 15 °C/min, from 50 to 310 °C and left in isothermal mode at 310 °C for 15 min; a flow of helium 1 mL/min. Chromatograms were registered in the mode of selective ion detection with m/z: 57 and 71. Concentration of n-alkanes was calculated as the average value for three parallel experiments. The determination error did not exceed 15%.

2.4. Evaluation of hydrocarbon-oxidizing activity of R. erythropolis

The hydrocarbon-oxidizing activity of R. erythropolis was determined after implementation of the main cultivation experiment in the presence of compound 2 for the seventh day. The degree of oil utilization under the action of the microorganisms was evaluated by the decrease of n-alkane C₆–C₃₃ homologs using a chromatographic method on the 7th day of the experiment. To isolate the n-alkane fraction, chloroform (4.5 mL) and squalane solution (0.5 mL in chloroform (0.3 mg/mL⁻¹)), used as the internal standard, were added to an experimental mixture. The mixture was vigorously shaken and allowed to stand until complete phase disengagement. Then an aliquot was collected from the lower phase (chloroform) and transferred to an autosampler flask of the chromatograph with a division of flow into the injector at a ratio of 1:10. The analysis was carried out under the following conditions: capillary column OPTIMA-17 ms, 30 m, internal diameter of 0.32 mm, the temperature of the injector was 280 °C; temperature of the column was 50 °C, 5 min, then a gradient of temperature of 15 °C/min, from 50 to 310 °C and left in isothermal mode at 310 °C for 15 min; a flow of helium 1 mL/min. Chromatograms were registered in the mode of selective ion detection with m/z: 57 and 71. Concentration of n-alkanes was calculated as the average value for three parallel experiments. The determination error did not exceed 15%.

Fig. 2. Changes in the number of hydrocarbon-oxidizing bacteria of the R. erythropolis (A) and P. fluorescens (B) during oil destruction in the presence different concentrations of protatranes 1–3.
3. Results

The investigations have shown that protatranes 1–3 exert a different effect on growth of two strains of the microorganisms (Fig. 2), which considerably differ in their physiological and biochemical properties from each other [35,38]. In addition, microorganisms of the genus Rhodococcus have previously shown higher growth rates and generation times than those of the genus Pseudomonas while growing on hydrocarbon substrates [40].

All three tested compounds influenced the growth rate of R. erythropolis strain (No. 4–08). Under conditions of periodic cultivation in mineral medium with oil but without addition of compounds 1–3 [control], a longer (up to 24 h) growth lag-phase was observed. Exponential growth of R. erythropolis began after 24 h, with its specific growth rate being increased by an order (from 0.002 h\(^{-1}\) to 0.02 h\(^{-1}\)). After introduction of compound 1 in concentrations of 10\(^{-4}\)–10\(^{-8}\) wt. %, the lag-phase decreased and growth rate ranged from 0.007-0.03 h\(^{-1}\). Further, by the 4th day of cultivation, in the presence of compound 1 (concentration 10\(^{-4}\)–10\(^{-8}\) wt. %, growth rate 0.03 h\(^{-1}\), generation time ~20.6 h), the number of the microorganisms increases by 7–9 times (Fig. 2a). At the same time, in the control sample on the 4th day the growth rate is 0.005 h\(^{-1}\) and generation time is 138 h. The introduction of compound 1 at a lower concentration (10\(^{-7}\), 10\(^{-8}\) wt. %) did not exert a significant impact on growth of the microorganism.

The effect of compound 2 on the growth rate of R. erythropolis was observed by the third day of the experiment (Fig. 2a). In this case, concentrations of 10\(^{-6}\)–10\(^{-8}\) wt. % were the most effective, with the number of microorganisms increasing by 2–2.8 times. Notably, the growth rate is 0.06–0.07 h\(^{-1}\), which corresponds to a generation time of 10 h, while for the control \(\mu = 0.02 \text{ h}^{-1}\) and the generation time is 35 h. At higher concentrations (10\(^{-4}\)–10\(^{-5}\) wt. %), growth of the microorganism is inhibited in comparison with the control.

When compound 2 is used at concentrations of 10\(^{-4}\)–10\(^{-5}\) wt. %, growth inhibition and low hydrocarbon-oxidizing activity is detected for R. erythropolis. If the bacteria are cultivated in the experimental medium containing 10\(^{-4}\)–10\(^{-6}\) wt. % of compound 2, the number of normal alkanes decreases by 38–50% during the 7 days of the experiment. Meanwhile, the application of compound 2 in a concentration of 10\(^{-7}\) wt. % increases the number of bacteria by 3 times and significantly decreases the number of n-alkanes (up to 74%). It should be noted that in the control samples containing nutrient medium, oil, and microorganisms without compound 2, the number of normal alkanes is reduced by 70%. Thus, most likely, the tested compound stimulates a growth of the microorganisms, but does not significantly affect their hydrocarbon-oxidizing activity (Fig. 3).

Compound 3 at a concentration of 10\(^{-4}\) wt. % appears to be the most effective biostimulant. The addition of this compound significantly affects the growth of microorganisms (Fig. 2a). For instance, in the presence of compound 3, the number of bacteria increases by 16 times on the 4th day of experiment. The growth rate is 0.04 h\(^{-1}\) and the generation time is 16 h, while for the control samples these parameters are 0.01 h\(^{-1}\) and 38 h, respectively. Lower or higher concentrations of this substance did not significantly influence the growth of the microorganisms.

The introduction of compounds 1–3 to the culture medium has a low effect on the growth of P. fluorescens strain (No. 5–05). In the presence of compound 1 at a concentration of 10\(^{-6}\)–10\(^{-7}\) wt. %, the growth rate (1st day of cultivation) increases to 0.02 h\(^{-1}\) in comparison with the control (0.004 h\(^{-1}\)). The increase in the number of microorganisms is observed only at concentrations of 10\(^{-4}\) and 10\(^{-6}\) wt. % and only by the seventh day of cultivation (Fig. 2b). At the same time, despite the increase in the number of the bacteria after 168 h of cultivation (concentration of compound 1 = 10\(^{-4}\) and 10\(^{-6}\) wt. %), the growth rate of the bacteria is low, being between 0.004 – 0.006 h\(^{-1}\) (generation time = 114–171 h) and comparable with that of the control sample (\(\mu = 0.004 \text{ h}^{-1}\), generation time ~ 175 h).

Upon addition of compound 2 at concentrations of 10\(^{-4}\) and 10\(^{-6}\) wt. %, the number of microorganisms increases only in the first 24 h of the experiment (Fig. 2b). When compound 2 is introduced at a concentration of 10\(^{-4}\) wt. %, the growth rate increases by an order (0.04 h\(^{-1}\)) as compared to the control (0.004 h\(^{-1}\)). After seven days of the experiment, the growth rate in the presence of compound 2 at various concentrations was comparable to that of the control.

The effect of compound 3 on characteristics of P. fluorescens strain (No. 5–05) is observed only at a concentration of 10\(^{-6}\) wt. %. In the control samples, which contain none of the tested compound, growth acceleration was found by the 1st day of the experiment (\(\mu = 0.01 \text{ h}^{-1}\)). In 48 h, the specific growth rate increases twice and reaches 0.02 h\(^{-1}\) (generation time ~31 h). In 72 h, the growth rate slows down, indicating that it has reached stationary phase. After the addition of compound 3 at a concentration of 10\(^{-6}\) wt. %, the number of the bacteria increases

![Fig. 3. Content of n-alkanes in oil before the experiment (µg/mL\(^{-1}\)) □ after seven days of cultivation of R. erythropolis without the addition protatranes 2 □ after seven days of cultivation of R. erythropolis in the presence of protatranes 2 in different concentrations □](image-url)
by the 3rd day of the experiment (Fig. 2b). Introduction of compound 3 to the medium inhibits the growth phase for this strain by one day.

4. Discussion

*Pseudomonas* spp. and *Rhodococcus* spp. are often isolated from hydrocarbon-contaminated sites and hydrocarbon-degrading cultures [41]. These two genera have a broad affinity for hydrocarbons and can degrade selected alkanes, alicyclics, thiophenes, and aromatics. Typically, *Rhodococcus* spp. are more hydrophobic and have a higher affinity for hydrocarbon-water interfaces than *Pseudomonas* spp., suggesting that these strains utilize different modes of hydrocarbon access [41,42]. In spite of the fact that *Rhodococcus* and *Pseudomonas* species are the most frequent participants in the processes surrounding hydrocarbon biodegradation, in marine ecosystems the obligate marine group of hydrocarbonoclastic bacteria (OMHCB) has been found. *Rhodococcus* sp. and *Pseudomonas* sp. cannot be referred to this group, since they belong to the heterotrophic community and are present both in contaminated and uncontaminated ecosystems. Hydrocarbons are biodegraded by *Rhodococcus* and *Pseudomonas* bacteria via syntrophic metabolism [43,44]. Unlike other heterotrophic microorganisms, *Pseudomonas* spp. and *Rhodococcus* spp. contain a number of enzymes capable of oxidizing hydrocarbons (alkanehydroxylases), and are able to absorb a hydrophobic substrate. The latter ability is closely connected to the structural peculiarities and metabolic organization of the hydrocarbon-oxidizing bacteria. Direct contact of cells with a substrate plays a crucial role in the processes of hydrocarbon oxidation. Therefore, the ability or inability of a microorganism to absorb hydrocarbons depends on the structure and composition of the cell wall and, first of all, is defined by the presence of a hydrophobic cellular surface [45,46]. The ability of *Rhodococcus* sp. to emulsify and degrade various hydrocarbons is mainly due to structural features of their cellular shell. The cell wall of these bacteria is highly lipophilic and has a high affinity for hydrophobic substrates, which is absent in other bacteria [47]. Apart from lipids, the cell wall of *Rhodococcus* sp. includes mycolic acids [48], which provide advantages to hydrocarbon-oxidizing bacteria in the course of substrate sorption. The formation of bioemulsifiers is also characteristic of *Pseudomonas* bacteria [11]. By emitting bioemulsifiers to the external environment, the bacteria reduce the hydrophobic properties of hydrocarbons and promote their solubilisation. However, a more complex cell wall structure, and the absence of teichoic and mycolic acids, hinders the intake of some substances into the cell. It is probable that the effect of the synthesized protatranes 1–3 on growth rate is defined by the structural peculiarities of the cell wall of the two different microorganisms.

As mentioned above, protatranes 1–3 can be referred to as PAIL [24–29]. Previously, it has been shown that some PAIL, having unusual physical and chemical properties and consisting of biologically active ammonium cations and proton acid anions, can penetrate faster through cellular membranes in comparison with other compounds [28]. It is assumed that successful transport of PAIL is due to the fact that they penetrate through a membrane in the form of hydrogen-bonded complexes. This renders ionic pairs and their aggregates “more neutral” in character, thus facilitating penetration through a model membrane. Possibly, the transport of biostimulators 1–3 inside *Rhodococcus* sp. bacteria occurs similarly.

A high specific biological activity of protatranes A, in particular 1–3, is explained by a favourable combination of several effects in their molecules. First, ethanolamines and arylchalogenyloblastic acids exert a so-called double action [49]. Second, protatranes have an unusual tricyclic structure containing the intramolecular bond N−H. Such a structure leads to a high dipole moment of the molecule and increased electronegativity of the equatorial oxygen atoms of the protatran skeleton. The positive end of the dipole is a nitrogen atom. Evidently, hemosorption of the protatranes on the surfaces of biological membranes, and their further penetration into a living cell, occurs due to the hydrogen bonds and dipole-dipole interaction with polar groups of proteins and lipids. An in-depth study of the mechanism of the physiological action of “atranes”, in particular “protatranes” A, on microorganisms, plants, fish and animals is the subject of our further research [50].

The results obtained show that protatranes A are promising biostimulators of the growth, development, and activities of oil destructive bacteria. Among the advantages of synthetic biostimulators 1–3 are their low cost, high solubility in water, stability upon storage, non-toxicity, and efficiency at low (1·10⁻⁶–1·10⁻⁸ wt. %) concentrations. The found positive effect of “protatranes” compounds on the growth rate of hydrocarbon-oxidizing microorganisms at low positive temperatures can be used for the development of environmentally benign methods for restoration of natural objects after their contamination with oil.

5. Conclusion

In conclusion, we have demonstrated that synthesised protatranes A compounds 1–3 are biostimulators of the growth of hydrocarbon-oxidizing *R. erythropolis* bacteria isolated from the area of oil seepage on Lake Baikal. The addition of compounds 1–3 to cultural medium at a low temperature (10°C) in micro-concentrations (10⁻⁵–10⁻⁸ wt. %) can significantly (by 2–16 times) increase the rate of *R. erythropolis* generation time, depending on the cultivation duration, concentration, and type of anion. The effect of compounds 1–3 on growth of *P. fluorescens* is less pronounced. Probably, the positive influence of biologically active protatranes 1–3 on *R. erythropolis* is due to structural peculiarities of the cell wall of these bacteria. The original composition and unique structure of compounds 1–3 promotes their penetration into the cell of oil destructive bacteria.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the State Task of the Ministry of Education and Science of the Russian FederationNo. 0345-2019-00007 (obtaining pure cultures of microorganisms, chromatographic analysis, experiments), the Russian Foundation for Basic Research (RFBR) and the Government of the Irkutsk Region in accordance with research project No. 17-43-380006 (synthesis of biostimulants). The study was carried out as part of the Integration Program of the Irkutsk Scientific Center of the SB RAS “Basic Research and Breakthrough Technologies as the Basis of the Advance Development of the Baikal Region and its Interregional Relations” (synthesis of biostimulants).

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