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SCREENING FOR CYTOTOXIC EFFECT OF MARINE ACTINOMYCETES FROM SEDIMENTS OF SOUTH COSTAL REGIONS OF INDIA

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ABSTRACT

Marine actinomycetes are known to be rich sources of new bio active compounds. However, only a very small proportion of microbial organisms living in this environment have been isolated and even fewer successfully grown in the laboratory. To facilitate this avenue of research, there is a need for new methods to collect, isolate and identify marine microorganisms as well as new source for the isolation. The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening disease cancer. Marine soil sample from different depths at two different sites were collected and all the samples were subjected for the analysis of the actinomycetes diversity. Actinomycetes were counted in five different soil depths, at two different sites of east cost. The actinomycetes counts were found maximum in the soil sample of luxuriant site than that in degrading site. The reason can be attributed to the level of nutrients available in the different marine ecosytem. Marine region is goldmine of biodiversity has been amply justified by richness of floral and faunal diversity and now also microbial diversity. This project investigation has established the rich antifungal actinomycetes diversity and promising bioactive potential of the actinomycetes isolate MNG3 of Rameswaram costal region. This study revealed that costal region of Rameswaram is a potential source for wide spectrum of antimicrobial and anticancer metabolite producing actinomycetes. Findings of the present study conclude that marine environment actinobacteria are the potential ecosystem for antagonistic actinobacteria which deserves for bioprospecting. Isolation of actinomycetes from marine sediment of Rameswaram is successfully achived. *In vitro* screening of the actinomycete strains for antimicrobial activity with special emphasis on anticancer activity shows positive results .and one of the most promising potential isolate was selected and with anticancer activity is completed and Characterization of the isolate by physico chmical property is also completed followed by International streptomyces project

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INTRODUCTION

Actinomycetes are filamentous Gram-positive Bacteria. The marine forms are a huge source of medically important compounds. Actinomycetes are the most common microbes on the planet, and are the source of almost 70% of the worlds naturally occurring antibiotics (Ambaranc et al., 2014). The discovery of most of the antibiotics stemmed from 60 years of research on soil-dwelling actinomycetes.

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Cancerous research using actinomycetes are very low in India Cancer is a term referring to a large group over a hundred different diseases that arise when defects in physiological regulation cause unrestrained proliferation of abnormal cells. In most cases, these clonal cells accumulate and multiply, forming tumours that may compress, invade and destroy normal tissues, weakening the vital functions of the body with devastating consequences including loss of quality of life and mortality. Nowadays, cancer is the second cause of death in the developed world, affecting one out of three individuals and resulting in one out of five deaths world-wide (Fernandez et al., 2002).

Goal of the current pharmacological therapies of cancer is to reduce the number of tumour cells and prevent their further clonel accumulation. Unfortunately, some forms of cancer are unresponsive or become resistant to conventional anti-cancer treatments, implying the need to discover and develop novel therapeutic agents with innovative mechanism of action for an effective systematic treatment of the diseases. The sea covering more than 70% of the surface of plant earth, contains an exceptional biological diversity, accounting for more than 95% of the whole biosphere (Fernandez et al., 2002). During evolution, marine organisms have developed complex adaptive and self-protecting mechanism to survive, often associated with the production of structurally unique chemical entities. This natural biochemical arsenal constitutes a promising source of innovative anti-cancer drugs; with a 100- fold higher potential to yield successful drug candidates than its terrestrial successful counterpart (Fernandez et al., 2002). This study is first attempt for anticancer activity form antimicrobial activity. It shows positive result from fungi and from cell lines.

MATERIALS AND METHODS

Marine sample collection: Soil sample was collected from Pamban bridge marine sediment located near mandapam, 3 km west of the Rameswaram town. The bridge is well protected and is located within the boat house premises. The mud sample was collected (with permission from boating authorities) from 8-10 points (periphery and the middle section of the sea shore) within the marine sediment using a mud sampler. All the sub-samples collected from the marine sediment were immediately transferred to a sterile polythene bottle sealed, labeled and transported to laboratory. In the laboratory the composite mud sample was spread in a clean aluminium tray and dried in a hot air oven for seven days at 40°C. The dry mud sample after removing large debris was transferred to a fresh clean polythene bag, secured, labeled and stored at 4°C until examined.

Isolation of actinomycetes using pour plate method: One-milliliter aliquots of each of the two dilutions (10^{-6},10^{-7}) were aseptically transferred to sterilized Petri plates. Two sets of five plates for each dilution were prepared. To one set of plates starch casein agar (45°C) (Kuster and Williams, 1964) was added at the rate of 20ml/plate and swirled gently for uniform distribution of the inoculum with the medium. To another set of plates molten (45°C) starch ammonium sulphate agar was added at the rate of 20ml/plate and mixed thoroughly. All the plates were incubated under laboratory conditions (25±2°C). Plates were examined for the appearance of actinomycete colonies from 2nd day onwards and up to 30-40 days. Total number of colonies in each plate and in each medium was recorded. This procedure was repeated for the other marine sediment sample too. Selected colonies from the plates were sub-cultured on PDA slopes and incubated for 7-10 days. When sufficient growth has occurred in the tubes the tubes were stored at 4^{o}C in a refrigerator.

Test organisms: The test organisms, six filamentous fungi *Rhizoctonia solani*, *Fusarium solani*, *Drechslera oryzae*, *Colletotrichum gloeosporioides*, and *Curvularia lunata* used in this screening program was procured from the Institute of Microbial Technique and Culture Collections, Chandigarh, Pune, India.

Dual culture screening: Four different isolates were point inoculated around the periphery of the plate at equidistance. All the plates were then incubated for ten days at 25±2°C. After ten days the plates were inoculated with a mycelial plug (5 mm dia.) of actively growing *Rhizoctoniasolani, Colletotrichum gloeospoioides, Drechslera oryzae* and *Fusarium oxysporium* and *Curvularia lunata* plate cultures. Separate plates were inoculated for each fungus. Plates were observed for growth inhibition after 3-8 eight days depending on the fungus. Reduction in radial growth was measured and recorded.

Preparation of culture filtrate: Culture filtrate (CF) containing inhibitory metabolites of the isolate as prepared by growing the isolate in Potato-Dextrose Broth (PDB). Fifty-milliliter portions of PDB (pH 7.2 before sterilization) was poured into six 250 ml conical flasks and sterilized in an autoclave for 20 minutes at 15psi. The broth was then inoculated with a 100µl of five days old broth culture and mixed well. All the inoculated flasks were incubated as static culture at room temperature (25±2°C) for twenty days. At the end of the incubation period two observations were made ie. type of growth – submerged growth or surface growth or both and colour of the culture broth. Then the broth was filtered through a handmade filter paper to separate the mycelial biomass and the liquid containing metabolites. The broth was then centrifuged at 3000 rpm for 10 minutes to remove spores and mycelial bits. The clear supernatant was used for bioassay. At this stage pH of the culture filtrate was also recorded in a digital pH meter. The Lethal activity of the cell lines were primarily tested with the active isolate only

Colony characterization: Characterization of the selected isolates was carried out according to the methods recommended by International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966). Spore chain and sporophore morphology of a mature colony were determined under light microscope. Colour of aerial surface growth, colour of substrate mycelium and diffusible pigments, other than melanin were also determined. Whenever diffusible colours other than brown or black were produced on any medium, the colour was also recorded. Production of melanin pigments on tyrosine agar (ISP-7) and tryptone-yeast extract agar (ISP-1) was also observed. The various media used and their compositions are given below:

Growth in different pH: Ability of the isolates to tolerate a wide range of pH was tested in PD broth. pH of the PD broth was adjusted with 0.1N NaOH or 0.1N HCl to get 4, 5, 6, 7, 8, 9, 10, 11 and 12 and sterilized following standard procedures. The liquid medium (15ml) in test tubes was inoculated with 100µl of spore suspension and incubated at 25±2°C for 15 days. After 15 days the tubes were scored for growth and recorded.

Growth in different temperatures: Growth in different temperatures was tested by incubating PDA slants inoculated with spore suspension of the test isolate at the following temperatures 20, 25, 28, 35, 40 and 50°C in an incubator for 15 days.

Sodium chloride tolerance: Salt tolerance studies were carried out on Glucose-yeast extract-malt extract medium as the basic medium. The NaCl concentrations (w/v) used were: 0%, 2%, 4%, 6%, 8%, 10%, 12%, 15%, 18% and 20%. The slants were inoculated by streaking the agar surface with a loop full of spore suspension of the isolate. The inoculated tubes

were incubated at 25±2°C for 20 days. After 20 days presence or absence of growth in different salt concentrations was recorded.

Utilisation of different carbon sources: Ability of the test strain, to use 20 different sugars as sole carbon sources for energy and growth was examined in carbon utilization medium suggested by Shirling and Gottlieb (1966). The various compounds were added to the liquid medium to get a final concentration of 1%. The inoculated tubes were incubated at 25±2°C for 20 days. The test strain was also inoculated on the basal medium (without any carbon source) which constituted negative control and a positive control with glucose (1%) was also included. A positive result was recorded when growth was greater than that in the negative control and that equal to or less than that in the negative control as negative.

RESULTS AND DISSCUSSION

The actinomycete isolates were obtained from the marine sediment samples in Starch casein agar (plate.1) Pure cultures were stored on PDA slops until use (Plate.2). All the 32 isolates were screened *in vitro* for antimicrobial and anticancer activity. Based on the intensity of the reaction of the test pathogen to the actinomycete isolates, culture filtrate and the width of the inhibition zones and changes of test organism or cell lines respectively, the response was grouped into four categories as shown in the Table 1.

Table 1. Summary of the antifungal activity of marine actinomycetes

Sl. No.	Nature of Response	Inhibition zone (mm)	No.of Isolates
1	Strong antibiosis	>7-10 mm	3 (9.4%)
2	Moderate antibiosis	3-6 mm	5 (15.6%)
3	Weak antibiosis	<3 mm	11 (34.4%)
4	No visible effect	Nil	13 (40.6%)

Among the five fungi tested *Fusarium solani* was totally resistant to the antagonist. *Fusarium solani* was the least sensitive with 14% inhibition. Out of the five remaining fungi *Rhizoctonia solani* was found to be highly susceptible to the antagonist with 75% growth suppression followed by *Drechslera oryzae* (40%), *Colletotrichum gloeosporioides* (23%) and *Curvularia lunata* (23%). (Plate- 3). Culture filtrate at 10% concentration reduced radial growth by 51%. When a sub-lethal dose of 2 µg/ml was integrated with 10% CF completely inhibited mycelial growth (100%). In another experiment growth of the biocontrol agent *Trichoderma harzianum* on PDA poisoned with 10% CF alone and in combination with the fungicide carbendazim at the rate of 2 µg/ml was tested. (Athoor. 2002 and Bardy. 2005).The CF had no adverse effect on the growth of the biocontrol agent but integration of CF (10%) with fungicide (2µg/ml) completely arrested mycelial growth. However, *Trichoderma* grew after 7 days and the colony remained white and no sporulation was noticed till 15th day. The same combination was tested for its effect on sclerotial germination too. The culture filtrate had no inhibitory effect on sclerotial germination. But the hyphal extension was slower on CF (10%) amended plates than in control plates. But integration of the fungicide at the above concentration completely inhibited sclerotial germination.

Plate 1. Isolation of marine actinomycetes on SCA

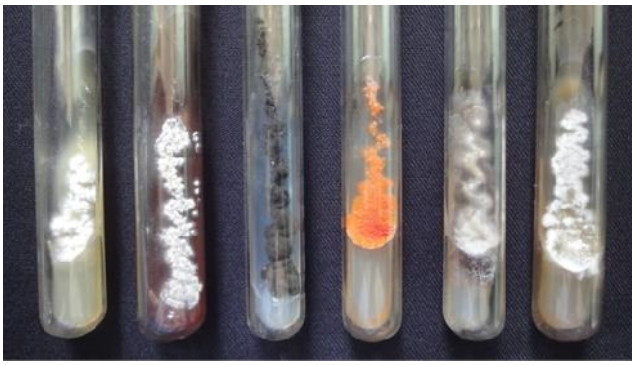
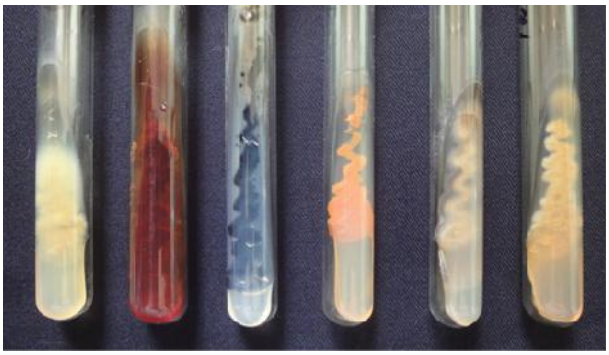


Plate 2. Pure culture on PDA slants



Reverse side of the Pure culture on PDA slants

The ability of the antagonist to tolerate and grow in the presence of a fungicide and an insecticide was also tested in broth culture. (Bernan 2004., Cragg.2013 The antagonist not only tolerated fungicide concentration ranging from 10 µg/ml to 60 µg/ml but also grew well in all the concentrations tested. It was also observed that growth was more in higher concentrations than in lower concentrations. Similar growth trend was noticed in tubes amended with the insecticide monocrotophos at the rate of 10 µg/ ml to 60 µg/ml. Here again growth was more in higher concentrations than in lower concentrations.

Cytotoxicity of the culture filtrate: Cytotoxicity study of the culture filtrate against various cell lines were observed it reveled the anticancer property of the actinomyctes. (Gangwar et al.2015).This culture filtrate was found more active against lung cancer cell lines then the other tested cell lines is presented in the table **(**Table. 2**)** the cell lines treated with 5µl of culture filtrate showed lysis of the cell lines as well as fungicidal activity of the tested organism (Elamvaghuthi et al. 2013) (plate 4).

In order to study whether the cytotocity of the culture filtrate accelerate the apotopic changes, cells were examined for nuclear condensation. In the tested cell lines and fungi, nuclear condensation and fungicidal effect were observed at 24h incubation stained with acridine orange/ethidium bromide/ acetocarmine. Whereas the untreated control cell were healthy. Finally an attempt was made to partially purify the inhibitory principle from the culture filtrate. The four fractions (aqueous, methanol, chloroform and ethyl acetate) were tested for their toxicity towards mycelial growth of the test (Ravikumar et al. 2012).

Table 2. Cell viability test of various cancer cell lines treated with the culture filtrate of active isolate

Cell lines	C.F. Conc. µl	% of live cells	% of Cytotoxicity
	0.5	101.13	-1.13
Colan cancer	1.0	96.62	3.38
	2.0	79.96	20.38
	5.0	76.84	23.16
Breast cancer	0.5	93.21	6.79
	1.0	92.85	7.15
	2.0	85.96	14.04
	5.0	80.97	19.03
Cervical cancer	0.5	99.00	1.00
	1.0	96.89	3.11
	2.0	82.53	17.47
	5.0	79.59	20.41
Lung cancer	0.5	85.53	14.47
	1.0	76.68	23.32
	2.0	62.72	37.28
	5.0	58.79	41.21

fungus *R. solani.* Among the four fractions tested methanol fraction was found to be active and inhibited mycelial growth to a certain extent than the other fractions. The methanol ane Eac fraction also found active on lung cancer cell lines. Morphological changes in the cell lines. A. control, B. treated with 5µl of culture filtate

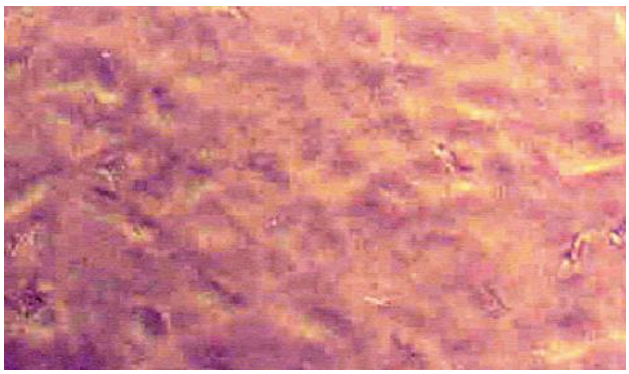


Fig: A

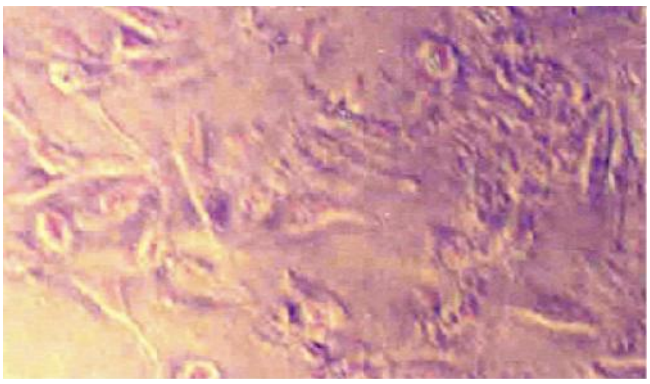


Fig: B

Plate. 4. Cytotoxicity of the actinomycetes culture filtrate MNG3

Colony cherectirization: Colony characterization of the isolate MNG3 was done on different media as recommended in the literature. The data is presented in (Table- 3.) The antagonistic actinomycete grew well only on PDA and Benett's agar. Moderate growth was recorded on ISP-5, ISP-7 and SCA. Less growth was noticed on ISP-1, ISP-3, ISP-4 and poor growth on ISP-2. Aerial mycelium was grey in all the media tested except in ISP-3, wherein it was white in colour. Basal mycelium was light yellow or with yellow shade on ISP-1, ISP-4, ISP-5, SCA and NA; colourless on ISP-7 and PDA and light brown in Benett's agar. On Czapek's agar growth was very poor and very slow with cream colour basal mycelium. Reverse side of the colony was in different shades of yellow in ISP-1, ISP-2, ISP-3, NA and colourless in ISP-4. Light yellow diffusible pigment was noticed in nutrient agar (NA) and in potao dextrose agar (PDA). No melanin pigment was produced by the isolate MNG3.

Physiological and biochemical properties of the isolate MNG3 are summarized in (Table-4). The colony grew well on medium without sodium chloride (0%), but even better growth was noticed at 2% NaCl amended medium. Between 4% to 8% salt concentration growth was moderate and at 10% growth was poor. Beyond 10% NaCl level no growth occurred. Effect of pH on growth of the antagonist was studied at a pH range of 4 to 12. The actinomycete could tolerate a pH range of 6 to 12. No visible growth at pH 4 and 5. Growth was poor at pH 6, moderate at pH 7, whereas equally good growth was recorded at pH 8 and 9. Between pH 10 to 12 moderate growth was noticed.

Temperature requirement for growth showed growth between 20ºC to 35 ºC with optimum at growth at 28ºC. The actinomycete showed no sign of growth at 40 ºC and 50ºC. The isolate MNG3did not produce melanin in both ISP-1 and ISP-7. But light yellow to yellowish brown diffusible pigment was noticed in Nutrient agar and PDA. Further it was noticed that the antagonist was catalase positive and could hydrolyze starch and casein. The pattern of utilization of different sugars as sole source of carbon is given in (Table 5). Among the 20 carbon sources tested the isolate readily utilized dextrose, dulcitol, lactose, maltose, mannitol, mannose and rhamnose for growth as sole source of carbon. Moderate growth was notice in adonitol, fructose, inulin, raffinose, salicin, sucrose, xylose, inositol and sorbitol amended media. A weak positive response was recorded in cellobiose, galactose, melibiose and trehalose amended media.

The above observations confirm the earlier report by Turhan and Grossman (1986), who observed that among the six soil borne fungi *R. solani* and *A. alternata* were relatively resistant against the antibiotic effects of the actinomycetes. Again in 1994, the same authors in another study observed that *R. solani* and *Pythium debaryanum* were least sensitive to *Myrothecium* isolates.From the 2 isolates with strong antagonistic activity one isolate MNG3 was selected for anticancer investigation. The few recent studies that focused on the chemical characterization of bioactive compounds produced by actinobacteria support the potential isolation of novel molecules with biological activity (Carr et al., 2012). Thus, an exploration program of isolation of bioactive molecules from actinobacteria from marine sediments certainly will result in the discovery of novel compounds with activity against cancer cell lines and microorganisms that are potentially pathogenic to humans.

Table 3. Colony characteristics of the Isolate MNG3

Medium	*Colony characters*					
	Growth	Aerial mycelium	Basal mycelium	Reverse side	Diffusible pigment	Melanin
ISP- 1	++	White - grey	Cream	Light yellow	-	-
ISP- 2	+	Light grey	very less Cream	Pale yellow	-	-
ISP- 3	++	White	Cream	Light yellow	-	-
ISP- 4	++	Grey	Light yellow	-	-	-
ISP- 5	+++	Grey	Cream	Dirty – pale green	-	-
ISP- 7	+++	Grey with white dots	Colourless	Dirty	-	-
CZ	+	White	Cream	Cream	-	-
SCA	+++	Grey	Light yellow	-	-	-
NA	++	Grey	Light yellow	Yellow	Light yellow	-
PDA	++++	Grey	Colourless	Brown	Light yellow	-
BA	++++	Grey	Dirty brown	Brown –dirty yellow	-	-

(++++) = Good growth, (+++) = Moderate growth, (++) = Less growth (+) = Poor growth, (±) = Doubtful, (-) = No growth

Table - 4. Physiological and biochemical properties of Isolate MNG3

Reaction	Response	Result
Melanin Reaction		
Medium ISP-1.	No browning	Negative
Medium ISP-7	No browning	Negative
Tyrosine Reaction		
Medium ISP-7	No browning	Negative
Soluble pigment		
Nutrient agar	Light Yellow	Positive
PDA	Light yellowish brown	Positive
Starch Hydrolysis	Zone appeared	Positive
Casein hydrolysis	Zone appeared	Positive
Catalase	Brisk effervescence	Positive
Growth at (°C)		
20°C	+	Poor growth
25°C	++	Moderate growth
28°C	+++	Good growth
35°C	+	Poor growth
40°C	-	No growth
50°C	-	No growth
NaCl Tolerance (%)		
0%	+++	Good growth
2%	++++	Good growth
4%	++	Moderate growth
	++	Moderate growth
8%	++	Moderate growth
10%	+	Poor growth
12%	-	No growth
15%	-	No growth
Growth at pH		
4	-	No growth
5	-	No growth
6	+	Poor growth
7	+++	Moderate growth
8	++++	Good growth
9	++++	Good growth
10	++	Moderate growth
11	++	Moderate growth
12	++	Moderate growth

(++++) = Good growth, (+++) = Moderate growth, (++) = Less growth (+) = Poor growth, (±) = Doubtful, (-) = No growth

Table 5. Carbon utilization pattern by Isolate MNG3

Utilization	Carbon source
Positive	Dextrose, Dulcitol, Lactose, Maltose, Mannitol, Mannose and Rhamnose (++++)
Moderate	Adonitol, fructose, Inulin, Raffinose, Salicin, Sucrose and Xylose (++), Inositol, Sorbitol, (+++)
Weak positive	Cellobiose, Galactose, Melibiose, Trehalose (+)

CONCLUSION

The isolate MNG3 was aerobic, Gram-positive and grew well on PDA and Bennet's agar. Aerial mycelium was grey; basal mycelium yellow; reverse side of the colony yellow. Light yellow diffusible pigment was produced; no melanin was produced. Tolerated 0-10% NaCl level but grew well at 2% NaCl concentration. It can grow in a wide range of pH 4-12, with optimum growth at pH 8 and 9. Growth in different temperatures showed that growth occurred between 20-35ºC, with optimum at 28ºC. The isolate also hydrolysed lipid, casein and starch and catslase positive. Utilised wide range of sugars as sole source of carbon.

Dextrose, dulcitol, lactose, maltose, mannitol, mannose, and rhamnose were used for growth. From the experimental data the actinomycete isolate is placed in the Genus *Streptomyces*. However, the species is yet to be confirmed. The data of this research work is primary attempt to identify potential marine actinomycetes isolate. It reveals promising effect against pathogens and cell lines It could be concluded that marine region south east coast of India is rich with Actinomyces. The isolates MNG3 from the investigated actinomycetes exhibited high antagonistic activity against the tested pathogens and cell lines particularly lung cancer cell lines. The phylogeny of the active isolate revels the Streptomyces sp are rich source for bioactive metabolite.

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