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POTENTIAL USE OF MARINE MICROORGANISMS FOR ANTIBIOTICS AND ENZYME PRODUCTION

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Abstract - Microorganisms were isolated from marine environment and cultivated under conditions pertinent to sea water. (1) Boron-containing polyether ionophore (Aplasmomycin) inhibitory to malarial plasmodium, (2) aminoglycoside antibiotics (Istamycin), (3) lytic enzyme of dental glucan and (4) immunopotentiating polysaccharide (Marinactan) inhibitory to tumor were found to be new and potential substances for use. The studies provided aspects on habitation and worth of marine microorganisms.

INTRODUCTION

Antibiotics are chemical compounds being produced by living organisms and inhibitory to other living organisms at low concentration. Thereby some scientists regard antibiotics as members of biologically active substances which include vitamins and hormones as well as toxic substances produced by organisms. Living organisms produce primary metabolites such as the tricarboxylic acids, for example, members of the TCA cyclic which generates energy for growth of living organisms. Unlike the primary metabolites, the majority of antibiotics are products being released as the results of secondary metabolic pathways which branches from primary metabolic pathways.

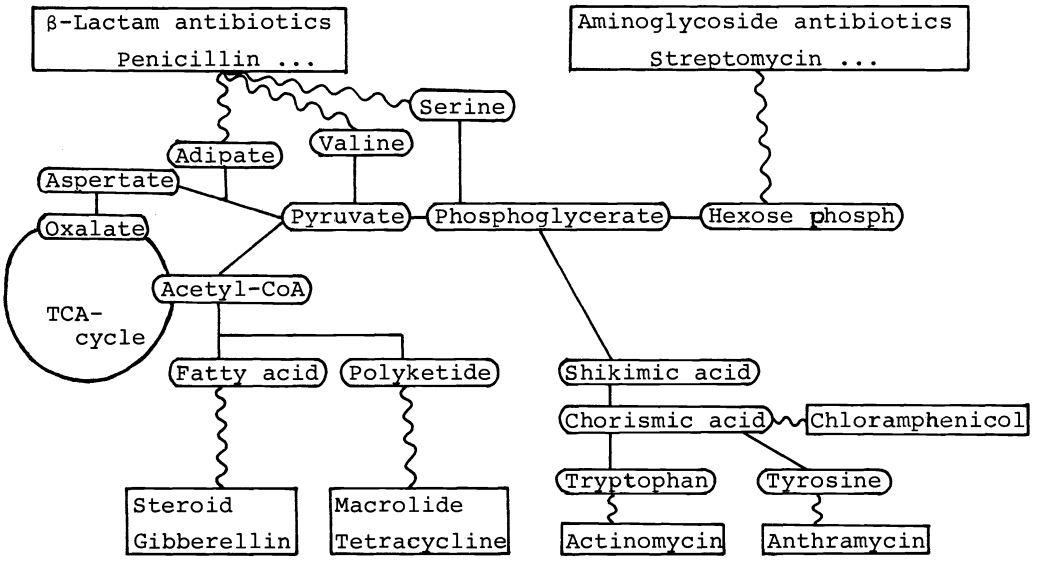


Fig. 1. Primary/secondary metabolites.
———: primary metabolism, ⬭ : primary metabolite,
∿∿∿: secondary metabolism, ▭ : secondary metabolite

Although the chemical structures of secondary metabolites are somewhat complicated, they are assumed to be derived from primary metabolites. Due to their complicated and diversified structure, they often exhibit activity

selectively against biological systems and become tools for biochemistry or oftenly chemotherapy. Practical chemotherapy requires chemical compounds having selective activity but also availability of sufficient amount necessary for therapy. Antibiotics which are in practical use at present satisfy the above requirements by their selective toxicity to pathogens and by reproducible cultivation of the producing microorganisms. The best example is provided by penicillin which is toxic selectively to bacteria but not to human beings and is produced reasonably by fermentation with a fungus. After wide use of penicillin, resistance to this antibiotic appears. To overcome the resistance problem, the inhibition of resistance by new substance which will be afforded by modification of the penicillin molecule or discovery of new antibiotics are needed. In addition to the above practical need for new substances, academic interests in the chemical structures shown by new antibiotics promote not only the search for more new antibiotics but also studies on their biosynthesis and their biochemical as well as biological activities. Consequently up to the present, nearly 4,000 antibiotics have been found mostly from terrestrial microorganisms and it is presumable that organisms living in unique environments may often provide new antibiotics.

Therefore, we started to search for new biologically active substances from the marine enrivonment which is quite different from terrestrial one. This attempt was supported by the following facts which were seen in literatures. (1) Sea water contains various biologically active substances such as vitamins, and many marine microorganisms are capable of producing various vitamins (Ref.1 & 2). (2) Sea water contains active agents inhibitory to organisms. This observation is demonstrated by the facts that (a) sea water is inhibitory to Gram-positive bacteria, (b) natural sea water is more inhibitory than artificial sea water, (c) heat-treated sea water shows reduced inhibitory activity compared with fresh sea water, (d) inhibitory activity of sea water is not eliminated by ultra-filtration which can eliminate phages without affecting salinity. This result suggests the inhibitory activity may not be due to phages or to salinity of sea water, but may be due to some antibacterial agents in sea water. (3) Some microorganisms isolated from sea water show antibacterial activity (Ref. 3). (4) Brominated pyrol compound is found from marine bacteria and shows strong inhibition against bacteria (Burkhold) (Ref.4 & 5). (5) Recent improvement of extraction methods as well as improved analytical instruments permit the efficient handling of very minor substances existing in miscellaneous materials and the efficient elucidation of precise chemical structures.

Population density of microorganisms in the sea

As many investigators have experienced and reported, microorganisms can be isolated from either sea water or bottom muds. The authors' experiments indicated that the population density of microorganisms varied with sea areas (coast, inlet, ocean or geographical location), seasons or depth of the sea. Bottom muds contained greater populations than sea water in general, and coastal areas contained denser populations than oceanic areas, and in particular coastal zones highly vegetated with sea weeds were high in population of microorganisms. This suggests coastal areas are preferable for various living organisms and that they are influenced heavily by terrestrial environments which contain many microorganisms, so that marine and terrestrial microorganisms can occur together. On the other hand, deep sea is an environment where sparse population of microorganisms will be found with the exception of microorganisms such as barophilic bacteria which can not be cultured unless a pressurized device is properly made.

Methods for isolation of microorganisms from marine environments

All methods which have been used for the isolation of microorganisms from terrestrial environments are applicable for the isolation from marine areas but some special devices such as media containing sea water, or hydrostatic pressure may be needed to isolate some marine microorganisms. The isolation employing different media, temperatures, or pH frequently provides different microorganisms. Since the methods used up to now are limited, a wide range of microorganisms remain to be isolated from marine environments. Oceanographic studies hitherto have provided many devices to collect sea water and sea mud, but the instruments employed for collection of sample are too large and not easily utilized for isolation of microorganisms. Many of them require a winch or carrier for their operation. To isolate the microorganisms, sea water less than 100 ml volume or only a few grams of mud are usually sufficient. To collect microorganisms from sea water, we concentrated

them on a filter such as millipore filter but the filtration often time, consuming due to viscous materials in sea water. Since sea mud contains many more microorganisms than sea water, we have devised a simple and handy instrument (Ref. 6) which is efficient to collect a few grams of mud from the sea bottom at depths less than 100 m. This device allowed routine collection of mud samples for isolation of microorganisms, particularly those in coastal sea area.

Remarks on marine microorganisms

Sea fish or sea weeds are simply regarded as native to the sea. Labyrinthula is a spindle-shaped protozoan that glides along filamentous tracks and forms a slime net occuring frequently on eelgrass grown in the sea. Dinoflagellates are algae, most of which live in marine environments and some of which are frequently found in red tides which occur in the sea. These organisms are regarded as native to the sea with few questions. On the other hand, microorganisms isolated from marine environments are not simply acknowledged as native to the sea because they have many features in terrestrial microorganisms and it is often difficult to distinguish them. For instance, many fungi which have common features with terrestrial ones are isolated from the sea and most of them are Ascomycetes which occur on submerged wood in sea or on sea algae. On the other hand, pressure-loving (barophilic) microorganisms which can be isolated only from very deep sea with high hydrostatic pressure, never being expected in terrestrial environment, may be regarded as native to the sea. Sea-water requiring microorganisms may have priority to live in the sea rather than in terrestrial environment. Rather frequent isolation of pseudomonas, vibrio, arthrobacter alteroromonas or flavobacteria suggests their dense population in the sea and they are presumed to be native to the sea. There is a tendency for more frequent isolation of salt-tolerant microorganisms from sea than from terrestrial environment. As above, although the clean definition of marine microorganisms is not made, they may be regarded as the microorganisms who are frequently found in the sea and have priority to live in sea or exhibit unique features favorable to the marine environment. For example, a pseudomonad isolated from the sea and produced an antibacterial metabolite consisting of bromine-rich molecule (Ref.4 & 5), which is not the usual metabolite of terrestrial organisms, can be regarded as a marine microorganism. An actinomycete isolated from the sea by us showed features almost identical with a terrestrial isolate of the same species with the exception of its ability to liquefy agar (a polymer produced by marine algae) and its salt tolerance. We regard this isolate as marine actinomycete, although the isolate did not require sea-water for the growth with which some one defines as marine.

Boron-containing ionophore

An actinomycete was isolated from shallow sea mud collected at Koajiro inlet of Sagami Bay and was designated as strain SS-20. This actinomycete, identified as Streptomyces griseus, a very common soil species, exhibited antimicrobial activity only when fermented in a medium containing very dilute nutrients and increased sodium chloride concentrations, but not when cultured in ordinary fermentation media for actinomycetes (Ref. 7).

When strain SS-20 was shake cultured in YE medium (yeast extract 0.4%, malt extract 1.0% and glucose 0.4%, pH 7.4), an ordinary medium for actinomycete fermentation, it did not produce any antimicrobial activity but did show activity in media in which YE medium was diluted with water and NaCl concentrations increased, as shown in Table 1. After a survey of media for optimal production of the active principle, the production increased 3-5 times in a medium Kobu-cha medium, containing glucose 1.0%, NaCl 1.5% and Kobu-cha which is dried powder of a sea-weed, Laminaria.

To isolate the active principle, spores of strain SS-20 were cultivated on an agar slant containing soluble starch 1.0%, casein 0.1%, agar 1.7% and artificial sea-water 500 ml plus distilled water 500 ml. Spores obtained after incubation at 27°C for 2-3 weeks were inoculated into 100 ml of the above Kobu-cha medium in 500 ml flask and shaken on a reciprocal shaker with 8 cm amplitude, 130 strokes per minute at 27°C for 72 hours. Twenty ml of this cultured broth were transferred to the Kobu-cha medium mentioned above and shake-cultured as above for 120 hours. The fermented broth was adjusted to pH 5.0 with diluted hydrochloric acid and filtered to remove the mycelium. The mycelium did not contain much of the active principle. Twenty liters of

TABLE 1. Effect of nutrients and NaCl on aplasmomycin production by Streptomyces griseus strain SS-20

Dilution	NaCl concentration and mcg/ml					
	0%	1%	2%	3%	4%	5%
YE	0	0	0	0	tr	tr
x 1/2	0	0	5	5	10	20
x 1/4	0	tr	15	15	20	30
x 1/8	0	tr	15	40	30	40
x 1/16	0	0	15	50	45	45
x 1/32	0	0	0	15	10	0

filtrate, thus obtained, were extracted with 10 liters of butyl acetate as shown in Fig. 2. The solvent layer was concentrated in vacuo at 40°C to give 420 mg of a yellowish colored syrup. The syrup was placed on a column (1.5 x 20 cm) filled with 30 g of neutral alumina. The charged column was

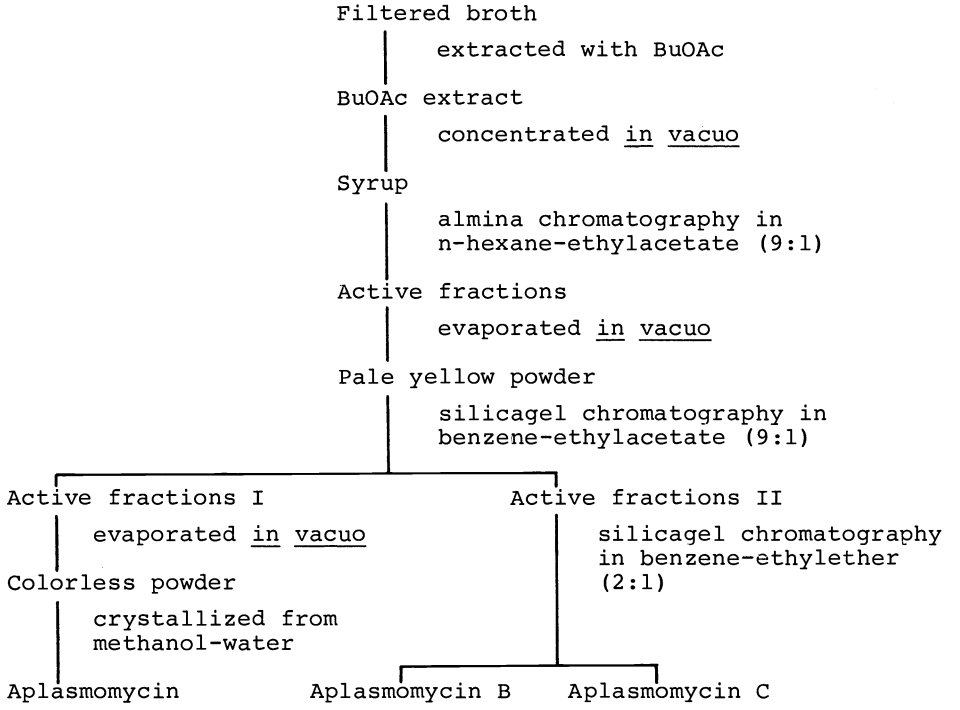


Fig. 2. Extraction of aplasmomycins

developed with a mixture of n-hexane and ethylacetate (9:1) and the eluted fractions with antimicrobial activity were collected. The combined fractions were concentrated in vacuo at 40°C to give a pale yellowish powder (230 mg). The powder was applied to the column (1.5 x 20 cm) filled with 30 g silicic acid. The charged column was developed by benzene and ethylacetate (9:1) and the eluted fractions with antimicrobial activity were collected. The active fractions were combined and then shaken with 2N sodium hydroxide. The solvent layer was washed with water and dehydrated with anhydrous sodium sulfate. After the organic solvent was evaporated in vacuo at 40°C, crystals (120 mg) appeared from a methanol-water solution.

This purified substance inhibited Gram-positive bacteria including mycobacteria in vitro at 0.78-6.25 mcg/ml and showed the acute toxicity (LD_{50}) in mice was 125 mg/kg by intraperitoneal injection. When it was administered

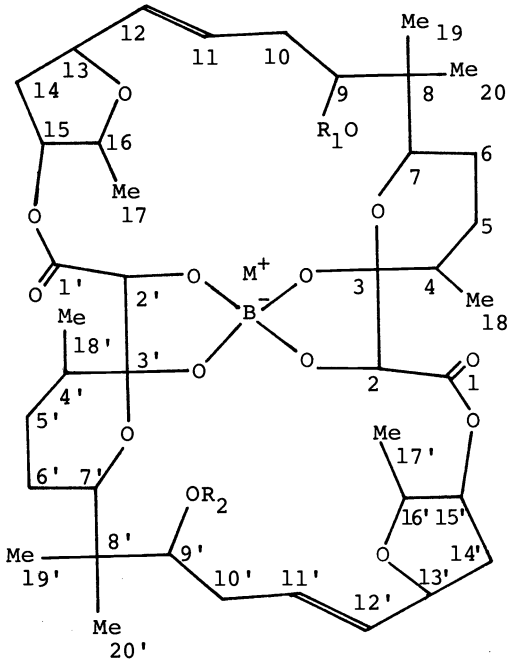
orally in 2 doses of 100 mg/kg each to mice infected intraperitoneally with the causative agent of malaria Plasmodium berghei (NK65), the number of plasmodium-containing red cells decreased and all treated mice survived, in contrast to the mice without treatment which died within 8 days and in which plasmodia were observed in more than half of red cells in the surviving mice. Because of this anti-plasmodial activity, it was named as aplasmomycin. As

TABLE 2. Physicochemical properties of aplasmomycins

Physicochemical properties		Aplasmomycin B		Aplasmomycin C		Deboro-aplasmomycin	
Melting point		238-241°C (dec.)		291-293°C (dec.)		183-186°C	
Elementary analysis (Na salt)		Found	Calcd.	Found	Calcd.	Found	Calcd.
	C	59.75	60.00	59.65	59.98	62.64	62.48
	H	7.05	7.14	7.06	7.26	8.40	8.39
	O	28.72	28.56	29.32	28.92	28.81	29.13
	B	1.37	1.31	1.40	1.24	—	—
	Na	3.11	2.74	2.57	2.61	0.05	0
Ultraviolet absorption		End absorption		End absorption		End absorption	
Optical rotation $[\alpha]_D$		+188° (c 0.21)		+134° (c 0.15)			
Mass spectra		840 (M^+), 798 (M^+-42), 784 (M^+-56)		882 (M^+), 840 (M^+-42), 826 (M^+-56), 798 (M^+-84)			
Molecular formula (Na salt)		$C_{42}H_{62}O_{15}BNa$		$C_{44}H_{64}O_{16}BNa$		$C_{40}H_{64}O_{14}$	
Infrared absorption		3400, 2920 1732, 1705		2920, 1731, 1707		3480, 2950, 1740	
Color reaction		sulfuric acid + ninhydrin - carminic acid +		sulfuric acid + ninhydrin - carminic acid +		sulfuric acid + carminic acid -	
TLC Rf (Kiesel gel $60F_{254}$, Merck) benzene:ethyl-acetate (1:1) ether		0.65		0.61		0.24	
		0.70		0.62		0.31	

shown in Table 2, pure aplasmomycin formed colorless needles, melting at 283-285°C with decomposition. It did not contain nitrogen, sulfur, phosphorous or halogen, and its sodium content could not be removed successfully because of its acid lability. It was soluble in many organic solvents but only slightly soluble in water. The high resolution mass spectrum showed $(M-1)^+$, m/e 789.3881. The spectrum showed peaks at m/e 814 which could be explained as a trace ion of K content. A positive color reaction was observed with sulfuric acid-vanillin (violet), but negative with ferric chloride, periodate-benzidine and red tetrazolium. Further studies (flame reaction with alcohol-green color, color reaction with carminic acid-red color and colorimetric determination with BF_4^- methylene blue) indicated that the antibiotic contained boron. The molecular formula is $C_{40}H_{60}O_{14}BNa$. The UV spectrum showed only end absorption and $[\alpha]_D^{22}$ was +225 (c 1.24, $CHCl_3$). Its infrared absorption spectrum showed a carbonyl band at 1740 cm^{-1} and no carboxylate band at 1600 cm^{-1} region. The pmr spectrum indicated absence of methoxyl group. ^{13}C-nmr spectrum in dioxane-d_8 showed 21 peaks, of which twenty indicated two equivalent carbons by each signal from their intensities and suggested a symmetric structure. The chemical shifts from TMS according to alkyl carbons were at δ13.2 (q), 16.5 (q), 19.4 (q), 21.4 (q), 25.7 (t), 29.4 (t), 32.7 (t), 33.6 (d), 36.4 (t) and 39.4 (s). The carbons attached to

oxygen were δ77.5 (d), 78.6 (d), 78.7 (d), 79.7 (d), 80.2 (d) and 106.7 (s). The olefinic carbons appeared at δ128.6 (d) and 133.0 (d) and carbonyl carbon at δ170.6 (s). A residual small signal at δ131.4 (s) indicated one carbon and relatively low chemical shift indicated the oxygen atoms attached to carbon atoms. Crystals of the silver salt of aplasmomycin were grown from an aqueous methanol solution as colorless hexagonal thick plates. Prof. Y. Iitaka and his colleagues of Tokyo University who have been cooperating with us used a crystal of 0.12 x 0.10 x 0.25 mm in size for X-ray crystalography. The result was in accordance with the chemical structure shown in Fig. 3.



I Aplasmomycin R_1, R_2=H

II Aplasmomycin B R_1=Ac, R_2=H

III Aplasmomycin C R_1, R_2=Ac

Fig. 3. Structure of aplasmomycins.

Further studies (Ref. 8) on the aplasmomycin-producing strain yielded aplasmomycin B and C, as two minor products of this strain. Structural studies on these substances revealed aplasmomycin B possesed acetyl group at C-9 or C-9' position of aplasmomycin and aplasmomycin C possesed at both positions, as indicated in Fig. 3.

Since it has been known that ionophore antibiotics have the ability to transport cation across lipid barriers in artificial and biological membranes, the three aplasmomycins were compared in terms of antibacterial activity, alkaline metal ion selectivity and K^+ transport ability. The results showed that the antibacterial activity of aplasmomycin B was nearly equal to aplasmomycin, while aplasmomycin C was weaker than aplasmomycin. Deboro-aplasmomycin was weaker than aplasmomycin C. As shown in Table 3, cation selectivity of these compounds were in order of $Rb^+ > K^+ > Cs^+ \sim Na^+ > Li^+$ and no affinity was found for divalent cations. Relative affinity of the three aplasmomycins did not directly correspond to their antibacterial activity. When aplasmomycin mediated K^+ transport across a bulk phase in a modified system of the Pressman method, aplasmomycin and aplasmomycin B catalyzed net transport of K^+ to almost the same extent, but aplasmomycin C and deboro-aplasmomycin were unable to transport K^+ ion. This was in good agreement with their antibacterial activity. Aplasmomycin is similar to a known boron-containing antibiotic, boromycin which was found from a terrestrial actinomycete, but unlike boromycin, aplasmomycin showed significant anti-plasmodial activity and almost no anticoccidial activity which are in contrast to boromycin.

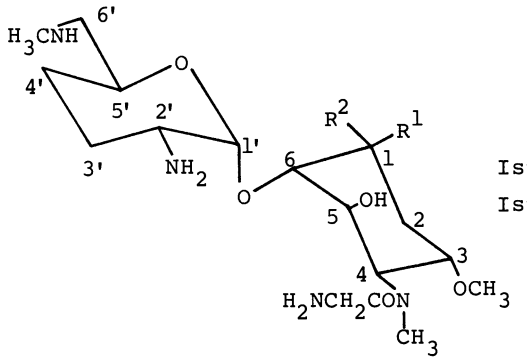
TABLE 3. Relative affinity of aplasmomycins for various cations and antibacterial activity

Cations	Aplasmomycin	Aplasmomycin B	Aplasmomycin C
Na^+	0.27	0.40	0.45
K^+	1	1	1
Rb^+	1.1	1.4	1.5
Cs^+	0.9	0.6	0.8
Li^+	0.12	0.15	0.11
Mg^{++}	10^{-2}	$<10^{-2}$	$<10^{-2}$
Ca^{++}	10^{-2}	$<10^{-2}$	$<10^{-2}$
Antibact. activity	+	+	-

Biosynthesis of aplasmomycin has been studied by Prof. G. Floss and his colleagues of Purdue University, U. S. A. and they find its skeleton structure is synthesized from acetates and glycerol, and its methyl groups are derived from methionine (Ref. 9). It will be interesting to know when and how boron is incorporated and binds the two symmetrical structures of aplasmomycin.

A new marine actinomycete and its aminoglycoside antibiotics (Ref. 10)
An actinomycete was isolated from a sea mud sample collected in shallow sea mud around Tenjin-island in Sagami-Bay at the Marine Biological Garden of the Yokosuka City Museum. Approximately 1 ml of a sea mud sample was suspended in 0.85% NaCl solution (4 ml), stirred vigorously and a 0.05 ml portion was spread on Maltose (1.0%)-Yeast Extract (0.4%) agar and/or Salts-Starch (ISP No. 4) agar supplemented with 20 mg/ml of antibiotics such as nystatin and kanamycin. Colonies were picked after incubating 4-10 days at 27°C. The isolate was chromogenic, produced light aqua blue aerial mycelium and utilized only glucose and inositol as the sole sources of carbohydrate on Pridham-Gottlieb's agar medium. The sporophore was rectiflexibilis and the surface of condial spores was smooth. These characteristics are not in any actinomycetes ever isolated from terrestrial environments. This was so new that it may establish a new species of the streptomycetes, Streptomyces tenjimariensis. An isolated strain designated SS-939 was cultivated with aeration in a 30 L jar fermentator which contained 15 L of a medium consisting of 2.0% starch, 0.2% glucose, 2.0% soybean meal, 0.2% sodium palmitate, 0.3% NaCl, 0.1% K_2HPO_4 and 0.1% $MgSO_4$-$7H_2O$. The fermentation was carried out at 27°C with agitation at 300 rpm and aeration at 15 L/min and stopped after 72 hours. The fermented broth was filtered at pH 2.0 and the acidic filtrate was neutralized. The antibiotics in the filtrate were adsorbed on a column of Amberlite IRC-50 (NH_4^+) and eluted with 1N aqueous ammonia. The active eluate (5,600 ml) was concentrated to 200 ml and the concentrate was passed through a column of Amberlite CG-50 (NH_4^+). After washing with water and 0.2N aqueous ammonia, the column was eluted with 0.4N aqueous ammonia and the eluate was divided into 18 ml fractions. Lyophilization of fractions 80-106 gave a crude powder of one antibiotic (240 mg named B) and that of fractions 107-124 gave a crude powder of another antibiotic (380 mg named A). Further purification was accomplished by silica gel (60 Silanised, E. Merck) column chromatography developed with the lower phase of chloroform-methanol-8.5% aqueous ammonia (2:1:1, v/v). 23 mg of A substance and 25 mg of B substance were obtained as their hemicarbonates. Both substances have positive ninhydrin and Rydon-Smith reactions.

After a survey of these two substances by melting point, elemental analysis, IR spectrum, UV spectrum, optical rotation, mass spectroscopy, CMR spectrum, high-voltage electrophoresis silica-gel thin-layer chromatography and degradation studies, the structure of two substances were elucidated as the new aminoglycoside antibiotics as shown in Fig. 4. Because of the structure,



	R^1	R^2
Istamycin A:	NH_2	H
Istamycin B:	H	NH_2

Fig. 4. Structure of istamycins

consisting of an amino-sugar and an aminocyclitol, these antibiotics were defined as belonging to the fortimicin-sporaricin group, but new attachments of amino or hydroxy radicals and side chains defined new aminoglycosides named istamycin A and B.

Istamycins showed strong inhibition against Gram-positive and -negative bacteria including those resistant to known aminoglycoside antibiotics except those having inactivating enzymes. LD_{50} in mice by the intravenous injection was 100-200 mg/kg for istamycin A and 80-160 mg/kg for istamycin B. Istamycin B was 2-4 times stronger than A in antimicrobial activity. The structure of istamycin A was confirmed by chemical synthesis starting from 3',4'-dideoxy neamine, in which the key step involved aziridine formation followed by a ring opening reaction to the 1,4-diaminocyclitol derivative. The 4-N-deglycine of both istamycin A and B had reduced antimicrobial activity. On the other hand, 3-O-demethylation of istamycin B extended the activity to Pseudomonas. The features of demethyl-istamycin are promising for practical as a drug, due to its low toxicity and effectiveness against resistant bacteria.

Marine occurence of actinomycetes

Actinomycetes were initially reported as terrestrial soil inhabitants or pathogens of terrestrial animals. There is a large number of reportd which describe a wide variety of actinomycetes, i.e. some are thermophilic, aquaphilic or salt-sensitive. So far, Streptomyces aureofaciens, which was originally found in a terrestrial soil habitat has not been found in the marine environment, and this may be explained by its inability to grow in salty water like sea water.

On the other hand, many thermophilic actinomycetes which grow frequently in soil composts with high temperatures like 50°C can be found in marine environment, even in the deep sea at more than 2,000 m. Thermoactinomyces vulgaris, a thermophilic actinomycete forms endospores tolerant to high temperatures and other physical conditions to which the endospore of Bacillus subtilis is tolerant, and thus can survive in the sea.

Micromonospora is an actinomycete which was originally found in terrestrial soil, but it can be found in sea mud at great depth. This actinomycete has monosporic spores submerged in agar media and can grow in conditions of reduced oxygen which are not favourable for Streptomyces. This characteristic may permit the survival of Micromonospora in the sea. Actinomycetes in terrestrial environments can be transferred to marine environments by various means such as irrigation or wind-blowing of soil particles in which actinomycetes grew, and they may survive as dormant forms or grow very slowly in the sea. In shallow sea areas, microorganisms are frequently transferred from land and survive in sea water for a period of time or settle down to the sea bottom resulting in a dense population of microorganisms. Some of them may be able to adapt to the sea environment and multiply. Strain SS-939 of Streptomyces tenjimariensis was first isolated by us in shallow sea area in 1978. Strains SS-989 and SS-1507 were isolated from the same area and found to produce istamycins in 1979 and 1980, respectively. After taxonomic studies, they both were identical with S.

tenjimariensis. However, their profiles of plasmid content are different from one strain to another as shown in Fig. 5. This suggests the survival and growth of S. tenjimariensis in the same area over a period of years, with a little shift of features such as plasmid. Thus, marine-habituated microorganisms of which features and changed a little from terrestrial ones will provide new metabolites different from those of terrestrial micro-organisms.

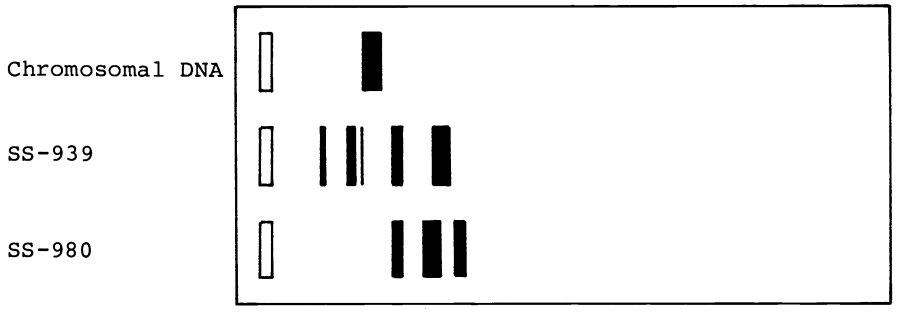


Fig. 5. Plasmid profile of istamycin producers

A new glucan-degrading enzyme of marine bacteria (Ref. 11)
When sea water is filtered with membrane filter, the filtration becomes difficult in a short time due to viscous materials in sea water. Polymers such as agar-agar are common in the marine environment. Sea weeds such as Laminaria produce slimy viscous materials on their surface. The cycling of these polymers requires that organisms exist that can decompose them, otherwise they would accumulate. Marine microorganisms may play an important role in this decomposition by producing enzymes for the degradation of marine polymers are often capable to liquify agar-agar, which terrestrial bacteria rarely can.

We attempted to isolate marine bacteria capable of degrading glucan, a biological polymer, produced by Streptococcus mutans, the cause of dental caries. Insoluble glucan (IG) produced by the oral streptococci adhers to the surface of teeth and leads to the development of carriogenic dental plaque. The glucan contains a high percentage of α-(1-3) linkages branching from a linear sugar polymer with α-(1-6) linkages and is resistant to the enzymatic hydrolysis by dextranases.

An isolate of bacteria, strain MT-G2 from marine mud in Tateyama, Chiba district was found to produce an enzyme hydrolyzing IG. The strain grew in diluted nutrient mediua such as 1/3 strength of heart infusion broth, but not in ordinary nutrient broth. The strain was identified as Bacillus circulans, although its growth was weaker than the type culture of B. circulans. Enzyme activity was measured under the following conditions; a reaction mixture (1.0 ml) containing 0.5 ml of 2% substrate (IG or other glucan), 0.25 ml of 0.1 M 2-(N-morpholino)ethanesulfonic acid-NaOH buffer (pH 6.2) and 0.25 ml of the enzyme solution was incubated for 60 min at 30°C, and then the reaction was stopped by heating in boiling water for 3 min. Reducing sugar released by the action of the enzyme was determined as glucose by the Somogyi-Nelson method. One unit of the enzyme was defined as the amount releasing 1 μmol of reducing sugar per min. under the above conditions.

The enzyme production required IG as an inducer and reached maximum at 6 days of incubation in the agar medium of 1/3 strength heart infusion. The strain grew but did not produce the enzyme in liquid medium. The enzyme was extracted from the cultured agar medium with 2 volumes of water. The extract was purified by ammonium sulfate fractionation, chromatography on DEAE-Sephadex eluted with KCl solution, chromatography on DE-32 cellulose eluted with acetate buffer and gel filtration on Sephadex G-150. The purified enzyme increased about 50 fold in specific activity. The optimum pH for activity of this enzyme was 6.2-6.7 and the optimum temperature was 35°C which was significantly lower than any other IG-lyzing enzymes. The enzyme activity was not influenced by Ca^{2+} which affects other IG-lyzing enzymes. The enzyme attacked glucans consisting of α-1,3 and/or α-1,6 linkages, but not those consisting of α-1,4 or β-1,3 linkages, as shown in

Table 4. The above properties indicated that the enzyme was a new α-glucanase and it has potential for liquefing IG on teeth.

TABLE 4. Substrate specificity of the enzyme

Substrate	Major linkage(s)	Activity
S. mutans E-49 IG	α-1,3; α-1,6	+
Nigeran	α-1,3	+
Soluble starch	α-1,4; α-1,6	±
Glycogen	α-1,6; α-1,4	+
Dextran T-70	α-1,6	+
Laminarin	β-1,3	±
Maltose	α-1,4	-
Isomaltose	α-1,6	+
Nigerose	α-1,3	+
Cellobiose	β-1,4	-
Gentiobiose	β-1,6	±

Antitumor polysaccharide with immunopotentiating activity (Ref. 12)
As described in the previous section, sea weeds produce viscous polymers. Not only higher organisms but also microorganisms may produce polymers such as polysaccharides.

We searched for marine microorganisms producing polysaccharides with antitumor activity against S-180 solid tumor in mice. The isolation was done with a medium consisting of polypeptone 0.2-0.5%, yeast extract 0.03-0.1%, agar 1.8% and artificial sea water (pH 7.2) incubated at 27°C for 1-3 days. Colonies appearing on isolation agar medium were picked and grown for polysaccharides production in medium consisting of glucose or sucrose 0.6%, polypeptone 0.5%, yeast extract 0.1% and artificial sea water (pH 7.2) at 27°C for 2 days. The fermented broth (1 L) was centrifuged to remove bacterial cells. An equal volume of ethanol was added to the supernatant and left overnight. The precipitate which occurred was filtered and repeatedly extracted with 100 ml of hot water. After the extract was centrifuged, the supernatant was dialyzed against water and 150 ml of ethanol added. The precipitate was dried under reduced pressure to obtain a crude polysaccharide preparation. The dried preparation was dissolved in water and injected intraperitoneally to ICR female mice of 5 weeks age which were inoculated with 3×10^6 cells of S-180 sarcoma subcutaneously. 5 and 20 mg per kg of the crude preparation were injected intraperitoneally once a day for 10 days after the day of tumor inoculaton. The weight of solid tumor which developed was measured after 5 weeks and the weight of treated mice was compared with that of untreated mice to calculate the inhibition rate, and surviving mice were examined for complete regression of tumor.

Among about 500 isolates, 20% of the strains produced a crude preparation of polysaccharide available for the antitumor test, and 5% of the tested strains had inhibitory activity. One strain designated as MP-55 was most significant in giving complete regression and was studied further.

The strain was isolated from sea weed collected at Sagami Bay and was identified as Flavobacterium uliginosum (with minor discrepancies in acid formation and GC-content). This strain required artificial sea water for the growth and the production of polysaccharide.

The crude preparation obtained as above was treated with chloroform and n-butanol to remove contaminating protein and then applied to a Sepharose CL-4B column chromatography and eluted with NaCl solution. The eluate was dialyzed to remove NaCl and ethanol added to yield a white purified preparation. This preparation showed only one peak by the above chromatography and the content of sugar as glucose was measured by phenol sulfuric acid method. Protein content measuring by the Lowry method was 0-0.4%. As shown in Table 5,

TABLE 5. Characteristics of MACT

Group	Heteroglycan
Produced by	Flavobacterium uliginosum MP-55
Form	White powder
Nature	Neutral
Solubility	Soluble in water Insoluble in organic solvents
Sugar composition	Glucose 69.7 ± 0.7% Mannose 19.8 ± 1.2% Fucose 10.8 ± 0.8%

gas-chromatography of this preparation detected fucose-mannose-glucose (0.15 ± 0.05 : 0.31 ± 0.05 : 1). It was a neutral heteroglycan and soluble in neutral or alkaline water but not in organic solvents. The ninhydrin reaction was negative. This heteroglycan was named as marinactan because of its marine origin. The purified marinactan showed a 75-95% inhibition rate and complete regression of the tumor was observed in 2-4 mice out of 7 mice treated, as shown in Table 6. When 25 mg/kg was intraperitoneally injected

TABLE 6. Antitumor activities of marinactan (MACT) from Flavobacterium uliginosum MP-55

Sample	Exp.	Dose mg/kg x day	Inhibition ratio (%)	Complete Regression
Crude MACT	I	10 x 10	90	0/3
	II	20 x 10	86	4/7
		50 x 10	86	1/7
	III	10 x 10	79	3/7
		20 x 10	90	3/7
		50 x 10	77	3/7
MACT		5 x 10	95	3/7
		20 x 10	92	2/6

once a day for 10 days into ICR female mice which were inoculated with 1×10^6 ascites cells of S-180, the treated mice survived for more than 30 days, while the untreated mice died an average of 15 days after the tumor inoculation. The antitumor effect with prolonged survival was also observed against mouse mammary tumor. It was observed to increase antibody forming cells in mouse spleen. This substance showed an increase of delayed-type hypersensitivity. In addition to these tests in vivo, it stimulated blastogenesis, shown by an increase of thymidine incorporation in cultured spleen cells, and a stimulation of activity of macrophages was observed in vitro.

Thus, this heteroglycan preparation had antitumor activity with immunopotentiating activity and has potential for use as a tumor-curing or suppressing agent.

CONCLUSION

Isolation of microorganisms from marine environment and their culture under unique conditions such as sea water containing media may privide new substances or enzymes which could be useful agents. Marine microorganisms are more practical source of useful agents than marine higher organisms, since they are readily collected and cultured. If an agent is produced extra-cellularly by fermentation of microorganisms, the agent is often easier in its purification than that contained in higher organisms.

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