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REVIEW

Construction of a natural product library containing secondary metabolites produced by actinomycetes

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To construct a natural product library for drug screening, we isolated secondary metabolites from a wide variety of actinomycetes cultured from marine sponges. The results suggested that marine sponges are a promising source of actinomycetes with the potential to produce new metabolites. Furthermore, we evaluated the chemical space occupied by our natural product library (CB library) by multidimensional principal component analysis and compared it with a commercially available compound library (ZINC library), which was randomly selected from the ZINC library (approximately 30 000 000 compounds). The CB library occupied a wider chemical space than the ZINC library. Bioactive compounds in the CB library possessed a wide chemical space that was not covered by ZINC library. These results indicate that the CB library mainly comprises secondary metabolites from actinomycetes, and it has great potential as a source of compounds for drug screening.
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INTRODUCTION

Natural products consisting of unique structures occupy a wider chemical space than synthetic compounds, and this observation holds true for clinical drugs as well.[1] Natural products are biosynthesized by several enzymes, and they preferentially bind to the drug-binding pockets of proteins.[2] Therefore, natural products are considered good sources of lead compounds for drug screening. We constructed a natural product library (CB library) to screen for bioactive agents targeting cancer, microbial diseases, viruses, immune disorders and so on. To construct the CB library, we isolated actinomycetes from diverse environmental substrates, including marine sponges, tunicates, lichens, marine sediments and mangrove soils in addition to the conventionally utilized terrestrial soils, and we screened their culture extracts. Furthermore, we collected purified compounds from actinomycete cultures that enabled us to perform drug-screening assays at a variety of drug concentrations to minimize false-positive results. The large natural product library constructed consisted of $>300\,000$ samples, including approximately 5500 purified natural compounds.

ACTINOMYCETES ISOLATED FROM MARINE SPONGES AND THEIR SECONDARY METABOLITES

To date, soil has been the primary source of actinomycetes. However, recently, the rate of novel compound discovery from these terrestrial strains has decreased significantly. To overcome this problem, we isolated actinomycetes from a variety of resources such as mangrove soils, marine sediments, marine organisms and lichens. We analyzed the secondary metabolites, including potential novel compounds, present in the cultures of the strains obtained using a UPLC-TOF-MS system (Waters, Milford, MA, USA). Microorganisms from marine habitats, particularly actinomycetes, constitute a promising untapped source of novel compounds that is now receiving special attention.[3,4] Among the marine organisms, marine sponges are of special interest in this regard because they are remarkable filter feeders; some of them can filter up to $24\,m^3\,kg^{-1}$ sponge in a day.[5] During filtration, they retain a large number of bacteria, thereby concentrating bacterial cells that are otherwise diluted in seawater. Therefore, in our study, we mainly targeted marine sponges as a source of actinomycetes to investigate their secondary metabolites in culture.

We isolated actinomycetes from 18 marine sponges that were chiefly obtained from an offshore area around Ishigaki Island, and the presence of actinomycetes in these marine sponges was investigated using both culture-dependent and culture-independent techniques. By using metagenome analysis of marine sponges involving 16S rRNA gene libraries, we showed that marine sponges contain an abundance and diversity of actinomycetes: we detected 10 species of *Haliclona*,[6] 4 species of *Cinachyra*, 10 species of *Petrosia* and 17 species of *Ulosa*.[7]

We isolated actinomycetes from marine sponges using two new media, namely, jewfish extract agar and clam extract agar[6] containing 50% seawater. Fishery products such as jewfish and clam are important sources of vitamins, minerals and proteins, and extracts from such fishery products therefore serve as complex undefined

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Table 1 Abundance and diversity of actinomycetes associated with marine sponges

Genus/sponge no.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*Total*
Actinokineospora																	1		1
Actinomadura			1			1		1								2		3	8
Astrosporangium		1																	1
Cellulomonas	1																		1
Gordonia										1						1			2
Microbispora		2	1	1				1						2		1		1	9
Micromonospora	5		4	2	1			1	2		2			3		2	1		23
Mycobacterium									3										3
Nocardia	17	1	2		2	3		4		2				2		6		3	42
Nocardiopsis	6		1							1									8
Nonomuraea			3		1									4	1	4		1	14
Promicromonospora					1														1
Rhodococcus	1	2	5		2	4	3	3	2					3		1			26
Rothia										1				1					2
Saccharopolyspora																1			1
Sphaerisporangium			1																1
Streptomyces	131	3	22	3	1	3	8	11	35				3	7	7	45		17	296
Streptosporangium		1	2																3
Williamsia	1																		1
Unknown[a]			5		1			1	4			2	1	1	2	2		1	20
Total	162	10	47	6	9	11	11	22	46	5	2	2	4	23	10	65	2	26	463

1, *Haliclona* sp.; 2, *Stylotella aurantium*; 3, *Cinachyra* sp.; 4, Demospongiae; 5, Demospongiae; 6, Demospongiae; 7, Demospongiae; 8, Demospongiae; 9, *Mycale* sp.; 10, Demospongiae; 11, *Dragmacidon* sp.; 12, *Petrosia* (*Strongylophora*) *strongylata*; 13, *Pseudoceratina purpurea*; 14, *Cinachyra* sp.; 15, Demospongiae; 16, Demospongiae; 17, Demospongiae; 18, Demospongi.
Numbers represent the number of isolates, obtained from a sponge sample, belonging to a given genus.
[a]Strains for which convincing sequence data is not available.

nutrition sources with diverse nutritive substances of marine origin. The 463 strains belonging to 19 actinomycete genera, which were identified by comparing their 16S rRNA gene sequences with the sequences in the EzTaxon[8] type strain database, were isolated from 18 marine sponges (Table 1).[9] Among these 18 sponge samples, *Streptomyces* was the most commonly distributed and abundant genus (present in 14 sponges), whereas members of the genera *Micromonospora, Nocardia* and *Rhodococcus* were also isolated from 10 sponges. Members of the genera *Streptomyces, Micromonospora* and *Rhodococcus* have been isolated as dominant groups in previous studies.[10,11] Our results indicated that like in soil, *Streptomyces* is also dominant in marine sponges. In addition, many actinomycetes were isolated from four marine sponges, including sponge number 1 (*Haliclona*), 3 (*Cinachyra*), 9 (*Mycale*) and 16 (*Demospongiae*, unidentified), as shown in Table 1. These results suggested that the number of isolates depends on the species of marine sponge. Within these strains, 24 candidate new species showed $<$98.5% 16S rRNA gene sequence similarity with the EzTaxon database in six genera (*Streptomyces*, *Nocardia*, *Nonomuraea*, *Mycobacterium*, *Rhodococcus* and *Sphaerisporangium*). Five new species isolated from *Haliclona* sp., that is, *Streptomyces haliclonae* NBRC 105049^T, *S. marinus* NBRC 105047^T, *S. rubrum* NBRC 105046^T, *S. spongiae* NBRC 106415^T and *S. tateyamensis* NBRC 105048^T have been previously reported by us.[6,12] An important property of these isolates is that 28 of 468 strains are dependent on seawater for growth. Eleven strains belonging to the genera *Streptomyces* (8 strains), *Rhodococcus* (2 strains) and *Nocardiopsis* (1 strain) required at least 50% (v/v) seawater for their growth, whereas an additional 17 strains showed better growth in the presence of 50% seawater (v/v).[9] To our knowledge, this is the first study identifying seawater-requiring strains of the genus *Rhodococcus*. In addition, the pigment production and spore formation properties were affected by seawater. The seawater requirement of these strains is suggestive of their marine origin or their adaptation to marine habitats. Our results indicated that diverse and unique actinomycetes were isolated from marine sponges. However, further development of the method for isolating actinomycetes from marine sponges is expected because the metagenomics of marine sponges implies the presence of non-culturable actinomycetes.

To construct a purified natural compound library, we analyzed the metabolites in the cultures of the isolated strains and purified 22 new compounds from 12 *Streptomyces* strains and 1 new compound from an *Actinomadura* species (Table 2). A number of these compounds displayed unique structures. For example, the structures of JBIR-34 and JBIR-35 isolated from *Streptomyces* sp. Sp080513GE-23 consisted of a modified indole moiety that originated from tryptophan, a trisubstituted oxazoline moiety, and L-serine and D-alanine residues.[13] No previous report has described a highly substituted indole skeleton with chlorine, methyl, and hydroxy functional groups. Furthermore, 2 3,5,6-trisubstituted 2(1*H*)-pyrazinones, namely JBIR-56 and JBIR-57, isolated from a culture of *Streptomyces* sp. SpD081030SC-03 exhibited an abnormal connection of amino acid units.[14] These results suggested that marine sponge-derived actinomycetes are a promising source of structurally unique compounds.

CHEMICAL SPACE OF THE CB LIBRARY

We performed a large number of drug screens using different assay systems. In some assay systems, the crude extract library, which is the traditional natural product library, did not work effectively. In particular, high-throughput screening systems using over 300 000 crude samples involved the construction of assay methods suitable for a natural product library and were extremely difficult. Therefore, we attempted to collect purified compounds possessing various skeletons from actinomycete cultures that would be adaptable to any assay system, including high-throughput screening. To achieve this, we established a high-throughput system for the detection of secondary metabolites from the cultures of actinomycetes by using a UPLC-

Table 2 New compounds isolated from marine sponge-derived actinomycetes

Strain	*New compounds*	*Reference*
Streptomyces sp. Sp080513GE-26	Anthracycline; tetracenoquinocin, 5-imino-aranciamycin, JBIR-43	36
Streptomyces sp. NBRC 105896	Teleocidin derivative; JBIR-31	24
Streptomyces sp. Sp080513GE-23	Modified indole-containing tetrapeptide; JBIR-34, JBIR-35	13
Streptomyces sp. Sp080513SC-24	Piperazic acid-containing hexapeptide; JBIR-39, JBIR-40	37
Streptomyces sp. SpC080624SC-11	Phenazine derivative; JBIR-46, JBIR-47, JBIR-48	38
Streptomyces sp. SpA080624GE-02	Phenazine derivative; JBIR-46, JBIR-47, JBIR-48	38
Streptomyces sp. SpD081030SC-03	Pyrazinone-containing peptide; JBIR-56, JBIR-57	14
Streptomyces sp. SpD081030ME-02	Salicylamide derivative; JBIR-58	39
Streptomyces sp. SpG090213JE-10	JBIR-64 (1,3,12-trihydroxydodeca-8,10-dien-5-one)	In preparation
Actinomadura sp. SpB081030SC-15	Diterpene; JBIR-65	40
Streptomyces sp. RM50	Piericidin derivative; JBIR-105, JBIR-106 (13-(*o*-tolyl)tridec-12-enoic acid)	In preparation
Streptomyces sp. Sp080513SC-30	JBIR-107 (5-acetamido-6-(4-(methyl(2-oxo-3-phenylpropyl)amino)phenyl)-4-oxohexanoic acid)	In preparation
Streptomyces sp. RM72	Trichostatin derivative; JBIR-109, JBIR-110, JBIR-111	41

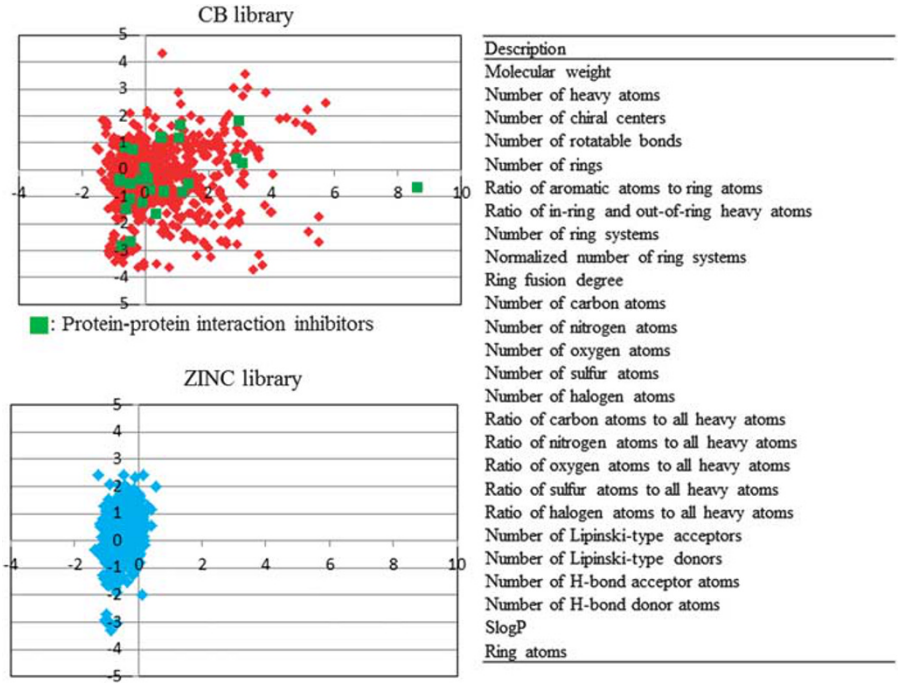

Figure 1 Chemical space occupied by the CB and ZINC libraries.

TOF-MS instrument. Briefly, we measured the data from approximately 1000 secondary metabolites with this system within 10 min per sample. We also used HR-MS, which facilitates the establishment of the molecular formula. The retention-time data of known compounds were registered as a mass spectra database by using the ChromaLynx database software (Waters). The culture extracts were similarly analyzed using the UPLC-TOF-MS system. These data were compared with the mass spectra database, and the peaks were identified. The unmatched peaks were selected as new candidates by molecular formula analysis using HR-MS. The corresponding compounds were isolated from the large cultures and stored in our library.

Although it has been reported that the chemical space of natural product libraries is wider than synthetic compound libraries,[1,15–17] we computed the chemical space occupied by the CB library and compared it with a commercially available compound library to evaluate the structural diversity of this library. We selected 915 compounds from the CB library; meanwhile, 915 compounds were randomly selected from the an available compound library, the ZINC database,[18] which consists of approximately 30 000 000 compounds (http://zinc.docking.org/) and six constructed data sets. We evaluated the chemical space by using multidimensional principal component analysis with 26 descriptors representing different chemical properties (Figure 1). The CB library occupied a wider chemical space than the ZINC library. These results indicated the several differences between the CB and ZINC libraries. First, the remarkable dissimilarity was attributable to the ring structure; the ring linkage of compounds in the CB library is complex, and fewer aromatic rings are present in their structures than those in the compounds of the ZINC library. A second dissimilarity involves the ratio of ring structures and side chains because the compounds in the CB library possess various side chains. These results were consistent with those of a previous report by Henkel *et al.*[15] In contrast, MW, asymmetric carbon number and

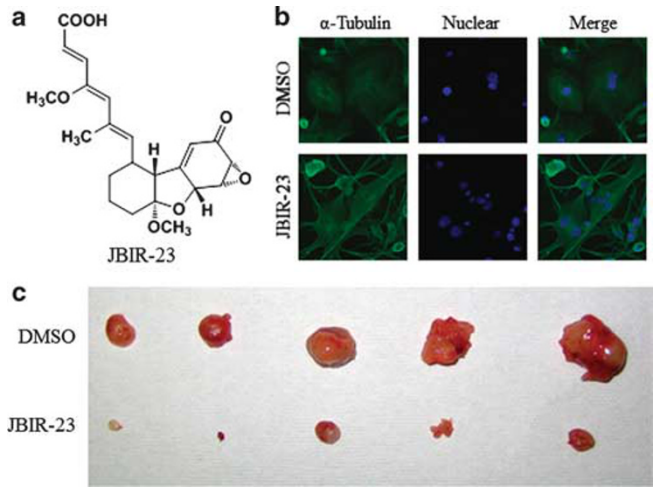

Figure 2 (**a**) Structure of JBIR-23. (**b**) Promotion of tubulin polymerization by JBIR-23 in MPM cells. (**c**) Antitumor activity of JBIR-23 in a xenograft model.

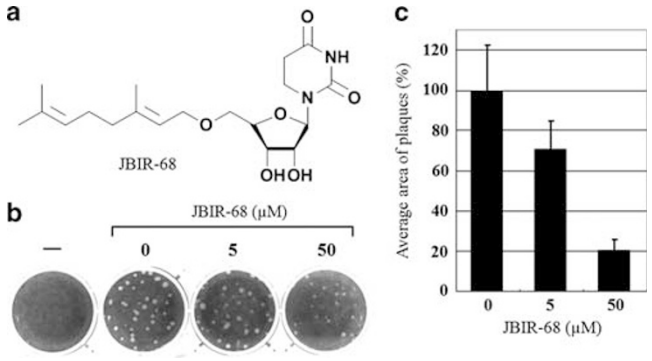

Figure 3 (**a**) Structure of JBIR-68. (**b**) JBIR-68-induced inhibition of plaque formation by influenza virus. (**c**) Average area of the plaq.

logP were less dissimilar between the compounds of the CB and ZINC libraries.

Protein–protein interactions (PPIs) have key roles in all cellular processes, and they have been validated as relevant pharmaceutical targets based on modern proteome analyses. Thus, screening of PPI regulators is a method to discover novel drugs. However, regulation of a typical PPI with a small molecule is generally considered difficult because approximately 750–1500 Å^2 of the protein's surface area is buried on either side of the interaction interface.[19,20] We therefore screened the CB library for PPI inhibitors because it is considered to possess a large chemical space and successfully discovered several compounds, some of which are novel.[21,22] We have plotted the target compounds in this chemical space occupied by the CB library, as shown in Figure 1. As a result, the PPI inhibitors have a large chemical space that cannot be covered by the ZINC library. Therefore, a natural product library such as the CB library is more suitable for the screening of PPI inhibitors than the available compound libraries. Taken together, our results showed that the CB library contains compounds possessing various skeletons.

BIOACTIVE COMPOUNDS FROM THE CB LIBRARY

In this section, we describe two novel bioactive compounds discovered in the CB library. First, we introduce a novel compound acting against malignant pleural mesothelioma (MPM), namely JBIR-23, isolated from *Streptomyces* sp. AK-AB27.[23] MPM, which is associated with exposure to asbestos fibers, is a rare and aggressive neoplasm that originates in the pleura and is highly invasive; it has a long latency period of 15–40 years, from asbestos exposure to the onset of symptoms.[24–26] Moreover, MPM is resistant to all conventional therapies, including chemotherapy, radiotherapy and surgery, and the prognosis for patients remains very poor.[24–26] It is therefore important to develop novel therapeutic agents against MPM. We isolated 11 new compounds that were cytotoxic to several human MPM cells.[23,27–32] Among these compounds, JBIR-23 possesses a unique structure, consisting of a dodecahydrodibenzo [*b,d*]furan skeleton (Figure 2a). This compound exerted its cytotoxic effect on MPM cells by promoting tubulin polymerization (Figure 2b) and G_2/M arrest, which led to the induction of apoptosis via the caspase pathway following phosphorylation of p38 mitogen-activated protein kinase and c-jun N-terminal kinase.[33] Furthermore, *in vivo* analysis showed that JBIR-23 prevented tumor growth in tumor-bearing nude mice without evident side effects (Figure 2c).[33] Taxanes, epothilones and other agents have been reported to promote tubulin polymerization, but the structural skeleton of JBIR-23 is not similar to any of these compounds. Therefore, JBIR-23 is a novel promoter of tubulin polymerization.

Second, the novel anti-influenza virus compound JBIR-68 was obtained from *Streptomyces* sp. RI18. The unique characteristic structure of JBIR-68 is a dihydrouridine with an ether-bonded geranyl residue at the C-5′ position in the ribose moiety (Figure 3a).[34] We evaluated the anti-influenza virus activity of JBIR-68 by using a plaque assay. JBIR-68 clearly reduced the number and area of influenza virus plaques (Figure 3b and c).[34] Therefore, JBIR-68 clearly inhibited the growth of influenza virus.

In addition to these examples, we isolated various new bioactive compounds, including anticancer agents, antimicrobial agents and PPI inhibitors from the CB library. Therefore, the wide chemical space of the CB library provides it with great potential as a source of compounds for drug screening.

CONCLUSION

We successfully constructed a huge natural product library for drug screening and showed that the library includes compounds possessing various skeletons and bioactivities. In addition, we isolated many new compounds produced by *Streptomyces* species derived from marine sponges to construct the library (Table 2). The results showed that marine sponges are now more attractive resources for the isolation of actinomycetes than soil samples. In our previous report, we suggested that *Streptomyces* produces compounds with diverse structures, and it is important to isolate actinomycetes from a wide variety of environmental substrates by using different methods to obtain new bioactive compounds.[35] Taken together, actinomycetes, particularly *Streptomyces*, should still be considered attractive sources of bioactive compounds.

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