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Anti-dormant mycobacterial activity and target analysis of nybomycin produced by a marine-derived *Streptomyces* sp.

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ABSTRACT

In the course of our search for anti-dormant Mycobacterial substances, nybomycin (**1**) was re-discovered from the culture broth of a marine-derived *Streptomyces* sp. on the bioassay-guided separation. Compound **1** showed anti-microbial activity against *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG with the MIC of 1.0 μg/mL under both actively growing aerobic conditions and dormancy inducing hypoxic conditions. Compound **1** is also effective to *Mycobacterium tuberculosis* including the clinically isolated strains. The mechanistic analysis indicated that **1** bound to DNA and induces a unique morphological change to mycobacterial bacilli leading the bacterial cell death.

1. Introduction

Tuberculosis (TB) is one of the most common causes of morbidity and mortality in HIV-positive adults living in poverty.[1] 8.9 million new TB cases and 1.5 million deaths by TB are estimated each year.[2] In addition, the requirement of long-term therapy for cure is a serious problem in the treatment of TB. Then, one of the major reasons for the extended chemotherapeutic regimens and wide epidemicity of TB is that the causative agent *Mycobacterium tuberculosis* has an ability to become dormant. Therefore, new lead compounds, which are effective against *M. tuberculosis* in both active and dormant states, are urgently needed. Although physiology of the latent *M. tuberculosis* infection is still unclear, hypoxic condition was found to induce dormant state of *Mycobacterium* sp., which has a drug susceptibility profile resembling that of the latent *M. tuberculosis* infection.[3–5] Based on these findings, we have established a screening system to search for substances that have anti-microbial activity against dormant mycobacteria and have isolated trichoderins[6] (new aminolipopeptides) from the culture broth of a marine sponge-derived fungus of *Trichoderma* sp., neamphamide B[7] (new cyclic depsipeptide) from a marine sponge of *Neamphius* sp., and 2-methoxy-3-oxoaaptamine[8] (new aaptamine class alkaloid) from a marine sponge of *Aaptos* sp. on the basis of bioassay-guided separation. In the continuous screening from marine organisms and marine-derived microorganisms, nybomycin (**1**) was re-discovered as an anti-dormant mycobacterial substance from the culture broth of a marine-derived *Streptomyces* sp. MS44. Nybomycin (**1**) was originally isolated from the terrestrial *Streptomyces* A717 by Strelitz et al. and demonstrated to show anti-phage activity and anti-microbial activity against Gram-negative and Gram-positive bacteria including *Mycobacterium smegmatis* under actively growing conditions.[9] However, the detailed anti-mycobacterial activity, especially under dormant state, and mode of action of **1** were remained unclear. In this paper, we studied in detail anti-microbial activity and mode of action of nybomycin (**1**).

2. Result and discussion

2.1. Anti-mycobacterial activity of nybomycin (1)

Dormant *M. tuberculosis* exists in the granulomas, where are known to be hypoxic environments. Wayne et al. clarified that oxygen depletion triggers the dormancy response such as resistance to isoniazid of anti-TB drug.[3,10] Based on these observations, we have established a screening system to search substances that have anti-microbial activity against dormant mycobacteria. In this assay

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system, the minimum inhibitory concentration (MIC) values of isoniazid against *M. smegmatis* and *Mycobacterium bovis* BCG were 2.5 μg/mL and 0.05 μg/mL under aerobic conditions respectively, whereas the MIC values of isoniazid against these strains were more than 25 μg/mL under hypoxic conditions as shown in Table 1. In the course of our screening from marine organisms and marine-derived microorganisms, nybomycin (**1**)[9,11] was re-discovered as an anti-dormant mycobacterial substance from the culture broth of a marine-derived *Streptomyces* sp. on the bioassay-guided separation (Fig. 1). Compound **1** exhibited potent anti-microbial activity against *M. smegmatis* and *M. bovis* BCG under both aerobic and hypoxic conditions with MIC of 1.0 μg/mL (Table 1). This result indicated that compound **1** is effective against *M. smegmatis* and *M. bovis* BCG in the both actively growing aerobic conditions and dormancy inducing hypoxic conditions. In addition, **1** was also effective against pathogenic strains of *M. tuberculosis* including clinically isolated strains with MIC ranging 4.2–6.3 μg/mL (Table 2). Thus, this result suggested that compound **1** would have a potent potential as anti-Tuberculosis drug.

2.2. Morphological change of nybomycin (1)-treated *M. smegmatis*

During bioassay, a unique morphological change was observed in the nybomycin (**1**)-treated *M. smegmatis* bacilli. As shown in Figure 2, the *M. smegmatis* bacilli treated with **1** elongated their cell bodies. Until now, a similar morphological change has been reported in the studies of several microorganisms.[12–14] For example, (a) the partial depletion of *pkn A* and *pkn B* of Ser/Thr protein kinases in *M. tuberculosis* induces elongation of their cell bodies;[12] (b) the deficiency of cell division gene *whmD* in *M. smegmatis* shows filamentous and aseptate morphology;[13] (c) The six-fold overexpression or reduction of cell division gene *ftsZ* in *M. smegmatis* results in an increase in cell length and a significant decrease in viability.[14] Furthermore, the inhibitors of DNA replication and transcription such as daunorubicin, nalidixic acid, mitomycin C, novobiocin, and ciprofloxacin also induce similar morphological change to *Escherichia coli*.[15] Therefore, we speculated that nybomycin (**1**) might bind to the molecules, which relate Ser/Thr protein kinases, cell division or DNA replication and transcription.

2.3. Analysis of target molecule of nybomycin (1)

In general, the transformant that overexpresses target protein for an anti-microbial compound shows resistance to the compound. On the basis of this concept, we have been identified the target molecules of several anti-microbial natural products by using 4000 strains of *M. smegmatis* transformed with genomic DNA library, which randomly overexpress the genome fragment of *M. bovis* BCG.[16–18] Then, we first applied this method for identifying target molecule of nybomycin (**1**), and four transformants, named ICHO2012–2015, were found to exhibit resistance to 4.0 μg/mL (4× MIC) concentration of compound **1** (Fig. 3A).

Table 1
MICs of compounds **1**–**3** against *M. smegmatis* and *M. bovis* BCG under aerobic and hypoxic conditions

Compounds	MIC (μg/mL)			
	M. smegmatis		*M. bovis* BCG	
	Aerobic	Hypoxic	Aerobic	Hypoxic
1	1.0	1.0	1.0	1.0
2	25	6.25	12.5	6.25
3	>200	>200	>200	>200
Isoniazid	2.5	25	0.05	>100

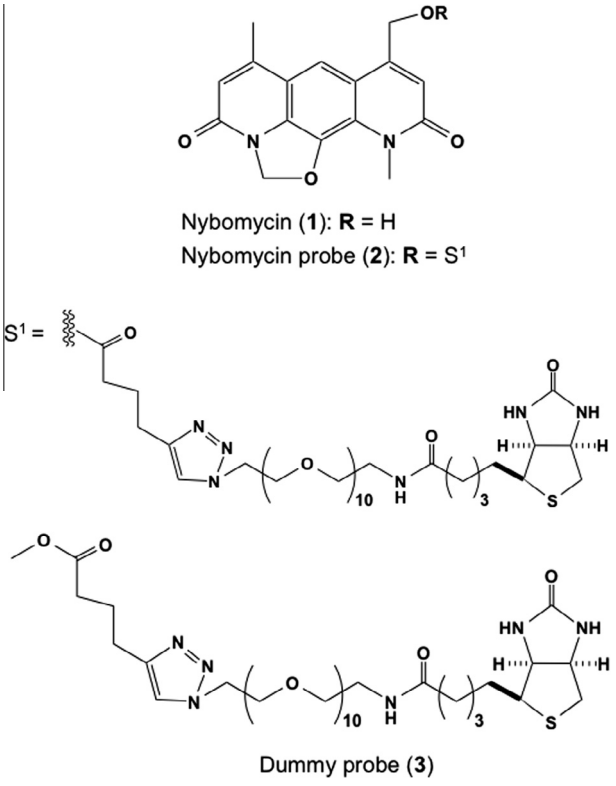

Figure 1. Chemical structures of nybomycin (**1**) and probes (**2** and **3**).

Table 2
MICs of nybomycin (**1**) against *M. tuberculosis* strains under aerobic conditions

Strains	MICs (μg/mL)
	1
M. tuberculosis H37Rv	4.2
M. tuberculosis T1413	6.3
M. tuberculosis T1538	5.2

Sequencing analyses of the cosmids isolated from each resistant transformant revealed that the cosmids of pYUB415_2012 and 2015, which were extracted from the ICHO2012 and 2015 transformants respectively, contained 29.9 kb (coordinates from 686.465 kb to 716.464 kb) of the same genome fragment. Then, the cosmid of pYUB415_2013 extracted from the ICHO2013 transformant included 34.5 kb (coordinates from 674.538 kb to 709.074 kb) of genome fragment (Fig. 4A). On the other hand, the cosmid pYUB415_2014 extracted from the ICHO2014 transformant contained 29.7 kb (coordinates from 4101.265 kb to 4130.915 kb) (Fig. 4B). Therefore, this result indicated that the gene giving resistance to compound **1** would be contained in the genome fragment of *M. bovis* BCG from 686.465 kb to 709.074 kb (22.6 kb, region 1) and in that from 4101.265 kb to 4130.915 (29.7 kb, region 2). However, there was no homologously and functionally similar gene between region 1 and region 2. Moreover, these regions did not include the gene encoding the multidrug resistance (MDR) protein, which reduced intracellular concentration of nybomycin (**1**). We then selected three gene regions (S1–S3) and two genes (S4 and S5) at random (Fig. 4) and investigated whether the transformants, which over-expresses each gene region or gene, shows resistance to compound **1** or not. As shown in Figure 3B, the ICHO1034 and ICHO1036 transformants, which overexpressed S1 region and S3 region respectively, grew on the agar plate

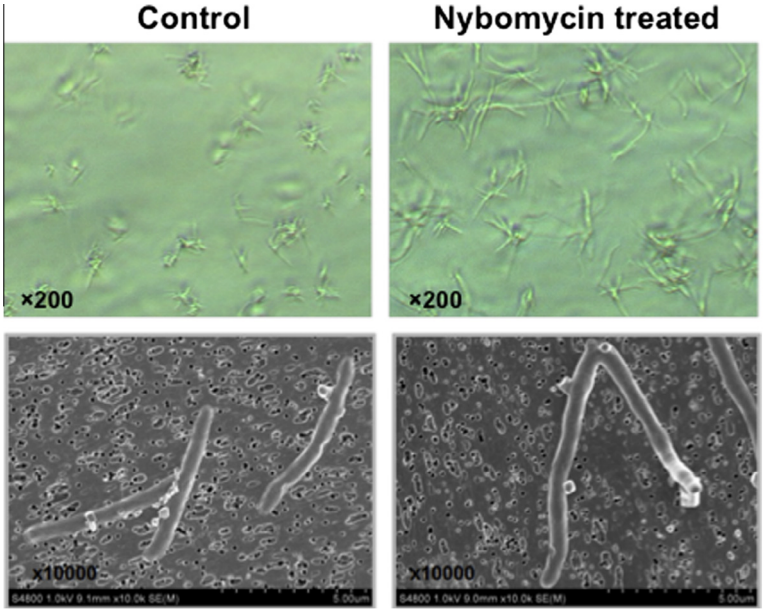

Figure 2. Morphology of nybomycin-treated mycobacterial bacilli. The phase-contrast microscopy (upper) and the scanning electron microscopy (lower) of *M. smegmatis* treated with or without 1.0 μg/mL of nybomycin (**1**) for 48 h.

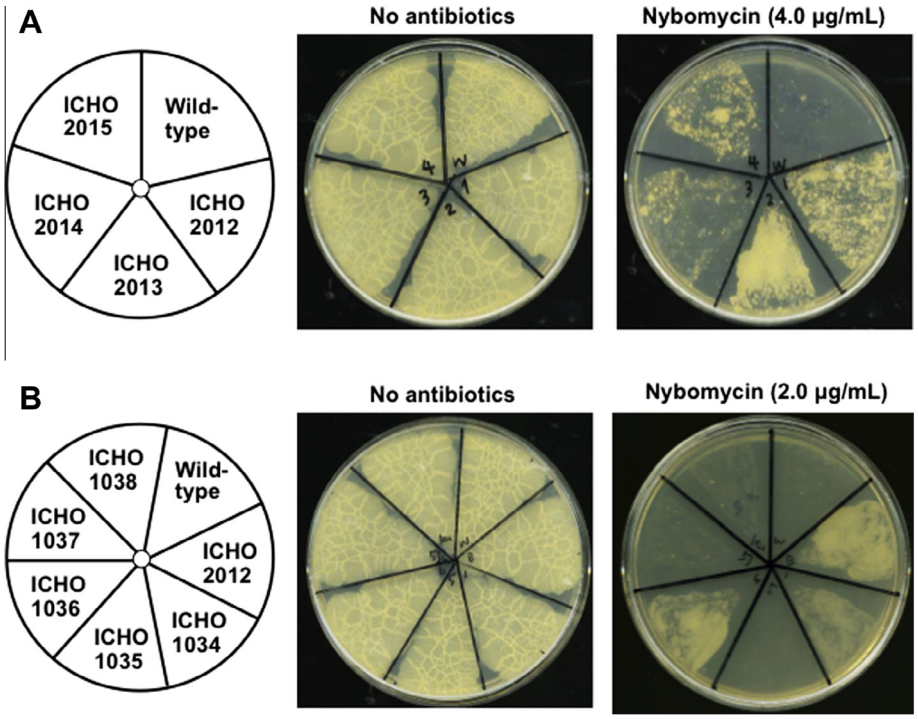

Figure 3. Growth of various transformants on the 7H10 agar containing nybomycin **(1)**. Growth of the nybomycin-resistant *M. smegmatis* transformed with the genomic DNA library of *M. bovis* BCG (A) and the transformants of *M. smegmatis* over-expressing candidate regions or genes conferring resistance to nybomycin (**1**) (B) on the 7H10 agar plate containing nybomycin (**1**). Each strain was cultured in the 7H9 broth, and cell number then was adjusted to 1×10^7 CFU/mL. The 10 μL aliquots of each culture were spread on the 7H10 agar plate in the presence or absence of indicated concentration of nybomycin (**1**).

containing 2.0 μg/mL (2× MIC) concentration of compound **1** similar to the ICHO2012 transformant overexpressing region 1. The ICHO1037 and ICHO1038 transformants, which overexpresses S4 gene and S5 gene respectively, exhibited a weak resistance to compound **1** as compared with ICHO2012 strain, whereas the ICHO1035 transformant overexpressing S2 region did not grow on the agar plate containing compound **1**. In addition, the five transformants, ICHO1034–1038, did not grow on the agar plate containing 4 μg/mL (4× MIC) of compound **1**, while the ICHO2012 strain grew under this conditions (Data not shown).

Recently, Hiramatsu et al. reported that nybomycin (**1**) was effective to the quinolone-resistant *Staphylococcus aureus* having

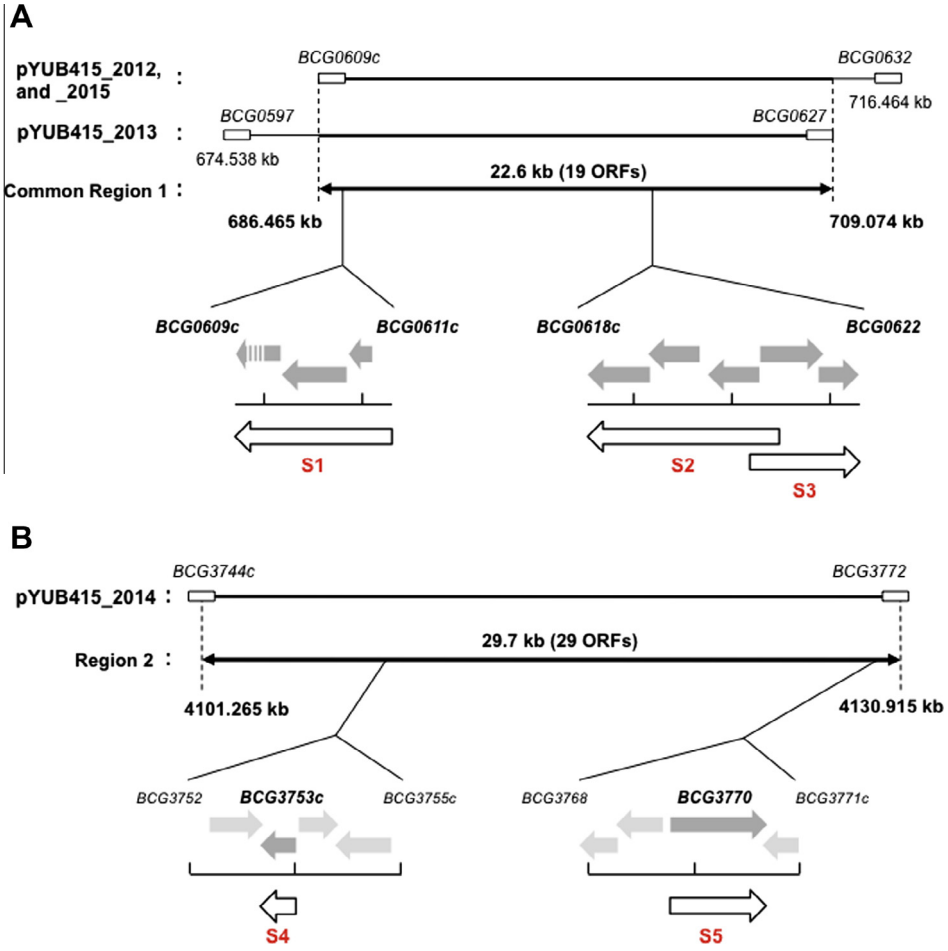

Figure 4. Gene regions of cosmids isolated from nybomycin-resistant transformants.

mutation in *gyrA* (type II topoisomerase) gene, whereas **1** was inactive against the strain having intact *gyrA* gene. Then, they supposed that compound **1** inhibited the DNA gyrase with altered GyrA subunit.[19] On the other hand, we isolated a spontaneous nybomycin (**1**)-resistant of *M. smegmatis* (MIC = 10 μg/mL), and analyzed gene mutation by whole genome sequencing. Our preliminary result indicated that the analyzed strain have the following two mutations in amino acid levels; L59P in MSMEG_6223 (tetR family transcriptional repressor) and A23V in MSMEG_6471 (glycine/D-amino acid oxidase). In addition, no mutation was observed in *pknA* gene (MSMEG_0006), *pknB* gene (MSMEG_5437), *ftsZ* gene (MSMEG_4222), *whmD* gene (MSMEG_1831), and two *gyrA* genes (MSMEG_0006 and _0456) in the spontaneous **1**-resistant strain (data not shown). From these results, we hypothesized that nybomycin (**1**) targets mycobacterial genome instead of particular protein.

In order to confirm this hypothesis, we synthesized nybomycin probe (**2**) and dummy probe (**3**) and investigated binding affinity of the probes **2** and **3** to several plasmid DNA (Fig. 1). As shown in Table 1, the probe **2** retained moderate anti-microbial activity against *M. smegmatis* and *M. bovis* BCG under both aerobic conditions and hypoxic conditions with MIC values ranging 6.25–25 μg/mL, whereas probe **3** did not show the activity. Then, the probe **2** bound to the plasmid DNA, which cloned S3 region into pMV206 (Fig. 5A, lanes 3 and 4), and this binding was partially inhibited by the addition of nybomycin (**1**) as a competitive inhibitor (Fig. 5A, lanes 5 and 6). Whereas no effect was observed in the case for addition of isoniazid, which targeted enoyl-ACP reductase (Fig. 5A, lanes 7 and 8). The avidin beads itself (Fig. 5A, lanes 1 and 2) and dummy probe (**3**) (Fig. 5A, lanes 9 and 10) did not bind to plasmid DNA. Interestingly, the plasmid pMV206 itself (Fig. 5B, lanes 7 and 8) and the pMV206 plasmid inserted the S2 region, which was contained in the non-resistant transformant (ICHO1035) (Fig. 5B, lanes 9 and 10. Fig. 3B), exhibited weak affinity to probe **2** in comparison with the pMV206 inserted the S3 region, which gives the resistance against compound **1** to *M. smegmatis* (Fig. 5B, lanes 11 and 12. Fig. 3B). This result indicated that nybomycin (**1**) directly binds to DNA. Moreover, nybomycin (**1**) basically has a DNA binding property, and its binding amount and affinity to DNA would be altered depending on DNA sequence.

3. Conclusion

Nybomycin (**1**) isolated from the culture broth of a marine-derived *Streptomyces* sp. showed potent anti-microbial activity against *Mycobacterium* sp. in both actively growing aerobic conditions and dormancy induced hypoxic conditions. In addition, compound **1** was also effective to pathogenic strains of *M. tuberculosis* including clinically isolated strains. This result proved the validity of nybomycin (**1**) as a new drug lead for Tuberculosis. The further study of target identification for compound **1** clarified that compound **1** directly binds to plasmid DNA with some selectivity. Nybomycin (**1**) might bind to mycobacterial genome to cause inhibition of DNA replication and transcription, so that the unique

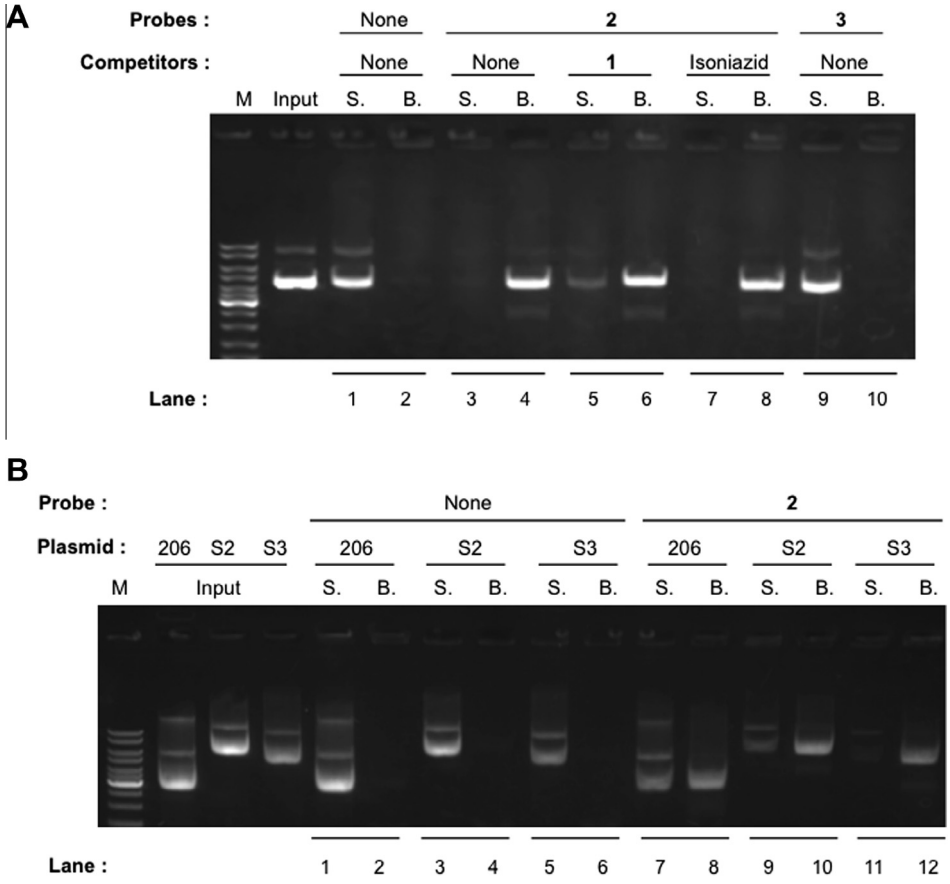

Figure 5. Binding of nybomycin probes **2** and **3** to DNA. (A) The probe **2** or **3** conjugated or unconjugated Dynabeads were suspended with the solution of the plasmid DNA (300 ng/0.1 mL) that cloned S3 region into pMV206, in the presence or absence of nybomycin (**1**, 60 μM) or isoniazid (60 μM) as competitive inhibitors. The each reaction mixture was then incubated at room temperature for 1 h, and the beads were separated by centrifugation. The resulting beads and supernatants were subjected to agarose gel electrophoresis, and the resolved DNAs were visualized after ethidium bromide staining. (B) The binding of probe **2** to the various kinds of plasmids was investigated using same protocol of the experiment A. The following is the meaning of abbreviations: **M**; Marker, **S**; Supernatant, **B**; Beads, **206**; Mock plasmid pMV206, **S2** and **S3**; pMV206 cloned S2 region or S3 region, respectively, as shown in Figure 4 and Table 3.

morphological change leading the bacterial cell death is induced. The further analysis of mode of action of nybomycin (**1**) is currently under way.

4. Experimental

4.1. General materials

Middlebrook 7H9 broth, Middlebrook 7H10 agar, Middlebrook OADC Enrichment, and Luria–Bertani (LB) broth were obtained from BD (Franklin Lakes, NJ). DNA restriction enzymes and T4 DNA ligase were obtained from New England BioLabs Inc. (Ipswich, MA). Expand High Fidelity PCR System (Roche Applied Science, Mannheim, Germany) was used for PCR. Streptavidin-conjugated Dynabeads (M-280) was purchased from Invitrogen (Carlsbad, CA). Isoniazid, hygromycin B, kanamycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma (St. Louis, MO) or Kishida Chemical Co., Ltd (Osaka, Japan).

The following instruments were used to obtain physical data: a JASCO P-2200 digital polarimeter (L = 50 mm) for specific rotations; a JEOL ECS-300 (^{1}H NMR: 300 MHz, ^{13}C NMR: 75 MHz), ECA-500 (^{1}H NMR: 500 MHz, ^{13}C NMR: 125 MHz) and a Varian NMR system (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) spectrometer for ^{1}H and ^{13}C NMR data using tetramethylsilane as an internal standard; a JASCO FT/IR-5300 infrared spectrometer for IR spectra; a Waters Q-Tof Ultima API mass spectrometer for ESI-TOF MS; HPLC was performed using a Hitachi L-6000 pump equipped with Hitachi L-4000H UV detector. Silica gel (Kanto, 40–100 μm) and pre-coated thin layer chromatography (TLC) plates (Merck, $60F_{254}$) were used for column chromatography and TLC. Spots on TLC plates were detected by spraying acidic *p*-anisaldehyde solution (*p*-anisaldehyde: 25 mL, *c*-H_2SO_4: 25 mL, AcOH: 5 mL, EtOH: 425 mL) or phosphomolybdic acid solution (phosphomolybdic acid: 25 g, EtOH: 500 mL) with subsequent heating.

4.2. Isolation of nybomycin (1) from a marine-derived *Streptomyces* sp.

The marine-derived *Streptomyces* sp. MS44 was isolated from the marine sediment, which was collected at Maizuru, Kyoto prefecture, Japan in 2000. The MS44 strain was cultured in the ISP1 + glucose medium (totally 10 L) consisting of 0.5% bacto tryptone, 0.3% yeast extract, and 0.5% glucose under shaking condition for 2 weeks at 30 °C. The 2 weeks old culture broth was extracted with 2-butanone twice to obtain a crude extract. The crude extract (4.0 g, MIC = 200 μg/mL against *M. smegmatis* under aerobic and hypoxic conditions) was partitioned with *n*-hexane (1 L) and 90% methanol (1 L). The resulting insoluble material (100 mg), which showed most potent anti-microbial activity against *M. smegmatis* under both aerobic and hypoxic conditions with MIC of 12.5 μg/mL, was washed several times with methanol. After

further partitioned with chloroform–methanol (ratio 3:1, 300 mL) and water (150 mL), the organic layer was concentrated in vacuo and finally recrystallized with TFA-water to obtain 14 mg of nybomycin (**1**) as white needle-like crystals. Nybomycin (**1**) was identified by ESI-TOF-MS and 2D-NMR analyses and comparison with authentic spectral data.[9,11,20]

4.3. Bacterial strains and culture

Nonpathogenic strains of *M. smegmatis* mc^{2}155 and *M. bovis* BCG Pasteur are kindly provided from Dr. William R. Jacobs, Jr. (Albert Einstein College of Medicine, New York, USA). *M. tuberculosis* T1413 and T1538 strains were clinically isolated at University of KwaZulu Natal as drug susceptible strains, while *M. tuberculosis* H37Rv is standard laboratory strain.

Escherichia coli DH5α was used for cloning and maintaining plasmid and was grown in LB liquid medium. Mycobacterial strains were grown in the Middlebrook 7H9 broth containing 10% OADC, 0.5% glycerol and 0.05% Tween 80 or on the Middlebrook 7H10 agar containing 10% OADC and 0.5% glycerol. *M. smegmatis* was grown in the LB liquid medium with 0.05% Tween 80 for competent cell preparation. The concentrations of antibiotics used were 150 μg/mL (hygromycin B), and 40 μg/mL (kanamycin) for *E. coli* strains, and 50 μg/mL (hygromycin B) and 20 μg/mL (kanamycin) for *Mycobacterium* strains.

4.4. Anti-microbial activity of compounds under aerobic and hypoxic conditions

MIC values against *M. smegmatis* of a fast growing strain and *M. bovis* BCG and *M. tuberculosis* of slow growing strains were determined using the established MTT method.[21] Mid-log phase bacilli [*M. smegmatis* (1×10^4 CFU/0.1 mL) or *M. bovis* BCG and *M. tuberculosis* (1×10^5 CFU/0.1 mL)] were inoculated in a 96-well plate, and then, serially diluted sample was added to the 96-well plate. In the case of aerobic condition, the bacteria were incubated at 37 °C for 36 h (for *M. smegmatis*) or for 7 days (for *M. bovis* BCG and *M. tuberculosis*). Alternatively, the hypoxic model was performed based on the protocol of Rustad et al. with minor modification.[22] The mycobacterial bacilli were grown in Middlebrook 7H9 broth at 37 °C under nitrogen atmosphere containing 0.2% oxygen until optical density reached 0.8 at 600 nm. Subsequently, the bacilli were inoculated to the 96-well plate at the same density under aerobic condition and incubated at 37 °C under nitrogen atmosphere containing 0.2% oxygen for 96 h (for *M. smegmatis*) or for 14 days (for *M. bovis* BCG). After incubation, 50 μL of MTT solution (0.5 mg/mL) was added to each well and incubated at 37 °C for additional 12 h under aerobic or hypoxic conditions. The optical density at 560 nm was measured to determine the MIC value.

4.5. Electron microscopic analysis of nybomycin-treated *M. smegmatis*

10 mL of *M. smegmatis* (1×10^6 CFU/mL) was incubated for 48 h at 37 °C in the presence or absence of 1.0 μg/mL nybomycin (**1**). The bacilli were collected by centrifugation and washed with 10 mL of PBS. The bacilli were re-suspended with 10 mL of PBS and diluted 256 times with PBS. The 100 μL of portion were transferred on the membrane (MILLIPORE, ISOPORE membrane filters 0.2 μm) using vacuum pump. The bacilli on the membrane were fixed with 2% osmium tetraoxide solution, and were applied to a silicon wafer slide and sputter-coated with gold before examination by an electronic microscope (JSM-5200, JEOL, Japan).

4.6. Syntheses of probes 2 and 3

4.6.1. Nybomycin probe (2)

White solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.55 (1H, s), 7.38 (1H, s), 6.82 (1H, s), 6.71 (1H, s), 6.48 (1H, s), 6.40 (2H, s), 5.88 (1H, s), 5.37 (2H, s), 5.18 (1H, s), 4.51 (3H, t-like), 4.35 (1H, t, J = 6.0 Hz), 3.95 (3H, s), 3.86 (2H, t, J = 5.2 Hz), 3.66–3.62 (36H, m), 3.55 (2H, t, J = 5.2 Hz), 3.44 (2H, t, J = 4.3 Hz), 3.17–3.13 (1H, m), 2.92 (1H, dd, J = 13.5, 5.4 Hz), 2.80–2.73 (3H, m), 2.54 (2H, t, J = 7.7 Hz), 2.50 (3H, s), 2.26–2.22 (2H, m), 2.10–2.04 (2H, m), 1.76–1.63 (4H, m), 1.44 (2H, t, J = 7.4 Hz). IR (KBr): 3266, 2922, 1798, 1740, 1669, 1634, 1456, 1352, 1248, 1101 cm^{-1}. MS (ESI-TOF) m/z: 1167 [M+Na]$^+$. HRMS (ESI-TOF) m/z: 1167.5260, calcd for $C_{54}H_{80}N_8O_{17}SNa$ [M+Na]$^+$; Found: 1167.5293. Synthesis of nybomycin probe (**2**) was described in detail at Supplementary data.

4.6.2. Dummy probe (3)

White solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.49 (1H, s), 6.79 (1H, s), 6.27 (1H, s), 5.39 (1H, s), 4.50 (3H, t, J = 5.2 Hz), 4.31 (1H, t, J = 6.1 Hz), 3.84 (2H, t, J = 5.2 Hz), 3.66 (3H, s), 3.66–3.63 (32H, m), 3.60 (4H, s), 3.55 (2H, t, J = 5.2 Hz), 3.47–3.38 (3H, m), 3.15–3.12 (1H, m), 2.89 (1H, dd, J = 12.8, 4.9 Hz), 2.75–2.74 (3H, m), 2.38 (2H, t, J = 7.4 Hz), 2.22 (2H, td, J = 7.3, 3.1 Hz), 2.00 (2H, quint, J = 7.4 Hz), 1.76–1.63 (4H, m), 1.46–1.41 (2H, m). IR (KBr): 3285, 2922, 2872, 1701, 1452, 1352, 1246, 1111 cm^{-1}. MS (ESI-TOF) m/z: 901 [M+Na]$^+$. HRMS (ESI-TOF) m/z: 901.4568, calcd for $C_{39}H_{70}N_6O_{14}SNa$ [M+Na]$^+$; Found: 901.4604. Synthesis of dummy probe (**3**) was described in detail at Supplementary data.

4.7. Binding affinity of probe 2 to DNA

The 150 μg of streptavidin conjugated Dynabeads (M-280) were mixed with probe **2** or probe **3** (260 pmol each) in the 100 μL of 5 mM Tris–HCl buffer (pH 7.5) supplement with 0.5 mM EDTA and 1 M NaCl (Binding buffer), and were incubated at room temperature for 30 min to form a probe **2** (or probe **3**) conjugated Dynabeads. After washing the beads with binding buffer, the probe **2** (or probe **3**) conjugated beads were re-suspended with the DNA solutions (300 ng/0.1 mL) in binding buffer in the presence or absence of nybomycin (**1**, 60 μM) or isoniazid (60 μM) as competitive inhibitors. The each reaction mixture was then incubated at rt for 1 h. After centrifugation (1500×*g*, 5 min), the supernatant was transferred to a new test tube, and the beads were washed with binding buffer 3 times. Next, the washed solutions were combined with the corresponding supernatant. The beads was mixed with DNA loading dye, boiled for 3 min, and cooled down at room temperature. The DNA in the supernatant was precipitated by the method of ethanol precipitation. Then, the resulting DNA was dissolved in the DNA loading dye. The samples from the beads or supernatants were subjected to agarose gel electrophoresis, and the resolved DNAs were visualized by ImageQuant LAS4010 Digital Imaging System (GE Healthcare Life Sciences) after ethidium bromide staining.

4.8. Construction of genomic DNA Library and transformation of *M. smegmatis*

The chromosomal DNA of *M. bovis* BCG was prepared using the hexadecyltrimethylammonium bromide (CTAB) method.[23] Chromosomal DNA was then digested with restriction endonuclease *Sau*3AI to produce approximately 30 kb DNA fragments. A genomic DNA library was constructed in the *E. coli*-*Mycobacterium* shuttle cosmid pYUB415 by using the double *cos* vector strategy as previously described.[24] Briefly, the left and right arms of pYUB415 were generated by digestion with restriction endonucleases of *Xba*I and *Bam*HI. The fragments of the genome

Table 3
List of the transformants of *M. smegmatis* and description of plasmids

Strains	Vectors	Description of plasmids	Sequence of primers (5′ to 3′) F: Forward primer, R: Reverse primer
ICHO1034	pMV206	Cloned S1 area[a] (685769–689308 bp of *M. bovis* BCG genome)[b]	F: GGTACCGTTCGGTGAACAGCCCAAG R: AAGCTTCGTTGTGCGGACATCACC
ICHO1035	pMV206	Cloned S2 area[a] (696695–700844 bp of *M. bovis* BCG genome)[b]	F: GGTACCAACGGGTGACCGTGAACTG R: ATCGATTCGATGCGTCGGCAGTC
ICHO1036	pMV206	Cloned S3 area[a] (700476–702854 bp of *M. bovis* BCG genome)[b]	F: TCTAGACGTTTGGCCACCTGGTAGC R: ATCGATCCGGGATGCTCCCTATTGC
ICHO1037	pMV261	Cloned S4 area (*BCG3753c* gene)[a] (4109204–4110235 bp of *M. bovis* BCG genome)[b]	F: AAGCTTTGCGCAGGGTGGAC R: GTTAACGTCGTGTCGCGAGTTTC
ICHO1038	pMV261	Cloned S5 area (*BCG3770* gene)[a] (4126836–4129396 bp of *M. bovis* BCG genome)[b]	F: AAGCTTACCATCCCGGAGCAAC R: GTTAACTCATGCCGCGGACC
ICHO2012	pYUB415	Cloned genome fragment of *M. bovis* BCG (686465–716464 bp)[b]	N/A Direct cloning of genome fragment
ICHO2013	pYUB415	Cloned genome fragment of *M. bovis* BCG (674538–709074 bp)[b]	N/A Direct cloning of genome fragment
ICHO2014	pYUB415	Cloned genome fragment of *M. bovis* BCG (4101265–4130915 bp)[b]	N/A Direct cloning of genome fragment
ICHO2015	pYUB415	Cloned genome fragment of *M. bovis* BCG (686465–716464 bp)[b]	N/A Direct cloning of genome fragment

[a] Name of area corresponds to Figure 4.
[b] Coordinate of *M. bovis* BCG genome is based on the database of BCGList (http://genolist.pasteur.fr/BCGList).

were ligated to both arms of pYUB415. The mixture of ligations were in vitro packaged with packaging mix of MaxPlax Lambda Packaging (Epicentre), and the resulting recombinant cosmids were transduced to *E. coli* HB101. The transformants of *E. coli* HB101 were selected on LB agar plates containing carbenicillin. Over 3×10^5 independent clones were pooled, and cosmids for transformation of *M. smegmatis* were obtained by large-scale DNA preparation by using a standard alkaline lysis method.

The generation of transformants of *M. smegmatis* with genomic DNA library as previously described.[16] Briefly, *M. smegmatis* were grown at 37 °C as described above until the optical density reached 0.8–1.0 at 600 nm. The cultures were centrifuged, and the resulting pellets were washed with 10% glycerol twice and re-suspended in the same solution (1/10th of the initial culture volume). The cell suspensions were mixed with the gnomic DNA library and electroporated (2500 V, 25 μF, 1000 Ω). The resulting suspensions were incubated at 37 °C for 4 h and then plated on Middlebrook 7H10 agar containing 50 μg/mL of hygromycin B.

4.9. Isolation of nybomycin-resistant clones from *M. smegmatis* transformed with the genomic DNA library and end sequencing of cosmids

Nybomycin-resistant clones were screened from over the 4×10^3 *M. smegmatis* transformed with the genomic DNA library by cultivating on the 7H10 agar containing 4.0 μg/mL concentration of nybomycin (**1**, 4× MIC). Subsequently, the resistant clones against nybomycin (**1**) were grown in the 7H9 broth containing hygromycin B, and the cosmids for end sequencing were isolated using the standard alkaline lysis method. The cosmids extracted from the nybomycin-resistant transformants were subjected to end sequencing (MACROGEN Japan, Tokyo, Japan). The primers P1 (5′-GTACGCCACCGCCTGGTTC-3′) and P2 (5′-GTGCCACCTGA CGTCTAAG-3′), which were designed based on the sequence of cosmid vector pYUB415, were used for end sequences of the cosmids. The obtained sequences were analyzed by BLAST search using the database of BCGList (http://genolist.pasteur.fr/BCGList/), TubercuList (http://genolist.pasteur.fr/TubercuList/), and Comprehensive Microbial Resource in J. Craig Venter Institute (http://cmr.tigr.org/cgi-bin/CMR/CmrHomePage.cgi).

4.10. Preparation of *M. smegmatis* over-expressing candidate genes that confer resistance to nybomycin (1)

The candidate gene regions or genes (Fig. 4) that confer resistance to nybomycin (**1**) were PCR amplified from the cosmid pYUB415_2013 or _2014 by using the primer pairs as shown in Table 3. PCR was performed using a program of 30 cycles of 94 °C for 15 s, 57 °C for 30 s, and 68 °C for 1 min/kb. Following cloning into pCR2.1-TOPO (Life Technologies, Carlsbad CA) and sequencing, the cloned PCR fragment was excised using the primer-introduced restriction sites and cloned into the mycobacterial expression vector pMV261 with a *hsp*60 promoter and kanamycin-resistant gene or promoter-less shuttle vector pMV206 containing the hygromycin B-resistant gene. Description of plasmids and name of transformants are shown in Table 3. *M. smegmatis* was transformed with the constructed plasmids, and the transformants were investigated susceptibility against nybomycin (**1**) using the above-mentioned methods.

4.11. Isolation of spontaneous nybomycin-resistant strain

M. smegmatis (1×10^7 CFU/mL) was incubated in Middlebrook 7H9 broth containing 5.0 μg/mL nybomycin (**1**, 5× MIC) at 37 °C for 3 days. The culture broth was plated on Middlebrook 7H10 agar containing 10 μg/mL nybomycin (**1**) and incubated at 37 °C for 3 days. Strains showing resistant against nybomycin (**1**) were picked up and incubated on Middlebrook 7H10 agar containing 10 μg/mL nybomycin (**1**) for further 3 days. The obtained strains were used for the experiments as spontaneous mutants. The analysis of whole genome sequencing was done by Hokkaido System Science Co., Ltd (Hokkaido, Japan) using Illumina MiSeq.

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Supplementary data

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