The Journal of Antibiotics (2013) 66, 663–667

www.nature.com/ja

ORIGINAL ARTICLE

Structure–activity relationship study of novel iminothiadiazolo-pyrimidinone antimicrobial agents

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An iminothiadiazolo-pyrimidinone derivative, 0002-04-KK, harboring a furan moiety, acts as an antimicrobial agent with a minimum inhibitory concentration (MIC) against *Staphylococcus aureus* of 25 μg ml^{-1}. Several derivatives of 0002-04-KK were synthesized and among them 0026-59-KK, harboring a nitrofuran moiety, had the most potent antimicrobial activity with an MIC of 6 μg ml^{-1}. Both 0002-04-KK and 0026-59-KK inhibited the biosynthesis of DNA, RNA and proteins. Peptidoglycan biosynthesis was inhibited by 0026-59-KK, and slightly inhibited by 0002-04-KK. Derivative 0002-04-KK showed bactericidal activity in contrast to the bacteriostatic activity of 0002-04-KK. Derivative 0002-04-KK had less toxicity in silkworms (lethal dose fifty (LD_{50}): >230 μg g^{-1}) than 0002-04-KK (LD_{50}: 100 μg g^{-1}). The bactericidal activity against *S. aureus* was because of the nitrofuran moiety. These findings suggest that iminothiadiazolo-pyrimidinone compounds could be used as lead molecules to develop antimicrobial agents.
The Journal of Antibiotics (2013) **66,** 663–667; doi:10.1038/ja.2013.69; published online 3 July 2013

Keywords: bactericidal; macromolecule; nitrofuran; novel antimicrobial; structure–activity relationship

INTRODUCTION

The search for antimicrobial agents against human pathogens has been ongoing for centuries and remains a challenging task. Antimicrobials launched into the clinical field have led to the rapid emergence of more microbes resistant to these antimicrobials. The emergence of strains resistant to recently introduced antimicrobials enhances the threat of infectious diseases spreading around the globe. Many notorious resistant pathogens, such as methicillin-resistant *Staphylococcus aureus*,[1,2] vancomycin-intermediate *S. aureus*,[3,4] vancomycin-resistant *Enterococcus faecalis*,[5] multidrug-resistant *Pseudomonas aeruginosa*,[6] and multidrug-resistant *Mycobacterium tuberculosis*[7] cause serious problems for health-care facilities as well as the community. Hence, the development of new antimicrobial agents continues to be an urgent issue. To combat resistant strains, new structural antimicrobial agents are required. Here we identified a novel antimicrobial agent and modified its structure to reduce toxicity and enhance antimicrobial activity.

RESULTS

Chemical library screening for antibacterial compounds against *S. aureus*

Among the 103 873 compounds in the chemical library, we identified 3383 candidates (3.3%) with antimicrobial activity against the *S. aureus* MSSA1 strain at a concentration of 100 μM. We selected 0002-04-KK (**1**), whose antibacterial activity against *S. aureus* MSSA1 was 25 μg ml^{-1}. As the structure of the compound was promising, further studies of the structure function relationship were performed. First, we confirmed the antimicrobial activity by the synthesis of **1**. Then, we evaluated the toxicity of **1** using silkworms. The median lethal dose (LD_{50}) value of the compound in the silkworm was 100 μg g^{-1}.

Structure–activity relationship of 1

To enhance the antibacterial activity and reduce the toxicity of **1**, we examined the structure–activity relationship (SAR). Toxicity was tested using silkworms as a model animal.[8] Iminothaidiazolo-pyrimidinone (Table 1) was used as the mother compound and structures at the R^1 and R^2 positions were modified to obtain nine different compounds. Figure 1 summarizes the synthesis of derivatives of **1**. Condensation of 2-amino-5-substituted-1,3,4-thiadiazole and ethyl cyanoacetate gave 5-imino-2-(substituted)-5*H*-(1,3,4)thiadiazolo(3,2–a)pyrimidin-7(6*H*)-one. Further reaction with aldehyde (R^2CHO) in the presence of a catalytic amount of Et_3N gave products of 28–70% yield. Table 1 shows the results of the SAR analysis. Compound 0026-59-KK (**2**) exhibited the strongest antibacterial activity against *S. aureus* and had the least toxicity in silkworms. Hence, this compound was selected for further evaluation.

Antimicrobial spectrum of 1 and 2

Antimicrobial activities of **1** and **2** were tested with various microorganisms including Gram-positive bacteria, Gram-negative bacteria

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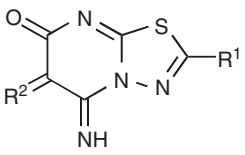
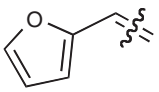
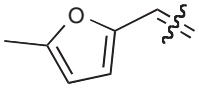
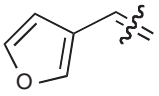
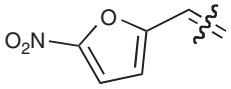
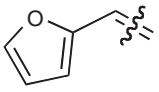
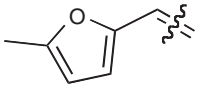
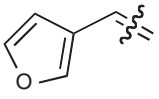
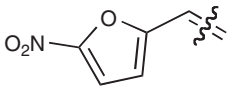
Received 23 March 2013; revised 28 May 2013; accepted 30 May 2013; published online 3 July 2013

Table 1 Structural activity relationship of the compounds

	R^1	R^2	Compound name	MIC ($\mu g\,ml^{-1}$)	LD_{50} ($\mu g\,g^{-1}$)	Mode of action
			0001-03-KK	25	60	
			0002-04-KK **(1)**	25	100	Bacteriostatic
			0017-22-KK	12.5	140	
			0021-27-KK	25	50	
			0027-56-KK **(4)**	25	>230	Bactericidal
			0005-08-KK	>400	>230	
			0006-09-KK **(3)**	25	160	Bacteriostatic
			0019-25-KK	12.5	30	
			0023-37-KK	400	>230	
			0026-59-KK **(2)**	6.3	>230	Bactericidal

Abbreviation: MIC, minimum inhibitory concentration.

and some fungi. Both compounds had antimicrobial activity against Gram-positive bacteria, including antibiotic-resistant strains such as methicillin-resistant *S. aureus* and vancomycin-resistant *E. faecalis*. Compound **2** showed more potent activity than **1** (Table 2). Neither compounds exerted any antimicrobial activity against Gram-negative bacteria. Compound **1** also had weak activity against some fungi, such as Candida and Cryptococcus, but not *Aspergillus niger*, whereas **2** exhibited no antifungal activity.

Mechanism of antibacterial action of 1 and 2

Pulse-labeling experiments for 30 min were performed. Both **1** and **2** inhibited the incorporation of radiolabeled precursors, [^{3}H]thymidine, [^{3}H]uridine and [^{35}S]methionine into acid-insoluble fractions in exponentially growing *S. aureus* (Figure 2). Incorporation of [^{3}H]N-acetyl glucosamine was inhibited by **2** and slightly inhibited by **1** (Figure 2). Interestingly, **2** showed bactericidal activity, whereas **1** did not; **1** merely exhibited bacteriostatic activity (Figure 3). The bactericidal activity of **2** was potent: it killed >99.9% of bacteria

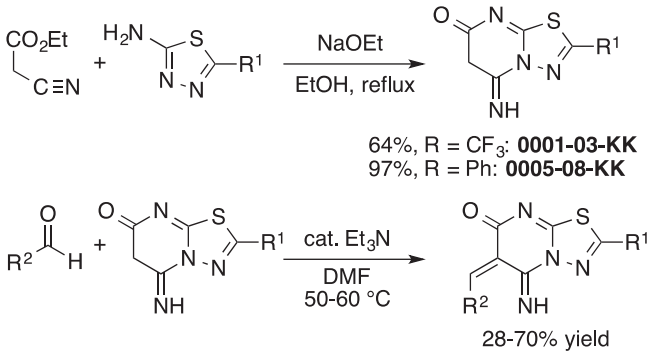


Figure 1 Scheme of synthesis of iminothiadiazolo-pyrimidinone derivatives.

Table 2 Minimum inhibitory concentration (MIC) of the compounds against microorganisms

	MIC (μg ml^{-1})	
Strain	*1*	*2*
S. aureus MSSA1	50	6.3
S. aureus RN4220	50	3.1
S. aureus NCTC8325-4	50	3.1
S. aureus Smith	50	6.3
Methicillin-resistant *S. aureus* MRSA3	50	6.3
Methicillin-resistant *S. aureus* MRSA4	50	6.3
Methicillin-resistant *S. aureus* MRSA6	50	6.3
Methicillin-resistant *S. aureus* MRSA8	50	6.3
Methicillin-resistant *S. aureus* MRSA9	25	6.3
Methicillin-resistant *S. aureus* MRSA11	50	3.1
Methicillin-resistant *S. aureus* MRSA12	50	6.3
Enterococcus faecalis EF1	100	25
Vancomycin-resistant *Enterococcus faecalis* EF5 (VRE)	100	12.5
Bacillus subtilis JCM2499	100	6.3
Bacillus cereus JCM20037	100	3.1
Streptococcus sanguinis JCM5708	>100	25
S. pyogenes SS1-9	>100	25
S. agalactiae JCM5671	>100	12.5
S. pneumoniae JCSC6523	50	1.5
Serratia marcescens 98-130-37	>100	>100
Escherichia coli W3110	>100	>100
Pseudomonas aeruginosa PAO1	>100	>100
Candida albicans ATCC10231	100	>100
C. tropicalis pK233	100	>100
Cryptococcus neoformans H99	100	>100
Aspergillus niger	>100	>100

within 30 min. The killing activity was comparable to that of daptomycin. To gain more insight into which moiety was responsible for the killing action, two other compounds, 0006-09-KK (**3**) and 0027-56-KK (**4**) were selected and a killing assay was performed. Compound **4** exhibited bactericidal activity, whereas **3** showed bacteriostatic activity (Figure 3).

DISCUSSION

In this study, we found novel iminothaidiazolo-pyrimidinone antimicrobial agents screened by an *in vitro* antimicrobial test from a chemical library. We successfully produced compounds with more potent antimicrobial activity against Gram-positive bacteria and less toxicity in a silkworm model. We previously demonstrated that the LD_{50} values of cytotoxic compounds in the silkworm model are similar to those in mammalian models.[9] Thus, in mammalian models the toxicity of synthesized compounds such as **2** would likely be reduced compared to that of the original screened **1**. Antimicrobial activity of the mother structure has not yet been reported.

SAR studies are necessary to develop antimicrobial agents for clinical purposes. Our preliminary SAR study suggested that **2** harboring a benzene ring and a nitrofuran moiety in the R^1 and R^2 positions, respectively, was the most effective against *S. aureus* and less toxic to silkworm larvae. Nitrofuran-containing compounds are reported to have cytotoxic and carcinogenic activities.[10,11] Although **2** has less acute toxicity, its long-term toxicity and carcinogenicity are unknown. The presence of hydrogen (0005-08-KK) and a furan ring (0023-37-KK) led to a decreased antibacterial activity (Table 1). In other cases, antibacterial activity was not affected, whereas toxicity was higher in trifluoro-methane containing groups. Closer comparison of the structures revealed that the trifluoro-methane moiety was responsible for the toxicity, except in 0019-25-KK (Table 1). The combination of trifluoro-methane and methylfuran moieties might be responsible for abolishing the toxicity owing to the counter action of the methylfuran moiety. The toxicity of the trifluoro-methane moiety was also verified by the antifungal activity possessed by **2**. These findings further support the usefulness of the silkworm model for identifying a low toxic modification based on the SAR. In the conventional method, the cytotoxicity of synthesized compounds is tested using an *in vitro* cell culture system, but this method does not reflect the pharmacokinetics of the tested compounds. Thus, the toxicity of compounds between individual and cultured cells does not always correspond. Testing the toxicity of many synthesized compounds using mammals, however, is not only costly but is also associated with ethical problems. The silkworm model solves these problems and accelerates the development of novel antimicrobial agents.

We found that **1** was bacteriostatic, whereas **2**, which is a chemically modified version of **1**, had potent bactericidal activity that killed *S. aureus* within 30 min. Inhibition of the incorporation of radiolabeled precursors into the macromolecules revealed that both **1** and **2** inhibited the biosynthesis of all the macromolecules: DNA, RNA and proteins. Although peptidoglycan synthesis was inhibited by **2,** it was only slightly inhibited by 1. Therefore, the potency of peptidoglycan synthesis inhibition was enhanced. The fact that **3** was bacteriostatic, whereas **4** was bactericidal, revealed that the nitrofuran moiety was responsible for the bactericidal activity. In the present study, SAR analysis led to the modification of a weak antimicrobial agent to a compound with potent antimicrobial activity; bacteriostatic activity was further improved to bactericidal activity. Further derivatization based on these results is required.

METHODS

Microbial strains and culture conditions

Gram-positive bacteria, including some resistant strains, Gram-negative bacteria and some fungi were used as test pathogens (Table 3). Bacterial cultures were prepared in either Luria Bertani medium (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}, pH 7.0) or Muller–Hinton Broth (MHB; Difco, Franklin Lakes, NJ, USA). Cation-adjusted MHB was used for antimicrobial susceptibility tests. For streptococcus species, cation-adjusted MHB with 2.5% lysed horse blood (Nippon Biotest Laboratories Inc, Tokyo, Japan), was used. Minimum inhibitory concentration (MIC) was determined as per the Clinical and Laboratory Standards Institute,[12] and Roswell Park Memorial Institute medium (Sigma Aldrich, St Louis, MO, USA) was used for testing antifungal susceptibility. Antifungal susceptibility was determined as per the Clinical and Laboratory Standards Institute.[9]

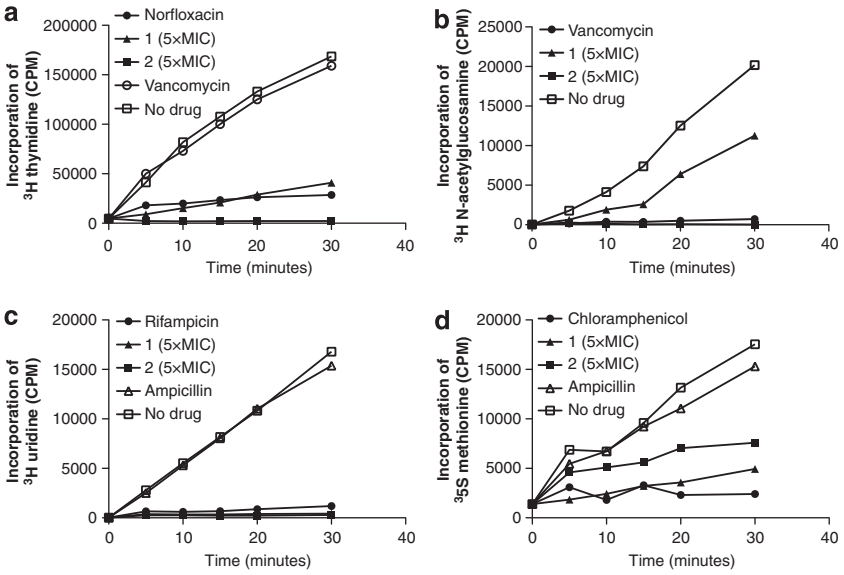


Figure 2 Effect of antimicrobial agents and comparator antibiotics on the incorporation of radiolabeled precursors in *Staphylococcus aureus*.

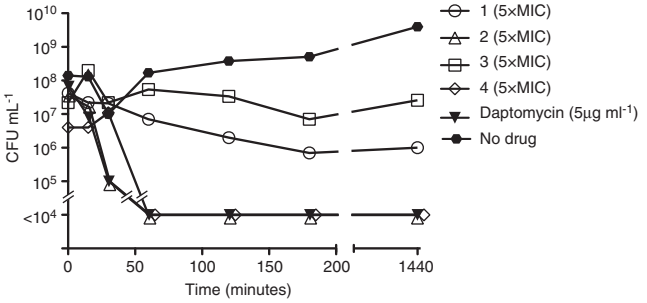


Figure 3 Effect of the compounds on *Staphylococcus aureus* viability.

Chemicals and antibiotics

All chemicals used were of analytical grade. Vancomycin, daptomycin and norfloxacin were purchased from Wako Pure Chemicals (Tokyo, Japan), Sequoia Research Products (Oxford, UK) and Sigma Aldrich, respectively. Rifampicin and chloramphenicol were obtained from Nacalai Tesque (Kyoto, Japan). Radiolabeled [^{3}H]*N*-acetylglucosamine was obtained from American Radiolabeled Chemicals (St Louis, MO, USA), [methyl-^{3}H]thymidine and [^{3}H]uridine were obtained from Moravek Biochemical (Brea, CA, USA) and [^{35}S]methionine was obtained from the Institute of Isotopes (Budapest, Hungary).

Chemical library and screening method

The chemical library of the Open Innovation Center for Drug Discovery at the University of Tokyo was screened for antibacterial activity against *S. aureus* MSSA1. Screening was based on MIC values against methicillin-susceptible *S. aureus* determined by broth dilution assay. Chemical compounds were screened with a criterion to obtain compounds with MIC values of $<100\,\mu M$.

Synthesis of iminothiadiazolo-pyrimidinone derivatives

Synthesis of 5-imino-2-(substituted)-5H-(1,3,4)thiadiazolo(3,2–a)pyrimidin-7(6H)-one (0001-03-KK and 0005-08-KK):

Sodium (140 mg) was dissolved into anhydrous ethanol (20 ml), and 2-amino-5-substituted-1,3,4-thiadiazole (5.9 mmol) and ethyl cyanoacetate (5.9 mmol) were added to the solution. The resulting solution was refluxed for 8 h, and then cooled to room temperature. The solution was poured onto

Table 3 Microbial strains used in this study

Strain	*Characteristics*
Staphylococcus aureus MSSA1	MET-susceptible, clinical isolate
S. aureus RN4220	MET-susceptible
S. aureus NCTC8325-4	MET-susceptible
S. aureus Smith	MET-susceptible
S. aureus MRSA3	OFXA, KAN, TET, ERM-resistant, clinical isolate
S. aureus MRSA4	OFXA, KAN, CHL, CYP-resistant, clinical isolate
S. aureus MRSA6	OFXA, FLX, KAN, TET, CYP, IM/CS-resistant, clinical isolate
S. aureus MRSA8	OFXA, FLX, KAN, ERM, CYP, IM/CS-resistant, clinical isolate
S. aureus MRSA9	OFXA, FLX, TET, ERM, CYP, IM/CS-resistant, clinical isolate
S. aureus MRSA11	OFXA, KAN, ERM, CYP, IM/CS-resistant, clinical isolate
S. aureus MRSA12	OFXA, FLX, KAN, ERM, IM/CS-resistant, clinical isolate
Enterococcus feacalis EF1	VM-susceptible
E. feacalis EF5	VM-resistant
Streptococcus sanguinis JCM 5708	
S. pyogenes SS1-9	
S. pneumoniae JCSC6523	
S. agalactiae JCM5671	
Bacillus subtilis JCM2499	
Bacillus cereus JCM20037	
Pseudomonas aeruginosa PAO1	
Escherichia coli W3110	
Serratia marcescens 98-130-37	
Candida albicans ATCC10231	
C. tropicalis pK233	
Cryptococcus neoformans H99	
Aspergillus niger	

Abbreviations: CHL, chloramphenicol; CYP, cyprofloxacin; ERM, erythromycin; FLX, flomoxef; IM/CS, imipenem/cilastatin sodium; KAN, kanamycin; MET, methicillin; OFXA, ofloxacin; TET, tetracycline; VM, vancomycin.

ice water and acidified with acetic acid. The precipitate was collected and washed with water to afford 0001-03-KK in 64% yield as a yellow solid and 0005-08-KK in 97% yield as a colorless solid.

Synthesis of compounds 0002-04-KK, 0019-25-KK, 0021-27-KK, 0027-56-KK, 0006-09-KK, 0017-22-KK, 0023-37-KK and 0026-59-KK:

Aldehyde (0.86 mmol) and one drop of Et_3N were added to a solution of thiadiazolopyrimidine 0001-03-KK or 0005-08-KK (0.82 mmol) in dimethylformamide (0.82 ml) and ethanol (0.82 ml). The reaction mixture was heated at 50–60 °C for 3 h and then kept standing at 25 °C for 12 h to obtain a precipitate. The resulting precipitate was collected by filtration and washed with dry diethyl ether to give products (0002-04-KK (**1**), 0019-25-KK, 0021-27-KK, 0027-56-KK (**4**), 0006-09-KK (**3**), 0017-22-KK, 0023-37-KK and 0026-59-KK (**2**)) of 28–70% yield. The physicochemical properties and structure elucidation details for the compounds are shown in the Supplementary Information.

Incorporation of [^{3}H]*N*-acetylglucosamine into cell wall peptidoglycans

Measurement of incorporated [^{3}H]*N*-acetylglucosamine was performed as previously described by Paudel *et al.*[12] Briefly, *S. aureus* NCTC8325-4 was cultured at 37 °C overnight in CGPY broth (Na_2HPO_4, 6 g; NaCl, 3 g; $MgCl_2.6H_2O$, 0.1 g; NH_4Cl, 2 g; Na_2SO_4, 0.15 g; KH_2PO_4, 3 g; bactopeptone, 10 g; yeast extract, 0.1 g and glucose, 5 g l^{-1}, pH 7.0). The culture was diluted 100-fold with the same medium and further cultured until an optical density $(OD)_{660}$ of 0.2 was reached. The culture was centrifuged at 8000 *g* for 10 min and the pellet was suspended in modified cell wall synthesis medium (KH_2PO_4, 6 g; K_2HPO_4, 6 g; NH_4Cl, 2 g; $MgSO_4$. $7H_2O$, 5 mg; $FeSO_4$, 5 mg; glucose, 100 mg; uracil, 40 mg; L-alanine, 50 mg; L-glutamic acid, 120 mg; L-lysine, 50 mg and chloramphenicol, 100 mg l^{-1}), pH 6.8) to obtain $OD_{660} = 0.1$. In the presence of 35 μCi [^{3}H]N-acetyl glucosamine, **1**, **2**, vancomycin, or norfloxacin (5 × MIC) was added to 1 ml of the cell suspension at time zero and incubated at 37 °C with shaking at 150 r.p.m. Samples were collected at the indicated times and an equal volume of 10% trichloroacetic acid was added. The mixture was incubated at 90 °C for 15 min, placed on ice for 30 min and filtered with a membrane filter (0.45 μm HA, Millipore, County Cork, Ireland) followed by washing with 5% trichloroacetic acid. Radioactivity was counted on a liquid scintillation counter (LS6000SE, Beckman Coulter, Brea, CA, USA) and expressed as c.p.m.

Incorporation of radiolabeled thymidine, uridine and methionine

Incorporation of thymidine, uridine, and methionine was measured as described previously.[10] Briefly, *S. aureus* NCTC8325-4 was grown overnight in Luria Bertani medium at 37 °C. The culture was diluted 200-fold in Luria Bertani medium and incubated at 37 °C until an OD_{600} of 0.3 was reached. Either 7 μCi [^{3}H]uridine, 70 μCi [^{3}H]thymidine or 20 μCi [^{35}S]methionine was added to the culture. Rifampicin, norfloxacin and chloramphenicol at a concentration of 5 × MIC were used to inhibit RNA, DNA and protein synthesis, respectively; and vancomycin or ampicillin (5 × MIC) was used as a negative control. Compounds **1** and **2** (5 × MIC) were used for all the assays. The MICs of rifampicin, norfloxacin, chloramphenicol, vancomycin and ampicillin were 0.004 μg ml^{-1}, 0.5 μg ml^{-1}, 4 μg ml^{-1}, 1 μg ml^{-1} and 0.03 μg ml^{-1}, respectively. At time zero, agents were added to exponentially growing cultures. Aliquots were collected at the indicated times, diluted twice with 5% trichloroacetic acid and the acid-insoluble fraction was obtained by filtration through glass fiber filters (Whatman, GE Healthcare, Maidstone, Kent, UK). Radioactivity retained on the filters was measured on a liquid scintillation counter and expressed as c.p.m.

Killing assay

The killing assay was performed as per the National Committee for Clinical Laboratory Standards.[11] Briefly, an overnight culture of *S. aureus* MSSA1 at 37 °C in MHB was diluted 1000 times with MHB medium and cultured for 2 h at 37 °C. For daptomycin, MHB was supplemented with 50 mg l^{-1} Ca^{2+}. Compounds **1**, **2** (5 × MIC), or daptomycin (5 × MIC; MIC: 1 μg ml^{-1}) was added to 1 ml of the culture and incubated at 37 °C for 0–24 h. Culture aliquots were collected at the indicated times, diluted and spread on Luria Bertani agar plates and incubated at 37 °C for 24 h. Cell viability was determined by the colony-forming units per ml. The lower limit of detection was 10^4 colony-forming units per ml.

Toxicity tests in silkworms

Fifth instar silkworm larvae were injected into the hemolymph with 0–400 μg of each compound and observed for 3 days. The LD_{50} was determined as the amount of chemical (μg g^{-1}) that killed 50% of the larvae.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the Chemical Library in the Open Innovation Center for Drug Discovery at the University of Tokyo for providing the compounds used in this study. We also thank Ms Matsuzawa, Ms Noguchi (Yoshino) and Ms Kyougoku for technical assistance. This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) and partly supported by JSPS Grant-in-Aid for Young Scientists (A) (24689008) from JSPS (HH) and Grant-in-Aid for Scientific Research on Innovative Areas (24102510) from MEXT (KS), and Platform for Drug Discovery, Informatics (MS).

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)