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ORIGINAL ARTICLE

Identification of novel deoxyribofuranosyl indole antimicrobial agents

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Three novel deoxyribofuranosyl indole derivatives, FG050227 (1), FG050223 (2) and FG050204 (3), were identified as antimicrobial agents effective against Gram-positive bacterial strains and some fungi. The MIC values of (1), (2) and (3) against methicillin-resistant *Staphylococcus aureus* were 3.0, 6.0 and 13 μg ml^{-1}, respectively. Compounds 1 and 3 had bactericidal activity against exponentially growing *S. aureus* and inhibited biosynthesis of peptidoglycan, protein, RNA and DNA. Compound 2 was bacteriostatic and inhibited the biosynthesis of protein and RNA. The results indicated that deoxyribofuranosyl indole derivatives could be potential lead compounds for the development of antimicrobial agents.
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Keywords: bactericidal; macromolecule synthesis; MRSA; novel antimicrobial; VRE

INTRODUCTION

The emergence of resistant strains against antimicrobial agents is a serious problem in the clinical field. *Staphylococcus aureus* strains resistant to penicillin[1] and methicillin[2] were identified as early as 2 years after their initial use. Vancomycin-resistant enterococcus (VRE) emerged in 1987,[3] a few years after vancomycin began to be used worldwide.[4] *S. aureus* that is resistant to relatively intermediate concentrations of vancomycin was first reported in 1997 in Japan.[5] Linezolide and daptomycin, the antibiotics effective against methicillin-resistant *Staphylococcus aures* (MRSA) and VRE, became ineffective within 1[6] and 2[7,8] years after approval by Food and Drug Administration in the USA, respectively. Therefore, the discovery of novel antimicrobial agents effective against multi-drug resistant bacteria is crucial. Toward this aim, we screened chemical compounds against *S. aureus*. Here, we report the identification of three novel deoxyribofuranosyl indole compounds as potential antimicrobial agents against Gram-positive bacteria, including MRSA and VRE, and some fungi.

MATERIALS AND METHODS

Microbial strains and culture conditions

The bacterial and fungal strains used in this study are summarized in Table 1. Bacterial cultures were prepared in either Luria Bertani (LB) medium (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}) or Muller–Hinton Broth (MHB) (DIFCO, Franklin Lakes, NJ, USA). For antimicrobial susceptibility tests, cation-adjusted MHB was used. Fungal suspensions were prepared in Roswell Park Memorial Institute medium (Sigma Aldrich, St Louis, MO, USA).

Chemicals and antibiotics

Vancomycin, daptomycin and norfloxacin were purchased from Wako Pure Chemicals (Osaka, Japan), Sequoia Research Products (Pangbourne, UK), and Sigma Aldrich, respectively. Rifampicin and chloramphenicol were obtained from Nacalai Tesque (Kyoto, Japan). Radiolabelled [^{3}H]*N*-acetylglucosamine was obtained from GE Healthcare (Chalfont St Giles, UK), [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). All the chemicals used were of analytical grade.

Chemical library and screening method

Our chemical library contains intermediates of several natural product synthesis projects, (for example, K252a,[9] ecteinascidin 743,[10] kainic acid,[11] etc). The compounds were screened based on MIC values against methicillin-susceptible *S. aureus*. MICs against bacteria and fungi were determined by broth dilution assay. [12,13]

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

Measurement of incorporated [^{3}H]*N*-acetylglucosamine was performed according to Maki *et al.*[14] *S. aureus* NCTC8325 was used for the assay in the presence of 35 μCi [^{3}H]*N*-acetyl glucosamine. Each of **1**, **2** and **3** (10 × MIC), or vancomycin (100 μg ml^{-1}), or norfloxacin (100 μg ml^{-1}) was applied to see their effect on the incorporation. The radioactivity was counted with a liquid scintillation counter (LS6000SE, Beckman Coulter, Carlsbad, CA, USA).

Incorporation of radiolabeled thymidine, uridine and methionine

S. aureus RN4220 was grown in LB medium overnight at 37 °C. The culture was diluted 200-fold in the same medium and incubated at 37 °C until an OD_{600} of

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Table 1 Microbial strains used in this study

Strain	*Characteristics*
Staphylococcus aureus MSSA1	MET susceptible, clinical isolate
S. aureus RN4220	MET susceptible
S. aureus NCTC8325	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
E. feacalis EF1	VM susceptible
E. feacalis EF5	VM resistant
Bacillus subtilis JCM2499	
Bacillus cereus JCM20037	
Pseudomonas aeruginosa PAO1	
Escherichia coli W3110	
Serratia marcences 98-130-37	
Candida albicans ATCC10231	
C. tropicalis pK233	
Cryptococcus neoformans H99	

Abbreviations: CHL, chloramphenicol; CYP, Cyprofloxacin; ERM, Erythromycin; FLX, Flomoxef; IM/CS, Imipenem/ Cilastatin sodium; KAN, Kanamycin; MET, Methicillin; OFXA, Ofloxacin; TET, Tetracycline; VM, Vancomycin.

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 and further incubated at 37 °C for 10 min. Rifampicin (100 μg ml^{-1}), norfloxacin (100 μg ml^{-1}) and chloramphenicol (100 μg ml^{-1}) were used as inhibitors of RNA, DNA and protein synthesis, respectively, and vancomycin (10 μg ml^{-1}) or ampicillin (100 μg ml^{-1}) was used as negative control. Compounds (**1**), (**2**) and (**3**) (10× MIC) were used for all the assays. Aliquots were collected at the indicated time, diluted 10 times by 5% TCA, and the acid insoluble fraction was obtained by filtration through glass fiber filters (Whatman, GE Healthcare, UK). Radioactivity retained on the filters was measured by a liquid scintillation counter.

Bactericidal assay

The bactericidal assay was performed as per NCCLS guidelines.[15] Briefly, overnight culture of *S. aureus* MSSA1 at 37 °C in MHB was diluted 1000 times with the same medium and cultured for 2 h at 37 °C. For daptomycin, MHB was supplemented with 50 mg l^{-1} Ca^{2+}. Compounds (**1**), (**2**) and (**3**) (5× MIC) or daptomycin (5 μg ml^{-1}) were added to 1 ml of the culture and incubated at 37 °C for 0 to 1 h. Culture aliquots were collected at indicated time, diluted and spread on LB agar plates and incubated at 37 °C for 24 h. Cell viability was determined by counting the CFU ml^{-1}. The lower limit of detection was 10^4 CFU ml^{-1}.

RESULTS

Screening for antibacterial compounds

A total of 400 compounds synthesized for the purpose of total synthesis of natural products in the laboratory of one of the authors (TF) were screened for antibacterial activity against *S. aureus*. Of these

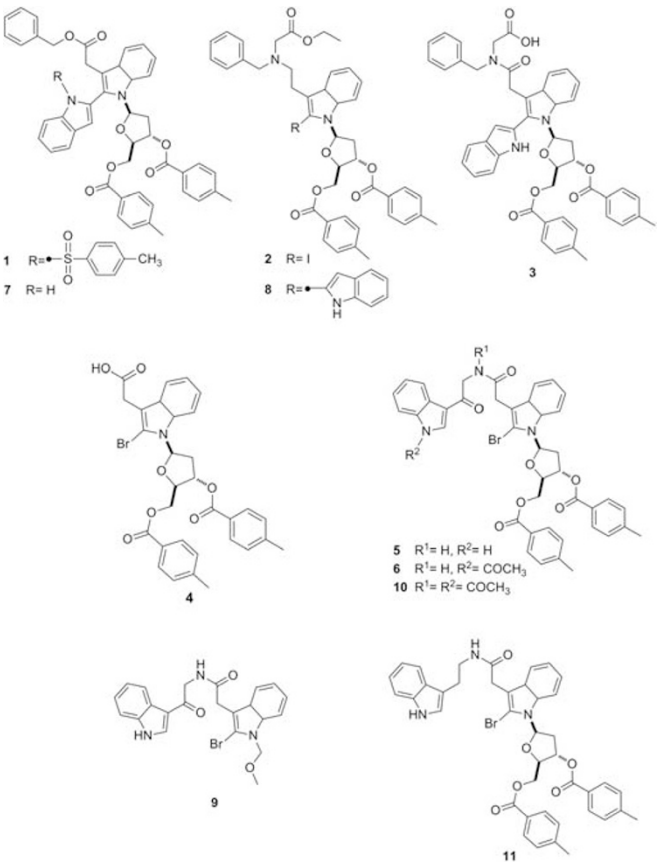

Figure 1 Compounds with similar structures selected from chemical library.

400 compounds, 47 were found to possess antibacterial activity against *S. aureus*. The MIC values against *S. aureus* (MSSA1) of these compounds were compared with select the best hit: FG050227 (**1**). Compounds with a structure similar to (**1**) (MIC: 3 μg ml^{-1}) were selected from the chemical library (Figure 1: **1–11**), from which two additional compounds FG050223 (**2**, MIC: 6 μg ml^{-1}), and FG050204 (**3**, MIC: 13 μg ml^{-1}) showed antibacterial activities, whereas (**4–11**) had no antibacterial activity at a concentration of 500 μM (Table 2).

Most of the compounds, except (**9**), had a deoxyribofuranosyl indole core. An additional indole ring is present next to deoxyribofuranosyl indole core in (**1**), (**3**), (**7**) and (**8**) with a sulfonyl group attached to it only in (**1**). The presence of iodine at the 2-position of the deoxyribofuranosyl indole core confers antibacterial activity (**2**), whereas an additional indole group in that position abolishes activity (**8**). Compound (**7**) similarly showed a loss of antimicrobial activity, whereas the addition of a sulfonyl group to the additional indole (**1**) restored activity. These findings suggest that the presence of an indole ring alone may reduce antibacterial activity, whereas the addition of a strong electron-withdrawing group, like a sulfonyl group, can recover the activity. Further comparison between (**3**), (**7**) and (**8**) revealed that even in the presence of the additional indole ring, modification of the upper group attached to deoxyribofuranosyl indole core can lead to antibacterial activity.

Antimicrobial spectrum of deoxyribofuranosyl indole compounds

Compounds (**1**), (**2**) and (**3**) were tested for antimicrobial activity against other microorganisms and were found to be effective against Gram-positive bacteria, including several clinical isolates of MRSA

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

	MIC ($\mu g\,ml^{-1}$)										
Strain	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Staphylococcus aureus MSSA1	3	6	13	>300	>380	>400	>360	>400	>230	>420	>370
S. aureus RN4220	3	6	13	–	–	–	–	–	–	–	–
S. aureus NCTC8325	3	6	13	–	–	–	–	–	–	–	–
S. aureus Smith	3	6	13	–	–	–	–	–	–	–	–
Methicillin resistant *S. aureus* MRSA3	3	6	13	–	–	–	–	–	–	–	–
Methicillin resistant *S. aureus* MRSA4	3	6	13	–	–	–	–	–	–	–	–
Methicillin resistant *S. aureus* MRSA6	3	6	13	–	–	–	–	–	–	–	–
Methicillin resistant *S. aureus* MRSA8	3	6	13	–	–	–	–	–	–	–	–
Methicillin resistant *S. aureus* MRSA9	3	6	13	–	–	–	–	–	–	–	–
Methicillin resistant *S. aureus* MRSA11	3	6	13	–	–	–	–	–	–	–	–
Methicillin resistant *S. aureus* MRSA12	3	6	13	–	–	–	–	–	–	–	–
Enterococcus faecals EF1	3	6	13	–	–	–	–	–	–	–	–
Vancomycin resistant *Enterococcus faecalis* EF5 (VRE)	3	6	13	–	–	–	–	–	–	–	–
Bacillus subtilis JCM2499	3	3	6	–	–	–	–	–	–	–	–
Bacillus cereus JCM20037	3	6	13	–	–	–	–	–	–	–	–
Serratia marcescens 98–130–37	>420	>390	>370	–	–	–	–	–	–	–	–
Escherichia coli W3110	>420	>390	>370	–	–	–	–	–	–	–	–
Pseudomonas aeruginosa PAO1	>420	>390	>370	–	–	–	–	–	–	–	–
Candida albicans ATCC10231	3	6	370	–	–	–	–	–	–	–	–
Candida tropicalis pK233	3	6	370	–	–	–	–	–	–	–	–
Cryptococcus neoformans H99	3	6	370	–	–	–	–	–	–	–	–

Abbreviation: ND, Not determined.

and VRE. The compounds were ineffective against Gram-negative bacteria (Table 2). Compounds (**1**) and (**2**) exerted antifungal activity against pathogenic true fungi like *Candida albicans, Candida tropicalis* and *Cryptococcus neoformans* with MIC values comparable to that against bacteria, whereas (**3**) showed much weaker antifungal activity compared with its antibacterial activity.

Effect on macromolecule biosynthesis in *S. aureus*

The incorporation of radiolabelled precursors into macromolecules peptidoglycan, DNA, RNA and protein was monitored on exponentially growing *S. aureus* with exposure to the compounds over a 3-h time period. Compounds (**1**) and (**3**) inhibited the incorporation of [^{3}H]*N*-acetylglucosamine (Figure 2a), [^{3}H]thymidine (Figure 2b), [^{3}H]uridine (Figure 2c) and [^{35}S]methionine (Figure 2d), suggesting that they inhibit the biosynthesis of peptidoglycan, DNA, RNA and protein. Compound (**2**) inhibited the incorporation of [^{3}H]uridine (Figure 2c) and [^{35}S]methionine (Figure 2d), whereas its inhibitory effect on the incorporation of [^{3}H]*N*-acetylglucosamine (Figure 2a) and [^{3}H]thymidine (Figure 2b) was not significant.

Bactericidal activity against *S. aureus*

Bactericidal activities of the compounds were determined by killing assays performed on exponentially growing *S. aureus* over a period of 1 h. Compounds (**1**) and (**3**) exerted potent bactericidal activity immediately after the addition of these compounds (Figure 3). The bactericidal activity of these two compounds was stronger than that of daptomycin. Compound (**2**) did not kill bacteria, indicating that action of this compound is bacteriostatic (Figure 3).

DISCUSSION

Three novel dihydrofuranosyl indole compounds exerted antimicrobial activity against Gram-positive pathogens including MRSA and VRE, as well as some fungi. The molecular targets of these compounds are expected to be different from that of vancomycin and methicillin as demonstrated by their similar potency against strains resistant and sensitive to vancomycin and methicillin. Although compounds (**1**) and (**2**) showed similar potency against bacteria as well as fungi, (**3**) showed stronger activity against bacteria than fungi. On the other hand, compound (**2**) differed from compounds (**1**) and (**3**) in the inhibition pattern of macromolecule synthesis, suggesting that these compounds have different cellular targets. This was further verified by the bacteriostatic activity of (**2**) as opposed to the bactericidal activity of (**1**) and (**3**). As (**1**) and (**3**) exhibited bactericidal activity with inhibition of all macromolecules unlike (**2**), which showed bacteriostatic activity with inhibition of RNA and protein syntheses, presence of the indole moiety at the 2-position of deoxyribofuranosyl indole core seems to be important for conferring the bactericidal activity. Bacteriostatic agents, unlike bactericidal agents, inhibit bacterial growth and do not kill bacteria, demanding frequent introduction of the agent, and are thus not the appropriate choice for the treatment of serious, life threatening infections.[16,17] In this study, structural modifications led to changes from bacteriostatic to bactericidal activity. This is an important finding and opens the doors to other structural modifications aimed at converting bacteriostatic agents to bactericidal and non-active compounds to active. These findings suggest that these novel compounds can be used as lead compounds for the development of antimicrobial agents.

The potent inhibition of macromolecule synthesis and rapid bactericidal activity of compounds **1** and **3** suggested that they, like daptomycin[18] and XF-73,[19] might damage bacterial membranes. Further experiments are required to gain more insight into the exact mechanism of action of these novel compounds.

In conclusion, these compounds are potential lead compounds for the development of antimicrobial agents. Further structural modifications can be made as required with suitable combinations to expect antimicrobial properties and bactericidal activities.

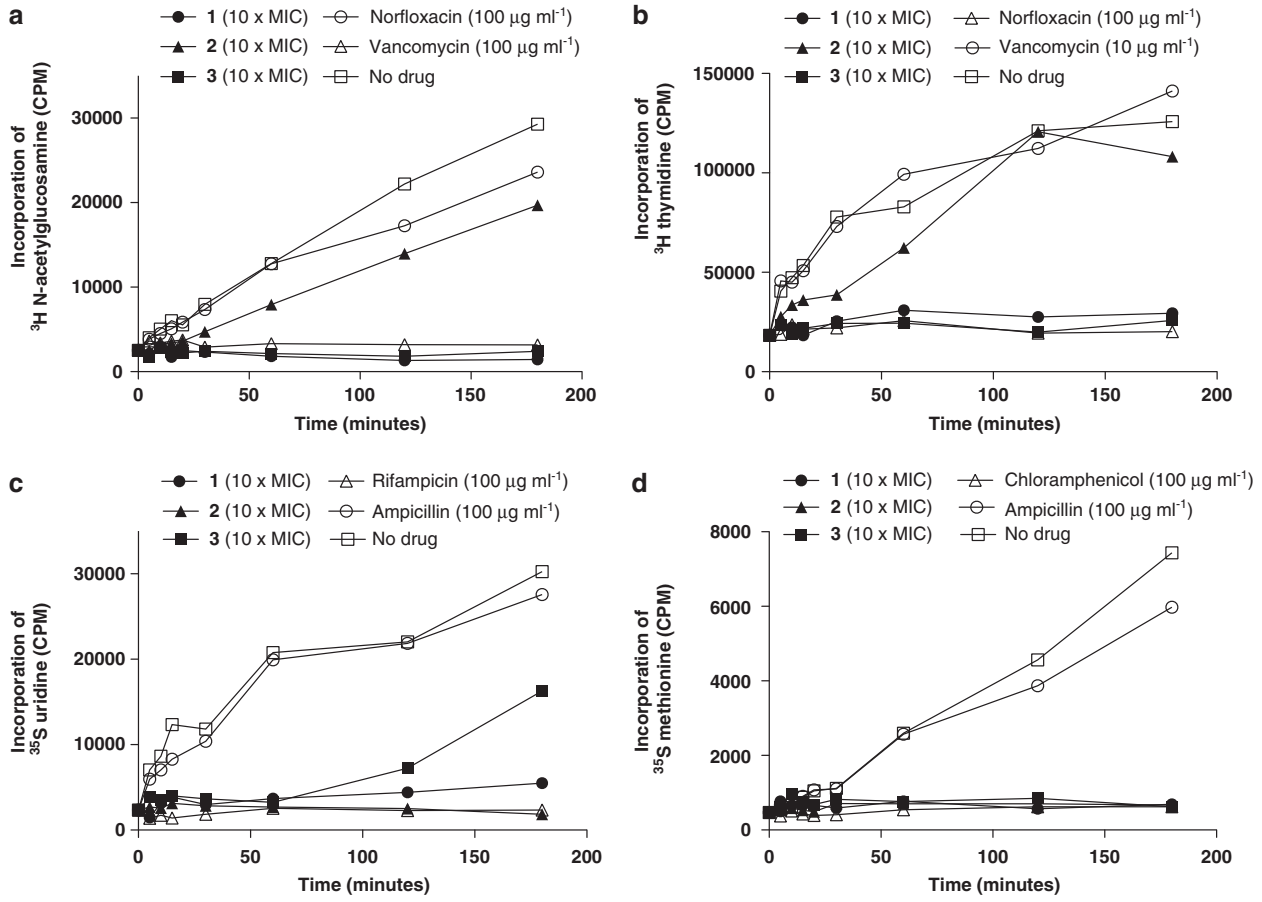

Figure 2 Effect of the antimicrobial agents and comparator antibiotics on the incorporation of radiolabelled precursors in *S. aureus*. Incorporation is shown as counts per minutes (CPM) measured by a liquid scintillation counter. Exponentially growing *S. aureus* cells were tested for the incorporation of radiolabelled precursors in the presence of the compounds and comparator antibiotics. At time zero, agents were added to exponentially growing cultures. Samples were collected at the indicated time intervals and radioactivity of acid insoluble fractions was measured. (**a**) [^{3}H] *N*-acetylglucosamine (**b**) [^{3}H] thymidine (**c**) [^{3}H] uridine (**d**) [^{35}S] methionine. Filled circles: **1** (10× MIC), filled triangles: **2** (10× MIC), filled squares: **3** (10× MIC), open circles: negative controls, open triangles: positive controls, open squares: No drug.

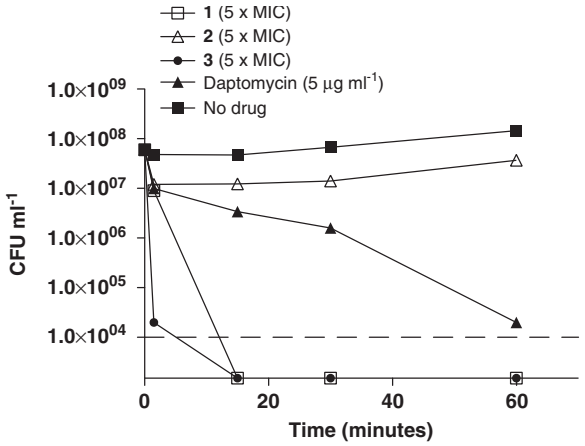

Figure 3 Effect on viability of *S. aureus*. Exponentially growing *S. aureus* ($\sim 10^8$ CFU ml^{-1}) was treated with compounds and a comparator antibiotic and cultured. Culture aliquots were collected at the indicated time, diluted and spread on agar plates, and incubated at 37 °C for 24 h. Cell viability was determined by counting the CFU ml^{-1}. Open squares: **1** (5× MIC), open triangles: **2** (5× MIC), filled circles: **3** (5× MIC), filled triangles: daptomycin (5 μg ml^{-1}), filled squares: No drug, dotted line: limit of detection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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