Neofiscalin A and fiscalin C are potential novel indole alkaloid alternatives for the treatment of multidrug-resistant Gram-positive bacterial infections

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ABSTRACT

Ten indole alkaloids were obtained from the marine sponge-associated fungus *Neosartorya siamensis* KUFA 0017. We studied the antimicrobial properties of these and of three other compounds previously isolated from the soil fungus *N. siamensis* KUFC 6349. Only neofiscalin A showed antimicrobial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE); with a minimum inhibitory concentration (MIC) of 8 μg mL^{-1} against both strains. Another compound, fiscalin C, presented synergistic activity against MRSA when combined with oxacillin, although alone showed no antibacterial effect. Moreover, neofiscalin A, when present at sub-MICs, hampered the ability of both MRSA and VRE strains to form a biofilm. Additionally, the biofilm inhibitory concentration values of neofiscalin A against the MRSA and VRE isolates were 96 and 80 μg mL^{-1}, respectively. At a concentration of 200 μg mL^{-1}, neofiscalin A was able to reduce the metabolic activity of the biofilms by ~50%. One important fact is that our results also showed that neofiscalin A had no cytotoxicity against a human brain capillary endothelial cell line.

Keywords: antimicrobial activity; multidrug-resistant Gram-positive; *Neosartorya siamensis*; neofiscalin A; fiscalin C

INTRODUCTION

The emergence and dissemination of antimicrobial resistance among bacteria threaten the usefulness of currently available antibiotics to treat infections. Multidrug-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), extended-spectrum beta-lactamase and carbapenamase-producing Enterobacteriaceae, are causes of significant concern not only due to high levels of resistance to antibiotics, but also due to the associated costs and human deaths that occur worldwide each year (Woodford and Livermore 2009; Lee Ventola 2015). There is, therefore, an urgent need for developing new and more effective antibiotics (Butler, Blaskovich and Cooper 2013).

Nature, itself, has been a source of antibiotics (Butler and Buss 2006). Based on this premise, our study focused on the identification of new potential antibiotics derived from terrestrial and marine-fungi of the genus *Neosartorya*. Several indole alkaloids have already been isolated from these fungi (Javasuriya *et al*. 2009; Yang *et al*. 2010; Kijjoa *et al*. 2011; Buttachon *et al*. 2012; Eamvijarn *et al*. 2012, 2013); however, none was tested for its antimicrobial activity.

Our work focuses on the study of the chemical constituents of two fungi, *Neosartorya siamensis* KUFA 0017 and *N. siamensis* KUFC 6349, which are deposited in a Thai collection in order to assess their antibacterial activity and their synergism with currently used antibiotics against multidrug-resistant bacteria.

MATERIALS AND METHODS

Fungi and extraction of secondary metabolites

Neosartorya siamensis (KUFA 0017) was isolated from the sea fan *Rumphella* spp., collected at a depth of 10 m from the coral reef of the Similan Islands (8˚39′09″N, 97˚38′27″E), Phang Nga Province, Southern Thailand, in 2010. The fungus was identified, and pure cultures were deposited as KUFA 0017 in the Fungal Collection of the Faculty of Agriculture at Kasetsart University in Bangkok, Thailand. The fungus *N. siamensis* KUFC 6349, which was isolated from forest soil at Samaesarn Island, Chonburi Province, Thailand, in November 2008, described by Buttachon *et al*. (2012) is also part of the same collection.

The general experimental procedures used in the extraction of secondary metabolites from *N. siamensis* KUFC 0017 were previously described (Buttachon *et al*. 2012; Gomes *et al*. 2014), resulting in the isolation of the indole alkaloids 3′-(4-oxoquinazolin-3-yl) spiro[1*H*-indole-3,5′-oxolane]-2, 2′-dione (**1**), nortryptoquivaline, tryptoquivaline (**2**), tryptoquivalines L (**3**), H (**4**) and F (**5**), fiscalin A (**7**), *epi*-fiscalin A (**8**), neofiscalin A (**9**), *epi*-neofiscalin A (**10**) and *epi*-fiscalin C (**12**) (Fig. 1), in addition to 2,4-dihydroxy-3-methylacetophenone and chevalone C. Compounds **1–5, 7–10** and **12** were evaluated together with tryptoquivaline O (**6**), fiscalin C (**11**) and sartorymensin (**13**), previously obtained from the soil fungus *N. siamensis* KUFC 6349 (Buttachon *et al*. 2012).

Bacterial strains, antimicrobial susceptibility testing and synergistic studies

The assays conducted using various bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *S. aureus* B1 and *E. faecalis* W1) to assess the antimicrobial properties of the compounds and their synergistic and antibiofilm activities have previously been described (Gomes *et al*. 2014). To assess the combined effect between the compounds and particular antibiotics, a screening susceptibility test using the disk diffusion method was conducted against two multidrug-resistant isolates, *S. aureus* B1 (a MRSA isolate) and *E. faecalis* W1 (a VRE isolate). Then, based on the results of that assay, the synergy between fiscalin C (**11**) and oxacillin or ampicillin (Sigma-Aldrich, St. Louis, MO, USA) was tested using a broth microdilution checkerboard method against the MRSA isolate (*S. aureus* B1). The determination of the minimum inhibitory concentration (MIC) using the microdilution method and the synergy assessment between compounds and antibiotics using the checkerboard method were performed in three independent experiments. The interpretation of the results obtained from the checkerboard assay was based on the calculation of the fractional inhibitory concentration (FIC) index (ΣFIC), which is the sum of the FIC of each drug. Thus, the FIC of drug A (FIC A) equaled the sum of MIC of drug A when in combination divided by the MIC of drug A alone, and the FIC of drug B (FIC B) equaled the MIC of drug B when in combination divided by the MIC of drug B alone. According to Odds (2003), a value of $\Sigma FIC \leq 0.5$ is indicative of synergic properties; $0.5 < \Sigma FIC \leq 4$ means there is no interaction, and a value of $\Sigma FIC > 4$ indicates antagonism.

Evaluation of the antibiofilm activity of neofiscalin A (9)

The inhibition of biofilm formation in the presence of neofiscalin A (**9**) was evaluated through the crystal violet assay. Various concentrations of neofiscalin A were tested (2× MIC, MIC, 1/2× MIC and 1/4× MIC) in two independent experiments carried out in triplicate for each of the concentrations. The statistical significance of the difference between controls and experimental groups was calculated using Student's t-test. *P* values < 0.05 are considered statistically significant. A microscopic analysis (fluorescence microscopy) of the biofilms of *S. aureus* B1 *and E. faecalis* W1 in the presence of the MIC and 1/4× MIC of neofiscalin A (**9**) was conducted, after staining the biofilms with the appropriate mixture of SYTO 9 and propidium iodide (LIVE/DEAD BacLight Bacterial Viability Kit—Life Technologies—Molecular Probes, Carlsbad, CA, USA).

The biofilm inhibitory (BIC) and biofilm eradication (BEC) concentrations were determined through two independent experiments. The metabolic activity of 24-h biofilms formed by *S. aureus* B1 (MRSA) and *E. faecalis* W1 (VRE) after treatment with neofiscalin A (**9**) for an additional period of 24 h was assessed in a single experiment using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Trafny *et al*. 2013).

Cytotoxicity assay for neofiscalin A (9)

The cytotoxicity of neofiscalin A (**9**) was evaluated using a culture of the human brain microvascular endothelial cell line hCMEC/D3, as previously described (Alves *et al*. 2015). Two experiments were conducted in triplicate and then averaged. In brief, 1% dimethyl sulfoxide (DMSO) was used as the negative control and 1% Triton X-100 as the positive control. Cytotoxicity was expressed as a percentage of cell viability, assuming 100% viability in the negative control.

RESULTS AND DISCUSSION

None of the compounds (Fig. 1) showed activity against the Gram-negative strains tested (MIC values higher than 256 μg mL^{-1}). However, neofiscalin A (**9**) presented antibacterial activity

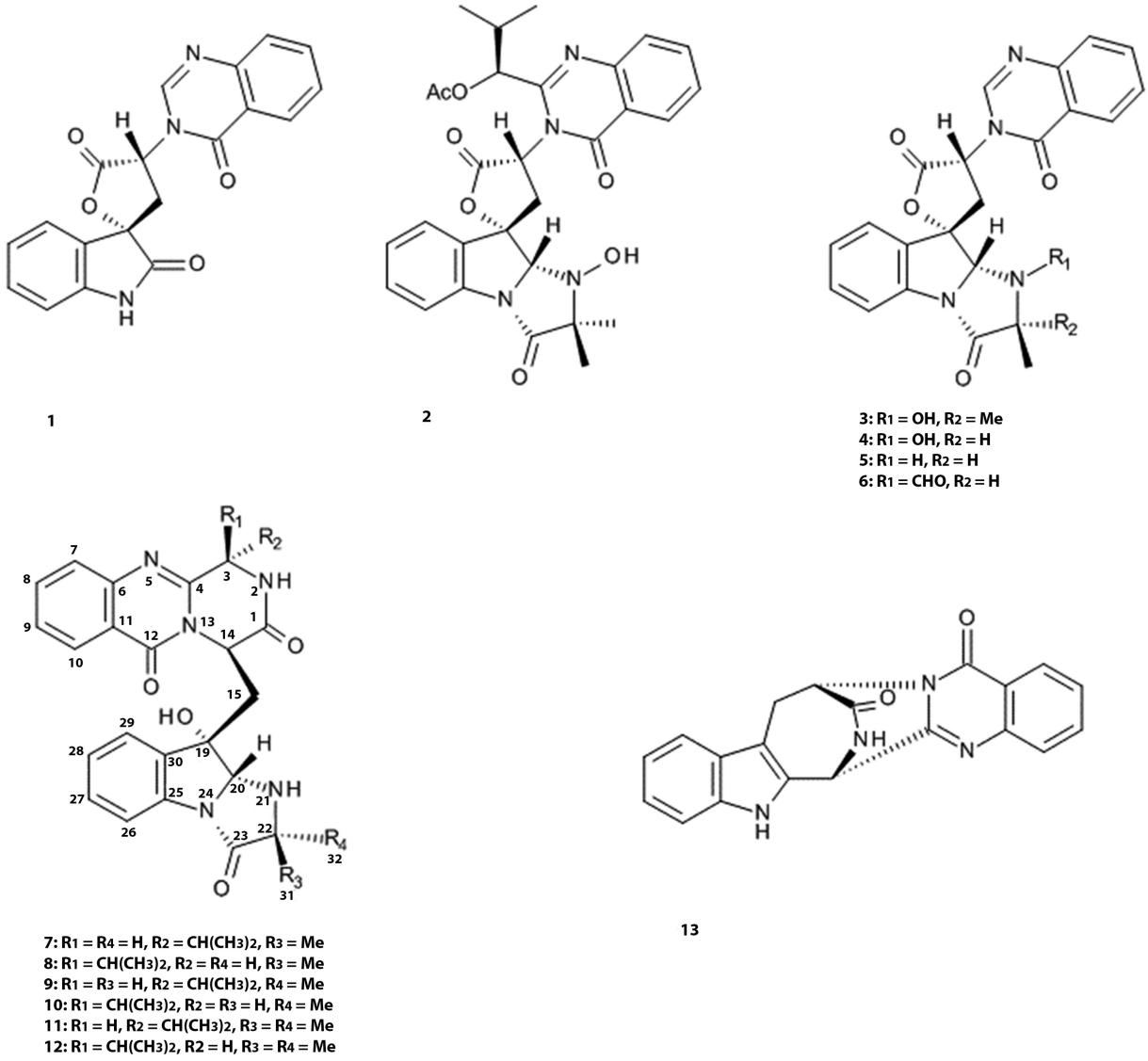

Figure 1. Secondary metabolites from *N. siamensis* KUFA 0017 (**1–5**, **7–10**, **12**) and from the soil fungus *N. siamensis* KUFC 6349 (**6**, **11** and **12**) (Buttachon *et al.* 2012).

against Gram-positive bacteria, showing a low MIC (8 μg mL^{-1}) against all strains of *Staphylococcus aureus* and *Enterococcus faecalis*. The minimum bactericidal concentration of neofiscalin A (**9**) was usually four times greater than the MIC (32 μg mL^{-1}); however, it was of 16 μg mL^{-1} against *S. aureus* ATCC 25923. These results attest the potential of neofiscalin A (**9**) to combat multidrug-resistant Gram-positive bacteria such as MRSA and VRE.

The analysis of the chemical structure of fiscalin A (**7**), *epi*-fiscalin A (**8**), neofiscalin A (**9**) and *epi*-neofiscalin A (**10**) revealed that they are diastereoisomers, differing from each other only in the absolute configuration of C-3 and C-22. However, the structures of the epimeric fiscalin C (**11**) and *epi*-fiscalin C (**12**) have two methyl groups instead of one methyl group on C-22, as in the structures of the fiscalin A series. The fact that only neofiscalin A (**9**) was active among the fiscalin analogs suggests that not only the substituent on C-22 (one methyl group) but also the stereochemistry of C-3 and C-22 (3*R*, 22*R*) may be required for antibacterial activity.

Regarding the synergistic effects inferred from the combination of compounds **1–13** with antibiotics to which the tested bacteria were resistant, it was observed (through the disk diffusion method) that fiscalin C (**11**) exhibited potential synergy with both ampicillin and oxacillin against the MRSA isolate (*S. aureus* B1). When not in combination, neither fiscalin C (**11**), ampicillin nor oxacillin produced any zone of inhibition on agar plates. However, when fiscalin C (**11**) was used in combination with ampicillin and oxacillin an inhibition zone was observed, of 8 and 10 mm, respectively. The checkerboard assay was performed by combining each antibiotic (ampicillin or oxacillin) with fiscalin C (**11**). Thus, from the FIC index values shown in Table 1 it can be observed that the association of fiscalin C (**11**) with oxacillin showed a synergistic effect (FIC index < 0.5), while the combination with ampicillin was indifferent ($0.5 <$ FIC index ≤ 4) (Odds 2003). These results may indicate that fiscalin C (**11**) and oxacillin may somehow interact. This interaction needs to be further studied to identify how the synergy occurs. Noteworthy, the structural features required for such synergy may be different from those needed for antibacterial activity. As only fiscalin C (**11**) exhibited a synergistic effect, it might be concluded that the presence of two methyl groups on C-22 and the 3*S* configuration are required for this activity.

Table 1. MIC values of fiscalin C (**11**) in combination with oxacillin and ampicillin and respective FIC index values obtained from the checkerboard method.

	MIC (μg mL^{-1})				
Strain	11	OX alone	11 with OX	OX with 11	FIC Index
S. aureus B1	> 512	128	16	16	<0.156
	11 alone	AMP alone	11 with AMP	AMP with 11	
S. aureus B1	> 512	128	512	128	>1

11: fiscalin C (**11**); OX: oxacillin; AMP: ampicillin; FIC index < 0.5: synergy.

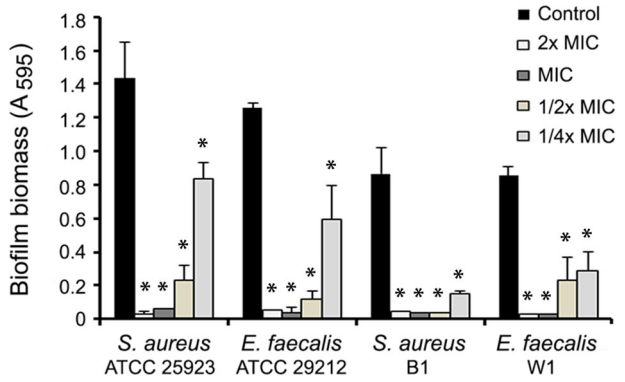

Figure 2. Biomass quantification through the crystal violet assay of biofilms formed by Gram-positive bacteria in presence of neofiscalin A (**9**). Biofilms were formed in the presence of different concentrations (ranging from 2× MIC to 1/4× MIC) of neofiscalin A (**9**). Error bars represent SD. Asterisks indicate that the difference between the concrete condition and the control is statistically significant ($P < 0.05$).

The effect of neofiscalin A (**9**) on the biofilm formation capability of *S. aureus* and *E. faecalis* was assessed at diverse concentrations (2× MIC, 1/2× MIC and 1/4× MIC) through biomass quantification after crystal violet staining. The results are shown in Fig. 2. At 2× MIC and MIC of neofiscalin A (**9**), none of the strains was able to form a biofilm. At the sub-inhibitory concentration of 1/2× MIC, a small increase in the biomass was verified but the strains did not form much biofilm. A lower sub-inhibitory concentration of 1/4× MIC allowed the formation of a larger biofilm, but smaller than that observed in the control group. In order to deepen the study of the effect of this compound on biofilm formation, a microscopic observation of the biofilm was performed by means of the live/dead staining method (Fig. 3). In the presence of neofiscalin A (**9**), at its MIC value, no growth and, consequently, no biofilm was produced (Fig. 3b). Nevertheless, at the concentration of 1/4× MIC a biofilm was formed (Fig. 3c), though it was not as large as in the control (Fig. 3a). In contrast, the sub-inhibitory concentrations of antibiotics, to which bacteria were continuously exposed, have been reported to greatly increase the biofilm formation (Hoffman *et al.* 2005).

The BIC values of neofiscalin A (**9**) against 24-h biofilms of *S. aureus* B1 and *E. faecalis* W1 were 96 and 80 μg mL^{-1}, respectively, but the BEC values were higher than 25× MIC, i.e. 200 μg mL^{-1} for both strains. Nevertheless, neofiscalin A (**9**) showed great potential in interfering with the viability of preformed biofilms of *S. aureus* and *E. faecalis*. Flemming *et al.* (2009) have shown that 24-h preformed staphylococcal biofilms subjected to treatment with 500 μg mL^{-1} of tetracycline were not eradicated, as most of the cells within the biofilms remained viable.

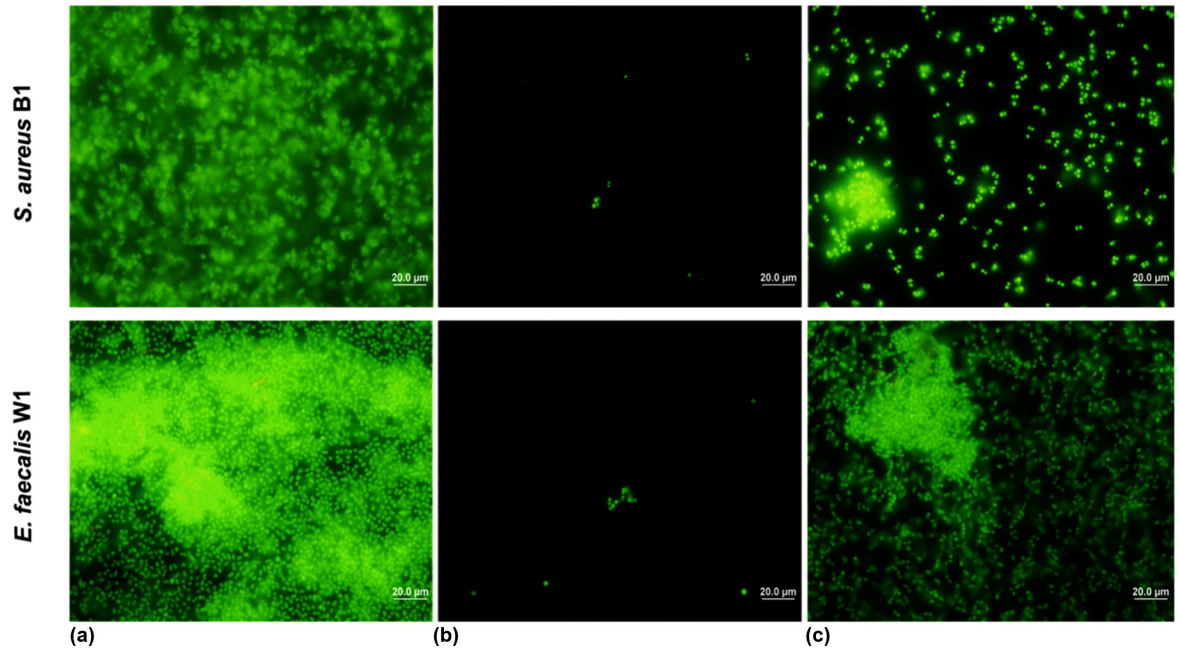

Figure 3. Microscopic visualization of 24-h biofilms formed in presence of neofiscalin A (**9**), after live/dead viability staining. Biofilm formation by *S. aureus* B1 (MRSA) and *E. faecalis* W1 (VRE) in absence (**a**), in presence of the MIC (**b**) and 1/4× of the MIC (**c**) of neofiscalin A (**9**).

The metabolic activity of 24-h biofilms of *S. aureus* B1 and *E. faecalis* W1 treated for another 24 h with 200 μg mL^{-1} of neofiscalin A (**9**) was reduced by nearly 50% (Fig. S1, Supporting Information).

No cytotoxicity was shown by neofiscalin A (**9**) at concentrations of 5, 10 and 20 μg mL^{-1} against a human brain endothelial cell line (hCMEC/D3). In other words, the percentage of cell viability was equal or greater than the viability obtained in the negative control, DMSO (Fig. S2, Supporting Information).

In conclusion, the marked antimicrobial activity (MIC = 8 μg mL^{-1}) of neofiscalin A (**9**) against MRSA and VRE strongly suggests that this natural product is a prime candidate for further chemical modifications aimed at the improvement of the antibacterial spectrum (changing peripheral chemical groups), as well as for additional *in vivo* tests to evaluate its efficacy, pharmacology and safety. Its potential as an antibiofilm agent should be pursued. Furthermore, fiscalin C (**11**) must be explored as an adjuvant in antimicrobial combined therapeutics.

The continued emergence of multidrug-resistant bacteria has drawn increasing attention in recent years. Currently, however, the main strategy used to combat the dearth of new effective antibiotics relies on the chemical modification of known antibiotics. If this approach fails, new classes of compounds that originate from natural sources can have great potential in research and development of new antibiotics.

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