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Identification of Arglecins B and C and Actinofuranosin A from a Termite Gut-Associated *Streptomyces* Species

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***Abstract*—**A high-throughput and automated ^{1}H NMR metabolic fingerprinting dereplication approach was used to accelerate the discovery of unknown bioactive secondary metabolites. The applied dereplication strategy accelerated the discovery of new natural products, provided rapid and competent identification and quantification of the known secondary metabolites and avoided time-consuming isolation procedures. The effectiveness of the technique was demonstrated by the isolation and elucidation of arglecins B (1), C (2) and actinofuranosin A (3) from a termite-gut associated *Streptomyces* sp. (USC 597) grown under solid state fermentation. The structures of these compounds were elucidated by extensive interpretation of ^{1}H, ^{13}C and 2D NMR spectroscopic data. These represent the first report of arglecin analogues isolated from a termite gut-associated *Streptomyces* species.

***Keywords*—**Actinomycetes, actinofuranosin, antibiotics, arglecins, NMR spectroscopy.

I. Introduction

NATURAL products are a privileged group of structures that have been evolutionarily selected to bind to biological macromolecules and represent the richest source of inspiration for the identification of not only chemotherapeutic agents but also lead compounds that can serve as the basis for the semi-synthesis or total synthesis of effective new drugs [1]-[3]. Since the discovery of actinomycin [4]-[7] natural products from actinomycetes have been extremely successful due to their biosynthetic ability stemming from their immense genome (> 5 Mb) which facilitates production of complex secondary metabolites [8]-[11]. It has been estimated that over the past 50 years only about 10^{7} (out of 10^{25}-10^{26}) actinomycetes have been screened for bioactive compounds [12], [13]. Although the rate of finding novel small molecules mostly from commonly screened actinomycete sources such as soil or marine sediments has declined in recent years [14], [15], they still present the most prolific source for biodiscovery [4], [16]-[18]. Therefore, the potential to isolate and identify new or novel compounds exceeds the rediscovery of well-known secondary metabolites from actinomycetes [12], [13], [18]. Large numbers of microbial natural products have eluded discovery most likely due to their limited biosynthesis that made their detection difficult under standard fermentation conditions [13], [19]. Thus, new approaches have been proposed to access this untapped microbial chemical diversity, including activation of silent and cryptic genes in particular during investigations of rare actinomycetes [20]-[25]. Selective isolation of rare and uncommon actinomycetes from extreme and understudied environments such as desert biomes, marine ecosystems, deep-sea sediments and insect-associated symbionts has been shown to be one of the most successful approaches [26]-[30]. Actinomycetes sourced from these habitats represent a rich source of novel strains with the potential to biosynthesize unique scaffolds which may be used as leads for the development of drug candidates [5], [16], [17], [23]. As part of the above outlined continuing global effort to discover new natural products from rare actinomycetes and extreme environments, a termite gut-associated *Streptomyces* sp. (USC-597) was selected from the University of the Sunshine Coast (USC) Microbial Library [31] to perform chemical and biological investigations. Herein, we report the isolation, structure elucidation and antibacterial activity of two arglecin analogues, namely, arglecins B (1) and C (2), one new nucleoside, named actinofuranosin A (3), and one known compound, 3H-pyrrolo[2,3-d]pyrimidine-5-carboxylic acid, 2-amino-4,7-dihydro-4-oxo-, methyl ester (4) (Fig. 1).

II. Results and Discussion

Comparative 16S rRNA gene sequence analysis revealed that the isolate USC-597 was a *Streptomyces* species occupying a distant phylogenetic position compared with the previously described species *Streptomyces puniceus* strain NBRC 12811 (Fig. 2). Arglecin B (1) was obtained as an optically inactive colourless amorphous solid. Analysis of the high resolution electrospray ionisation mass spectroscopy (HRESIMS) spectrum showed a quasimolecular ion $[M+H]^{+}$, m/z 266.1867, corresponding to the molecular formula $C_{14}H_{24}N_3O_2$ (calcd. for $C_{14}H_{24}N_3O_2$, 266.1868). The proton nuclear magnetic resonance (^{1}H NMR) spectrum of 1 in MeOH-d_4 revealed 10 resonances which corresponded to three sp^{3}-hybridized methyls

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at δ_H 1.92 (3H, s, H-17), and 0.93 (6H, d, J = 7.1 Hz, H-9 and H-10), one sp^3-hybridized methine at δ_H 2.15 (1H, m, H-8), five sp^3-hybridized methylenes at δ_H 2.58 (2H, d, J = 7.1 Hz, H-7), 3.19 (2H, t, J = 6.8, H-14), 2.52 (2H, t, J = 7.7 Hz, H-11), 1.66 (2H, m, H-12), and 1.54 (2H, m, H-13), and one sp^2-hybridized methine at δ_H 7.16 (1H, s, H-5) (Table I).

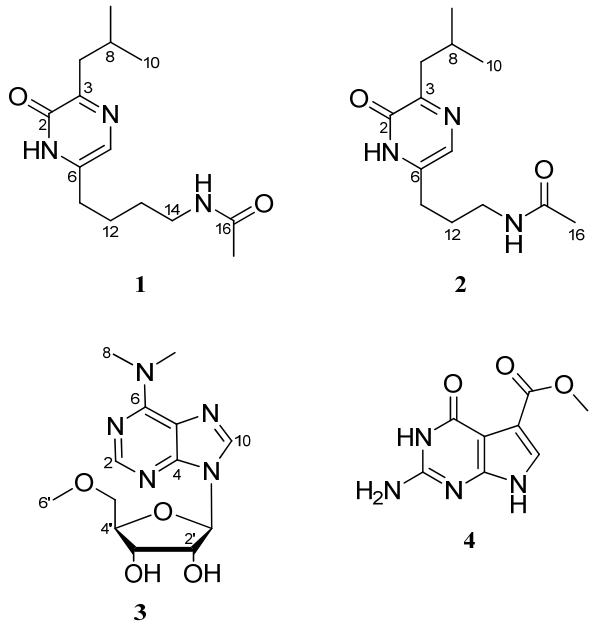

Fig. 1 Structures of compounds (1-4)

The ^{13}C NMR spectrum of 1 (Table II) showed the presence of three methyls at δ_C 22.5 (C-17), and 22.9 (C-9 and C-10), one methine at δ_C 28.1 (C-8), five methylenes at δ_C 42.5 (C-7), 39.9 (C-14), 30.8 (C-11), 27.1 (C-12), and 29.8 (C-13), three olefinic carbons at δ_C122.6 (C-5), 158.3 (C-2), and 141.1 (C-6), and two quaternary carbons at δ_C 173.3 (C-16), 157.7 (C-3).

The Correlated Spectroscopy (COSY) spectrum showed two partial structures depicted in Fig. 2 which were comprised of an isobutyl spin system and an n-butyl side chain attached to a secondary amine. The presence of the secondary amine at δ_H 7.77 (NH, brt, J = 6.8, H-15) attached to the methylene at δ_H 3.19 (t, J = 6.8, H-14) was further confirmed by strong COSY correlations between these protons when the spectrum of compound 1 was recorded in dimethyl sulfoxide-d_6 (DMSO-d_6) (Data not shown). Crucial Heteronuclear Multiple Bond Correlations (HMBC) displayed in Fig. 2 were used to complete the structure of 1. The methylene pair at δ_H 2.58 (H-7) showed correlations to C-2, C-3, C-8, C-9, and C-10. Moreover, HMBC correlations from the methylene pair at δ_H 2.52 (H-11) to the olefinic carbons at δ_C 122.5 (C-5) and 141.1 (C-6) suggested that the butyl side chain may be attached to a pyrimidine ring system [32]. HMBC correlations from the methyl at δ_H 1.92 (H-17) and from the methylene pair resonances at δ_H 1.54 (H-13) to the carbonyl carbon at δ_C 173.3 (C-16) indicated the presence of a terminal acetyl group. According to the index of hydrogen deficiency (IHD) calculated from the molecular formula of 1 ($C_{14}H_{24}N_3O_2$), it was determined that a core ring comprising two degrees of unsaturation needed to be established. HMBC correlations from the methines at δ_H 7.16 (H-5) and 2.15 (H-8) to the sp^2-hybridized carbon at δ_C 157.7 (C-3) as well as correlations from the methylene pair at δ_H 2.52 (H-11) to the olefinic carbons at δ_C 122.6 (C-5) and 141.1 (C-6) were indicative of a 2(1H)-pyrazinone core.

Upon comparison of the NMR spectroscopic data, with that of known natural products containing similar core structures exemplified by 2(1H)-pyrazinones (isolated from *Aspergillus* and *Streptomyces* species), ma'edamines A and B (isolated from the marine sponge *Suberea*) and dragmacidin D (isolated from the deep-sea sponge *Dragmacidon*) [32]-[37], the presence of a 3,5,6-trisubstitued 2(1H)-pyrazinone core was confirmed. The structure of compound 1 was therefore concluded to be N-[4-(3-isobutyl-2-oxo-pyrazin-2(1H)-one-6-yl)butyl]acetamide.

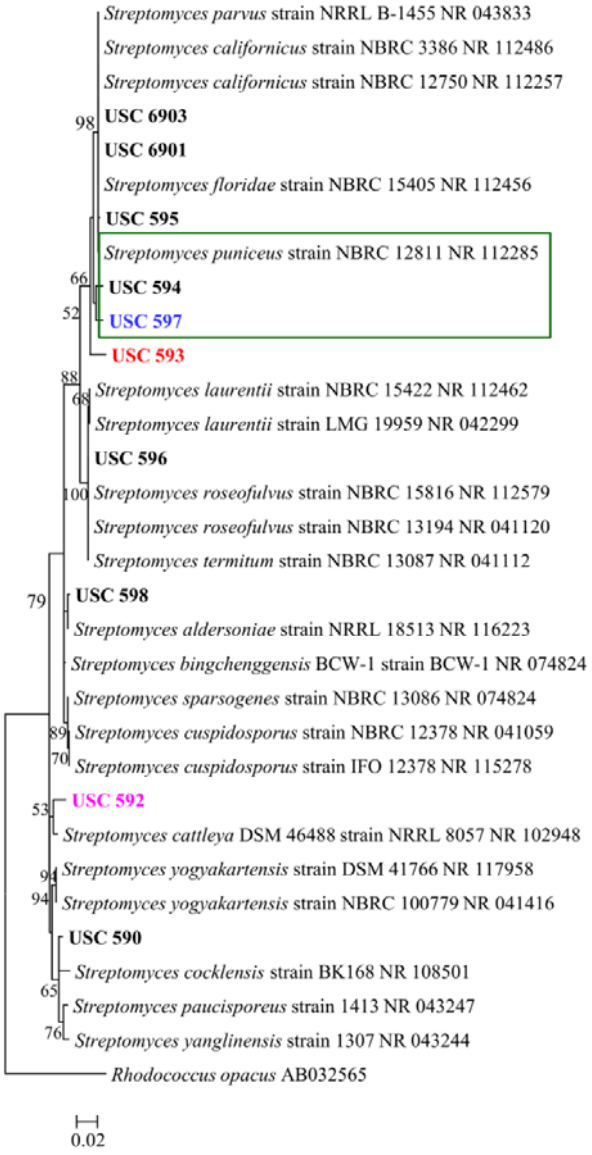

Fig. 2 Neighbor-joining phylogenetic tree based on partial 16S rDNA sequences of 10 *Streptomyces* strains isolated from termite gut

Arglecin B (2) was obtained as an optically inactive colorless

amorphous solid. Its molecular formula was determined to be $C_{13}H_{22}N_3O_2$ $[M+H]^+$, m/z 252.1715 (calcd. for $C_{13}H_{22}N_3O_2$, 252.1712) based on HRESIMS measurements.

Comparison of NMR spectral data of 1 with that of 2 in MeOH-d_4 (Tables I and II) revealed that compound 2 possessed a similar skeleton to 1 except that the n-butyl side chain in 1 was substituted for an n-propyl side chain in 2. The structure of arglecin C was further confirmed by interpretation of the 2D NMR spectra to be N-[4-(3-isobutyl-2-oxo-pyrazin-2(1H)-one-6-yl)propyl]acetamide.

Actinofuranosin A (3) was isolated as an optically active colorless amorphous solid ($[\alpha]_D$ -35.2, c 0.1, MeOH). The molecular formula of $C_{13}H_{20}N_5O_4$ $[M+H]^+$, m/z 310.1509 (calcd. for $C_{13}H_{20}N_5O_4$, 310.1515) was determined on the basis of the (+)-HRESIMS and NMR measurements (Table I).

The ^{1}H NMR spectrum of 3 in MeOH-d_4 displayed ten resonances which corresponded to three sp^3-hybridized methyls, one at δ_H 3.44 (3H, s, H-6') and two at 3.50 (6H, s, H-7 and H-8), one sp^3-hybridized diastereotopic methylene pair at δ_H 3.65 (1H, dd, J = 10.8, 3.7 Hz, H-5a'), 3.73 (1H, dd, J = 10.8, 3.0 Hz, H-5b'), four sp^3-hybridized methines at 4.18 (1H, dd, J = 8.0, 3.7 Hz, H-4'), 4.32 (1H, t, J = 4.7 Hz, H-3'), 4.52 (1H, t, J = 4.7 Hz, H-2') and 6.06 (1H, d, J = 4.7, H-1') [38], and two sp^2-hybridized methines at δ_H 8.21 (1H, s, H-2) and 8.26 (1H, s, H-10).

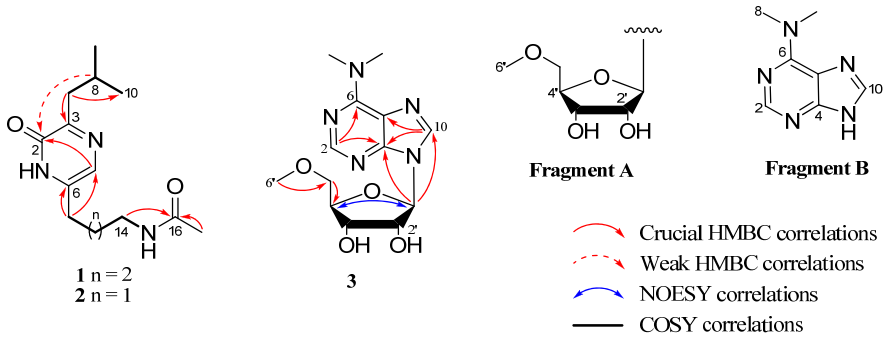

Fig. 3 Fragments found during the elucidation process and crucial COSY and HMBC correlations for arglecins A and B (**1**-**2**) and actinofuranosin A (**3**)

TABLE I
^{1}H NMR SPECTROSCOPIC DATA FOR ARGLECINS B, C AND ACTINOFURANOSIN A (**1**-**3**) IN MEOH-D_4

Position	**1**[a]	**2**[b]	**3**[c]
	δ_H [ppm] (J in Hz)	δ_H [ppm] (J in Hz)	δ_H [ppm] (J in Hz)
2			8.21 (s)
3			
4			
5	7.16 (s)	7.20 (s)	
6			
7	2.58 (d, 7.1)	2.60 (d, 7.0)	3.50 (s)
8	2.15 (m)	2.17 (m)	3.50 (s)
9	0.93 (d, 7.1)	0.95 (d,7.0)	
10	0.93 (d, 7.1)	0.95 (d, 7.0)	8.26 (s)
11	2.52 (t, 7.7)	2.54 (t, 7.7)	
12	1.66 (m)	1.84 (m)	
13	1.54 (m)		
14	3.19 (t, 6.8)	3.23 (t, 6.8)	
16			
17	1.92 (s)	1.96 (s)	
1'			6.06 (d, 4.7)
2'			4.52 (t, 4.7)
3'			4.32 (t,4.7)
4'			4.18 (dd, 8.2, 3.5)
5'			3.73 (dd, 10.8, 2.9) 3.65 (dd, 3.5, 10.8)
6'			3.44 (s)

[a] Proton resonances were acquired at 600 MHz
[b] Proton resonances were acquired at 900 MHz
[c] Proton resonances were acquired at 600 MHz

The ^{13}C NMR spectrum of 3 exhibited eleven resonances comprised of sp^3-hybridized methyls one at δ_C 59.5 (C-6') and the other at δ_C 38.9 (C-7 and C-8), one sp^3-hybridized methylene at δ_C 73.2 (C-5'), four sp^3-hybridized methines at δ_C 85.0 (C-4'), 71.8 (C-3'), 76.2 (C-2') and 89.7 (C-1'), two sp^2-hybridized methines at δ_C 153.2 (C-2) and 138.8 (1H, s, H-10) and two quaternary carbons at δ_C 149.8 (C-4) and 119.5 (C-5).

Interpretation of 1D and 2D NMR data allowed for the identification of two partial structures depicted in Fig. 3. Fragment-A, showed characteristic proton resonances indicative of a N-ribofuranose moiety, with the configuration of the anomeric proton assigned to be β based on a trans diaxial relationship of H-1' and H-2' coupling constant. Moreover, reciprocal HMBC correlations between the methyl at δ_H 3.44 (H-6') to the sp^3-hybridized methine carbon at δ_C 73.2 (C-5') and from the methine pair at δ_H 3.65 (H-5a') and 3.73 (H-5b') to the sp^3-hybridized methyl at (C-6') suggested a naturally occurring methylation of the ribofuranose at C-5'.

Detailed analysis of the NMR spectroscopic data of fragment-B, indicated the presence of a purine ring system. HMBC correlations from the methine at δ_H 8.21 (1H, s, H-2) to the olefinic carbons at δ_C 149.8 (C-4) and 119.5 (C-6) as well as HMBC correlations from the methine at δ_H 8.26 (1H, s, H-10) to the quaternary carbons at δ_C 149.8 (C-4) and 154.8 (C-5) were consistent with the presence of the aglycone N,N-dimethyl-10H-purin-6-amine.

The structural assignment was further confirmed by NMR data comparison with related synthetic compounds in DMSO-d_6 solvent and natural products containing the same residue

such as puromycin [7], [24], [39]-[41]. Additionally, crucial HMBC correlations shown in Fig. 3 from the anomeric proton at δ_H 6.06 (H-1') to the olefinic and quaternary carbons at δ_C 138.8 (C-10) and 149.8 (C-4), respectively, unequivocally positioned the furanose ring at N-11 [39]. The relative configuration of 3 was determined by total synthesis (Fig. 4). Synthesis of actinofuranosin A (3) started from the commercial precursor 6-chloropurine riboside (Merck). Initially the 2,3-cis diol was selectively protected under common conditions employing 2,2-dimethoxypropane and a catalytic amount of camphorsulfonic acid to yield 455 mg of 5 (98% yield).

Subsequent amination was achieved through microwave assisted replacement of the 6-chloro substituent with dimethylamine and resulted in a nearly quantitative formation of 6 (196 mg, 98% yield). Since 6-alkyl residues of similar structures are known to undergo C-alkylations under harsh conditions, such as sodium hydride promoted alkylations, the 5'-hydroxy function was methylated with methyl iodide in the presence of potassium t-butanolate in good yield (7, 47.5 mg, 95% yield). Cleavage of the isopropylidene acetal was performed under standard conditions to yield the final synthetic product 3 [42] (9.5 mg, 95% yield) which allowed the configuration of 3 to be established as N-*β*-D-ribofuranose-5-(methoxymethol)-N,N-dimethyl-10H-purin-6-amine (Fig. 5).

The ^{13}C-NMR signals for the N,N-dimethyl amine moiety of both the natural product and the synthetic compound were found to be separated, but weak and broad at room temperature. Thus, variable temperature (VT) NMR experiments were performed on the synthetic compound using DMSO-d_6. At a temperature of 100°C the peaks coalesced to finally sharpen into a single peak at δ_C 37.6 due to unhindered rotation (Fig. 6).

TABLE II
^{13}C AND HMBC NMR SPECTROSCOPIC DATA FOR ARGLECINS A AND B (1-2) AND ACTINOFURANOSIN A (3) IN MEOH-D_4

	1[a]		**2**[b]		**3**[d]	
Position	δ_C [ppm]	HMBC	δ_C [ppm]	HMBC	δ_C [ppm]	HMBC
2	158.3		159.0		153.2	C-4, C-6
3	157.3		158.2			
4					149.8	
5	122.6	C-2, C-6	122.5	ND[c]	119.5	
6	141.1		140.3		154.8	
7	42.5	C-2,C-3, C-8, C-9, C-10	42.1	C-2,C-3, C-8, C-9, C-10	38.9	
8	28.1	C-3, C-7, C-9, C-10	27.7	C-3, C-7, C-9, C-10	38.9	
9	22.9	C-7, C-8, C-9	22.6	C-7, C-8, C-10		
10	22.9	C-5, C-6, C-12, C-13	22.6	C-7, C-8, C-9	138.8	C-4, C-5
11	30.8	C-5, C-6, C-12, C-14	28.2	C-5, C-6, C-12, C-14		
12	27.1	C-11, C-14	29.2	C-11, C-14		
13	29.8	C-12, C-13, C-14				
14	39.9	C-12, C-13, C-16	39.2	C-12, C-16		
16	173.3		173.7			
17	22.5	C-16	22.2	C-16		
1'					89.7	C-2', C-10
2'					76.2	C-4'
3'					71.8	C-1', C-5'
4'					85.0	C-3'
5'					73.2	C-3', C-4', C-6'
6'					59.5	C-5'

[a] Carbon resonances were acquired at 225 MHz
[b] Carbon resonances were acquired at 150 MHz
[c] No signal detected
[d] Carbon resonances were acquired at 150 MHz

Compounds 1-4 were tested for their antibacterial activity against cultures of *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) using the well diffusion method. Although no inhibitory activity was detected for compounds 1-4 at concentrations as high as 100 µg/mL, weak antitubercular activity with a MIC value of 100 µg/mL was detected against *Mycobacterium bovis* bacillus (Calmette-Guérin (BCG), 1173P2) strain transformed with green fluorescent protein (GFP) using the constitutive expression plasmid pUV3583c with direct readout of fluorescence as a measure of bacterial growth [43].

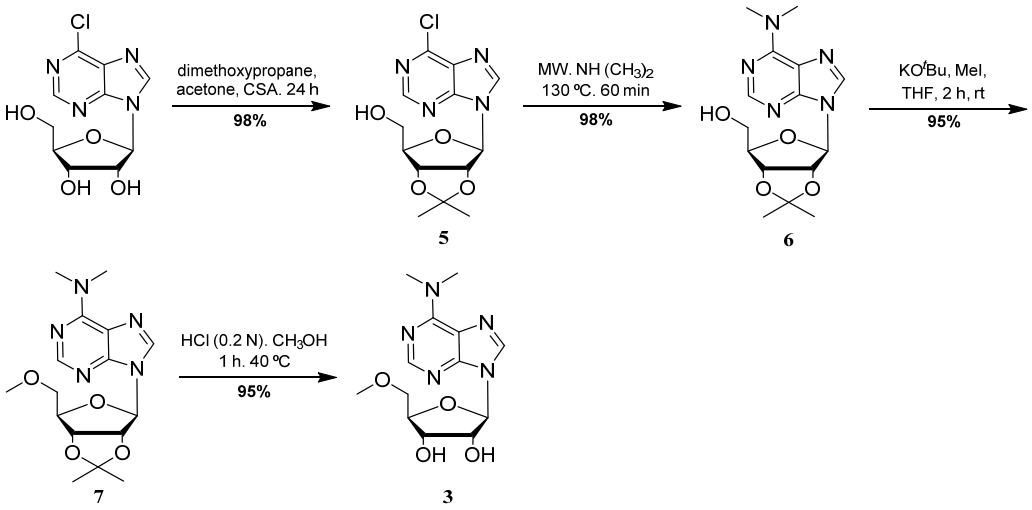

Fig. 4 Total synthesis of actinofuranosin A (**3**)

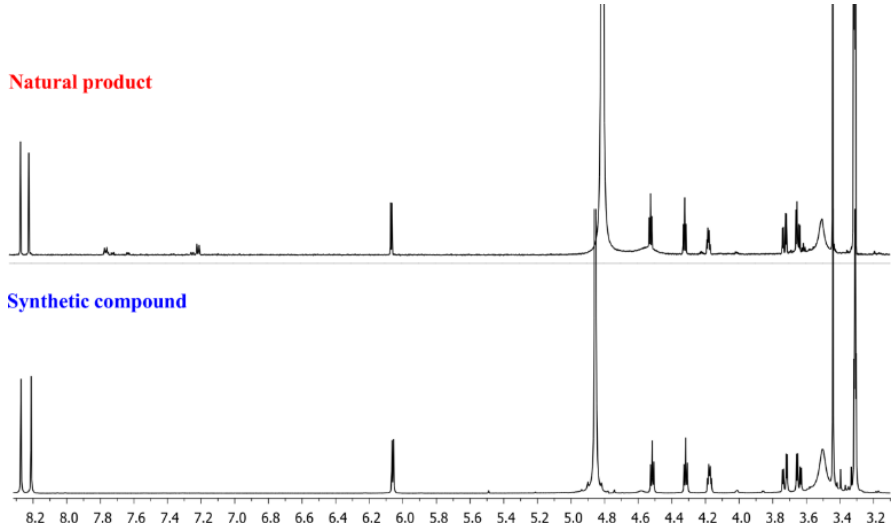

Fig. 5 Total synthesis of actinofuranosin A (**3**)

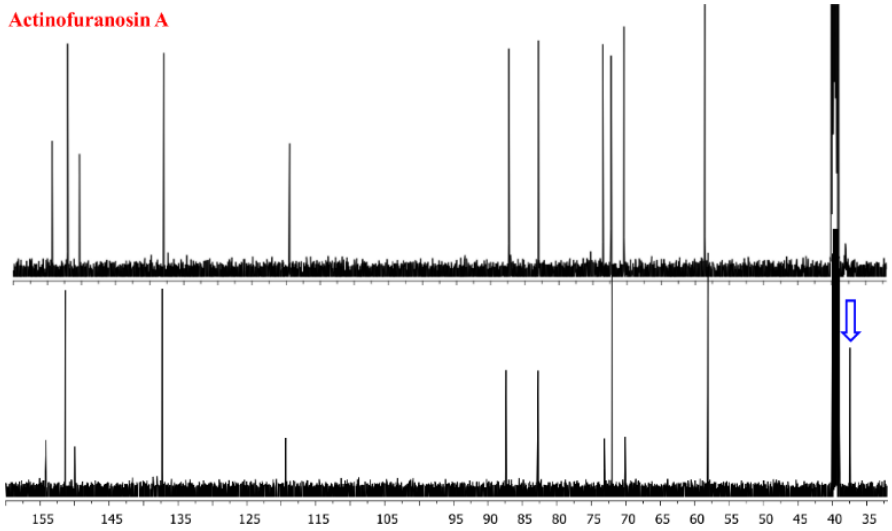

Fig. 6 Total synthesis of actinofuranosin A (**3**)

III. Discussion

The discovery of streptothricin (sourced from the actinomycete *Streptomyces lavendulae*) in 1942, the first microbial natural product with broad antimicrobial spectrum, and streptomycin (sourced from the actinomycete *Streptomyces griseus*) two years later, triggered the systematic screening of the genus *Streptomyces* for the identification of novel antimicrobial compounds. For the next 17 years, the discovery of novel antibiotics increased almost exponentially and then continued to rise at a lesser linear rate, reaching its peak in the 1970s [18], [44]. Actinomycetes are the richest source of clinically significant natural products exhibiting antimicrobial, anticancer, immunosuppressant, immunostimulant and enzyme inhibition properties, approximately 12,000 of all described bioactive metabolites have been isolated from the cluster of these bacteria [28], [45]. The genus *Streptomyces* has been identified as the largest producer of microbial secondary metabolites, accounting for approximately 80% of all isolated bioactive molecules [12], [13]. Mathematical modeling studies have estimated that this genus could synthesize more than 100,000 new or novel compounds with potential antimicrobial activities [18], [28]. During the last decades intensive screening programs were carried out worldwide to access to the large actinomycete biodiversity. Large numbers of samples from a wide range of geographical locations and habitats were processed and millions of strains were isolated and screened in industrial laboratories and research centers [8].

Therefore, the rate of discovering commercially relevant bioactive small molecules from common actinomycete sources has decreased as this practice frequently conducts to the costly re-isolation of known compounds [14], [45]. New approaches have been developed to address the problem of rediscovery of microbial compounds [8], [45]. One of these strategies involves the screening and selective isolation of novel/rare actinomycete taxa sourced from unique and underexplored environments. Novel actinomycete strains producing new structurally diverse bioactive natural products have been discovered from desert biomes, marine ecosystems, deep-sea sediments and insect-associated actinomycetes [23], [46]-[48]. Carr et al. [23] reported the discovery of the two novel small molecules, namely, microtermolides A and B isolated from a *Streptomyces* sp. strain associated with fungus-growing termites. Furthermore, the previously unreported 26-membered polyene macrocyclic lactam (sceliphrolactam) isolated from a wasp-associated *Streptomyces* sp., displayed an antifungal minimum inhibitory concentration (MIC) of 4 μg/mL against amphotericin B-resistant *Candida albicans*. And, the polyene peroxide mycangimycin isolated from the pine beetle-associated fungus *Dendroctonus frontalis* exhibited strong antifungal activity against the antagonistic ascomycetes, *Ophiostoma minus* (MIC = 1.2 μg/mL), *Saccharomyces cerevisiae* (MIC = 0.4 μg/mL) and *Candida albicans* ATCC 10231 (MIC = 0.2 μg/mL) [46], [49]. Therefore, this study aimed to identify new microbial natural products from a *Streptomyces* species (USC-597), an intestinal tract symbiont of the Australian wood-feeding termite *Coptotermes lacteous* (Froggatt). Culture extracts were HPLC fractionated following an in-house methodology. To access to the unique components of the drug-like natural products of the strain *Streptomyces* sp. (USC-597), a NMR metabolic fingerprinting approach was established and conducted to the isolation and structure elucidation of two arglecin analogues, namely, arglecins B (**1**) and C (**2**), one new nucleoside, named actinofuranosin A (**3**), and one known compound, 3H-pyrrolo[2,3-d]pyrimidine-5-carboxylic acid, 2-amino-4,7-dihydro-4-oxo-, methyl ester (**4**) (Fig. 1). Although similar compounds as the arglecin analogues were recently reported by Chen et al. [50] from *Streptomyces* sp. (ZZ446) isolated from coastal soils. This study represents the first report of arglecins derived natural products discovered from a termite gut-associated *Streptomyces* species. Thus, further chemical and biological investigations should be performed specially on rare actinomycete strains isolated from the gut of termites and other eusocial insects [5], [17], [28], [51].

Strain Fermentation and Isolation of Arglecin B, C and Actinofuranosin A

Cultures of the producing strain *Streptomyces* sp. (USC-597) were grown in 40 Petri dishes (100 x 15 mm) containing GYES medium (glucose 10.0 g, yeast extract 2.50 g, corn starch 2.50 g, NaCl 1.25 g, $CaCO_3$ 0.75 g, agar 20.0 g, dH_2O 1L) [52] for 15 days at 28 °C, after which, cultures comprising *Streptomyces* mycelium were cut into small squares and soaked in Ethyl acetate (EtOAc) (Merck) (600 mL) overnight. The EtOAc extract was concentrated to dryness *in vacuo* to yield 52.3 mg of the crude extract. A portion of this extract (44.3 mg) was subjected to reverse-phase HPLC using a combination of C_{18} and C_8 semi-preparative columns to afford 1.0 mg (2.2% crude weight) of arglecin B (**1**), 1.2 mg (2.7% crude weight) of arglecin C (**2**), 1.7 mg (3.8% crude weight) of actinofuranosin A (**3**) and 2.4 mg (5.4% crude weight) of 3H-pyrrolo[2,3-d]pyrimidine-5-carboxylic acid, 2-amino-4,7-dihydro-4-oxo-, methyl ester (**4**) [53], [54].

The producing strain *Streptomyces* sp. (USC 597) was first grown on the following listed solid media: OMA medium (Oatmeal 20.0 g, yeast extract 3.0 g, agar bacteriological 20.0 g, dH_2O 1L), LFA medium (Lupin flour 5.0 g, peptone 100.0 mg, glucose 1.0 g, agar bacteriological 20.0 g, dH_2O 1L), RFA medium (Rye flour 5.0 g, peptone 100.0 mg, glucose 1.0 g, agar bacteriological 20.0 g, dH_2O 1L) and GYES medium. GYES medium supported the detection of potential new natural products thus, this medium was further evaluated by incubating the actively growing isolate *Streptomyces* sp. (USC 597) for 15 days at 28 °C. Following incubation, the culture was removed together with the agar and soaked overnight in EtOAc. The EtOAc was dried under reduced pressure to yield a dark brown solid extract (52.3 mg). A portion of the crude extract (44.3 mg) was run down a Phenomenex Onyx Monolitic (100 x 10 mm) C_{18} column. Isocratic HPLC conditions of H_2O/MeOH (90%/10%) were initially employed for 10 min, followed by a linear gradient to 100% MeOH over 40 min, then an isocratic condition of 100% MeOH was run for 10 additional minutes, all at a flow rate of 9 mL/min. 60 fractions were collected from 0 to 60 min (60 x 1 min), and then analyzed by (+)-LR-ESIMS.

Fraction 20 yielded 1.0 mg of 1 (2.2% crude weight), fraction 18 yielded 1.2 mg of 2 (2.7% crude weight). Compound **1** was further purified by semi-prep HPLC using a Thermo Scientific BDS Hypersil C_8 column (250 X 10 mm). Isocratic conditions of MeOH/H_2O (26%/84%) were employed for 10 min, and then a linear gradient to 33% MeOH was run over 20 min at a flow rate of 4 mL/min. Compound **2** was also purified by semi-prep HPLC using a Thermo Scientific BDS Hypersil C_8 column (250 X 10 mm). Isocratic conditions of MeOH/H_2O (31%/79%) were employed for 10 min, and then a linear gradient to 36% MeOH was run over 20 min at a flow rate of 4 mL/min to yield 1.2 mg of **2** (2.7% crude weight). Compound **3** was isolated from HPLC fraction 21 and further purified by semi-prep HPLC using a Thermo Scientific BDS Hypersil C_8 column (250 x 10 mm). Isocratic conditions of MeOH/H_2O (27%/83%) were employed for 10 min, and then a linear gradient to 40% MeOH was run over 20 min at a flow rate of 4 mL/min to afford 1.7 mg of 37 (3.8% dry wt).

The UV spectra were recorded on a JASCO Varian-650 UV/Vis spectrophotometer. A Varian INOVA 500 or 600 MHz Unity spectrometer set at a constant temperature of 30 °C were used to record NMR spectra. The 600 MHz spectrometer was fitted with a triple resonance cold probe to enhance the sensitivity of the experiments, allowing the identification in small yields of the 3 new metabolites. The solvent peaks at δ_H 2.50 and δ_C 39.52 for DMSO-d_6 served as reference points for the ^{1}H and ^{13}C NMR chemical shifts, respectively [55]. LR-ESIMS were recorded on a Waters Alliance 2790 HPLC system equipped with a 996 PDA detector, and an Alltech ELSD that was attached to a Water ZQ ESI mass spectrometer. All HR-ESIMS were recorded on an Agilent Q-TOF 6520 mass spectrometer. A Waters 600 pump equipped with Waters 996 PDA detector and Gilson 715 liquid handler were used for all HPLC work. Semipreparative HPLC separations were carried out either with a Phenomenex Onyx Monolitic (100 x 10 mm) C_{18} column or a Thermo Scientific BDS Hypersil C_8 column (250 X 10 mm). HPLC and spectrophotometric grade solvents were used to performed chromatography, UV, and MS experiments (RCI Lab-Scan, Bangkok, Thailand), ultra-pure water was obtained using a Millipore Milli-Q filtration system [55].

Antibacterial Susceptibility Test

Compounds **1**-**4** were tested for their antibacterial activity against cultures of *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 using the well diffusion method as described by [56], [57]. Ethanol (96%) was used as a positive control. *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 inocula (McFarland turbidity standard 2) were prepared in Mueller-Hinton broth (NCIPD) and were plated onto Mueller-Hinton agar with 5% sheep blood and 1% Isovitalex in three directions by sterile swabs. Wells (7 mm diameter) were punched in the plates using a sterile stainless-steel borer. The wells were filled with 30, 60, 90 and 100 µL of compounds 1-3 (containing 30, 60, 90 and 100 µg/mL, respectively of compounds 1-4 per well). The control plates were filled with 30 µL of ethanol (96%) per well. The plates were then incubated under microaerophilic conditions at 35 °C for 72 h. The diameters of the inhibitory zones were measured in millimeters. After 72 h of incubation, no inhibitory activity was detected for any of the tested compounds.

Antitubercular Essay

Compounds **1**-**4** were also tested for their antitubercular activity against *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Pasteur 1173P2 strain transformed with green fluorescent protein (GFP) constitutive expression plasmid pUV3583c with direct readout of fluorescence as a measure of bacterial growth. BCG was grown at 37 °C to mid log phase in Middle brook 7H9 broth (Becton Dickinson) supplemented with 10% OADC enrichment (Becton Dickinson) 0.05% tween-80 and 0.2% glycerol, which then adjusted to OD_{600} = 0.025 with culture medium as bacterial suspension. Aliquots (80 µL) of the bacterial suspension were added to each well of the 96-well microplates (clear flat-bottom), followed by adding compounds (2 µL in DMSO), which were serially two-fold diluted. Isoniazid served as positive control and DMSO as negative control. The plate was incubated at 37 °C for 3 days, and GFP fluorescence was measured with Multi-label Plate Reader using the bottom read mode, with excitation at 485 nm and emission at 535 nm. MIC is defined as the minimum concentration of drug that inhibits more than 90% of bacterial growth reflected by fluorescence value [43].

IV. Conclusion

Chemical and biological explorations of untapped microbial communities have revealed a plethora of bioactive compounds with potential therapeutic applications and provide further evidence that promising lead and drug-like compounds can be isolated and elucidated from termite gut-associated *Streptomyces* species. The discovery of novel chemical scaffolds has been possible due to the continuous effort of the scientific community to develop state-of-the-art analytical dereplication strategies that allow the identification of compounds on the microgram scale. In this study, it was proven that accessing to the ^{1}H NMR data at the initial steps of the dereplication was highly discriminating for the recognition of small molecules with unusual spectral patterns, as the structural information could be obtained and interpreted in relatively short periods of time. Thus, NMR metabolomics fingerprints is an effective method that detects and prioritize in quantitative fashion, all bioactive natural products synthesized by a microorganism.

APPENDIX

Fig. 7 Three new microbial natural products isolated from the gut of the wood-feeding termite Coptotermes lacteous (Froggatt)

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