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NMR fingerprints, an integrated approach to uncover the unique components of the drug-like natural product metabolome of termite gut-associated *Streptomyces* species.

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A new NMR-based method to access to the unique components of the drug-like natural product metabolome of termite-gut associated actinomycetes has been developed. This approach was used to accelerate the identification of lead-like enhanced fractions containing small molecules with unique spectral patterns. The effectiveness of the approach was demonstrated by the isolation and identification of six new natural products, namely, actinoglycosidines A (**1**) and B (**2**), actinopolymorphol D (**3**), niveamycin A (**7**), B (**8**) and C (**9**).

Introduction

Natural products (NP) and their derivatives have historically played a vital role in drug discovery by serving as an invaluable source of therapeutic agents and potential drug leads.[1-3] NP structures usually exhibit a wide range of pharmacophores, high degree of stereochemistry and have advantages over synthetic compounds of being chemically diverse within biologically relevant 'chemical space' and therefore are likely to be substrates for many of the transporter systems that can deliver the compounds to their intracellular site of action.[4-6] However, due to technological challenges and the emergence of combinatorial chemistry, NP-based drug discovery has diminished in the last two decades and has been shifted from Nature to synthetic libraries.[7] In order to improve research competitiveness, more innovative and productive strategies are needed to rapidly identify novel lead structures from natural sources.[8-10] We have previously reported a strategy to front-load NP extracts with lead-and drug-like molecules to facilitate the NP-based drug discovery process.[11] The approach consisted of the generation of lead-like enhanced (LLE) fractions containing components with desirable physicochemical properties.[11] A subset of 18,453 biota samples, sourced from the in-house Nature Bank repository was used to generate a drug-like natural product library comprising 202,983 LLE fractions. The filter used to maximize the recovery of the desired molecules was partition coefficient (log P < 5). This optimised method facilitated the isolation of NP occupying mid-polarity physicochemical space, an essential property for oral bioavailability and cell permeability.[11] Subsequently, a ^{1}H NMR metabolic fingerprinting approach was developed to uncover and reveal unique spectral patterns of the drug-like natural product metabolome of an Australian marine sponge and allowed the identification of one novel compound iotrochotazine A, that may become a useful tool to investigate the mechanisms underlying Parkinson's disease.[12]

Herein, we applied the NMR fingerprint methodology for the identification of new drug-like NPs in cultures of termite gut-associated actinomycetes. It has been argued that the likelihood of finding new structurally diverse small molecules from underexplored environments such as desert biomes, marine ecosystems, deep-sea sediments and insect-associated actinomycetes is relatively high as they may be valuable sources of novel *Streptomyces* species and other rare actinomycetes with the capacity to produce complex molecules with a variety of biological activities.[13-22] Hence, twenty one actinomycete species isolated from the gut of the wood-feeding termite *Coptotermes lacteus* (Froggatt)[16, 23, 24] were grown in solid media (four Petri dishes, 100 x 15 mm) using four different solid culture conditions, namely, oatmeal agar (OMA), lupin flour agar (LFA), rye flour agar (RFA), and glucose yeast extract agar (GYES) and analysed to determine if the variation of media components could induce the production of new natural products (Figure 1). The actinomycete cultures were incubated at 28°C for 15 days, and then the agar containing the cells and mycelium was cut into small squares and soaked overnight in EtOAc. The EtOAc extracts were dried under reduced pressure to yield between 10 to 15 mg for each culture condition and were subsequently subjected to metabolic fingerprinting analysis. Each extract was fractionated onto five lead-like enhanced (LLE) fractions following published methodology.[11] A data set of 420 LLE fractions was manually examined for the occurrence of unique chemical profiles (i.e. non-repetitive or unique NMR resonances and distinctive ESIMS ion peaks). Based on this approach, five strains showing unique chemotypes were selected to be grown in 40-60

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Fig. 1 Colony morphologies of *Streptomyces* sp. USC 592 in four different solid culture conditions.

Petri dishes (100 x 15 mm) containing OMA, RFA or GYES solid media.

The present study describes in detail the ^{1}H NMR fingerprint method used to identify six new drug-like natural products, namely, actinoglycosidines A (**1**) and B (**2**), actinopolymorphol D (**3**), and niveamycins A (**7**), B (**8**) and C (**9**); together with five co-occurring known compounds, namely, BE-54017-derivative 4 (**4**), BE-54017 (**5**), 2-amino-6-methoxy-9H-pyrrolo[2,3-d]pyrimidine-7-carbonitrile (**6**) and WS-5995 A (**10**) and B (**11**) (Figure 2).

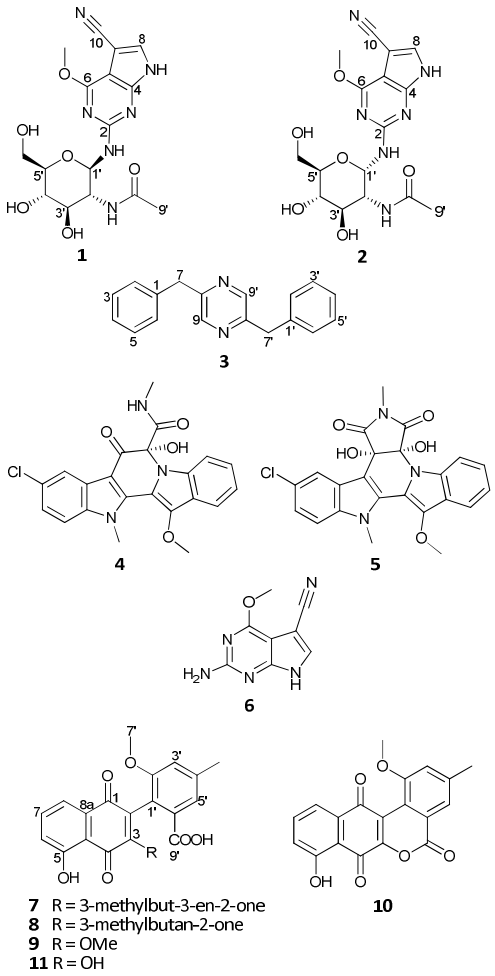

Fig. 2 Isolated natural products from two termite-associated actinomycete strains, S*treptomyces* sp. USC 592 and *Streptomyces* sp. USC 593.

Results and discussion

The metabolic fingerprinting approach consisted of the generation, through RP-HPLC, of five LLE fractions (Figure 3) for each of the eighty four crude extracts (21 strains/4 crude extracts: OMA, LFA, RFA, GYES) using parameters such as log *P* < 5 that permitted the retention of molecules with lead and drug-like properties.[11, 25] The resulting HPLC chromatograms were examined one by one for the presence of constituents within the drug-like region showing non-redundant retention times. Performing this analysis before characterising the LLE fractions by high-field NMR spectroscopy and LC-MS, allowed us to obtain not only a higher degree of chemical diversity but also focused on the fractions that were more likely to contain new microbial natural products. Therefore, the number of LLE fractions selected for further investigations was reduced from 420 to 150 (10 strains/3 solid cultures). As a secondary measure of project selection for large-scale culture and natural product isolation and identification, only strains with well resolved NMR fingerprints were selected in order to enable rapid, NMR-guided isolation follow-up work. Table 1 summarises the chemical profiles of two *Streptomyces* strains USC 592 and USC 593, which were selected to be grown in larger quantities on solid media.

The actinomycete strain, *Streptomyces* sp. USC 592, mostly showed unique ^{1}H NMR spectral fingerprints in the LLE fractions generated from the GYES crude extract. The GYES-extract sourced LLE fraction 1 showed unique proton signals (in MeOD-d_4) in the aromatic region at δ_H 7.61 (s) and 7.56 (s); as well as resonances at δ_H 5.28 (d, *J* = 9.6 Hz), 4.07 (s), 4.06 (s), 3.85 (m), 3.69 (m), 3.58 (m), and 3.41 (m) suggesting the presence of a sugar moiety (Figure 4). Similar proton resonances as those described in LLE 1 were found on LLE 2, except for the downfield shift of the sp^2-hybridized methine from δ_H 7.56 (s) to 7.59 (s) and a very low intensity proton signal at δ_H 6.01 (d, *J* = 5.0 Hz) which suggested the presence of an additional anomeric proton. LC-MS data of both LLE 1 and 2 indicated the presence of one molecular ion at 393.14 $[M+H]^+$ which after exhaustive searching of the Dictionary of Natural Products (DNP) database did not show any hits containing neither the molecular ion nor the distinctive NMR resonances.

LLE fraction 4 showed resonances at δ_H 2.62 (d, *J* = 4.7 Hz) and 8.54 (q, *J* = 4.7 Hz), indicative of the presence of a methyl group attached to a secondary amine (see Supporting Information). In addition, this fraction exhibited resonances characteristic of an aromatic ring system at δ_H 8.17 (s), 8.00 (d, *J* = 2.3 Hz), 7.80 (d, *J* = 8.2 Hz), 7.73 (d, *J* = 8.7 Hz), 7.59 (d, *J* = 8.2 Hz), 7.39 (dd, *J* = 8.7, 2.3 Hz), 7.25 (dd, *J* = 8.2, 1.1 Hz) and 7.12 (dd, *J* = 8.2, 1.1 Hz). LC-MS data analysis revealed a quasimolecular ion at 401.61 $[M+H]^+$ which did not correspond to any of the known compounds reported in the DNP from the genus *Streptomyces*. Although the NMR fingerprint of LLE fraction 5 (see Supporting Information) presented background noise that interfered with the recognition of its spectral patterns, it was possible to identify NMR resonances at δ_H 8.49 (s), 8.11 (d, *J* = 8.5 Hz), 7.93 (d, *J* = 2.0 Hz), 7.70 (d, *J* = 8.0 Hz), 7.57 (d, *J* = 8.7 Hz) and 7.28-7.14 (m) indicating that one of the compounds in this fraction has a similar aromatic scaffold to the LLE fraction 4 constituents.

Furthermore, NMR resonances in the aromatic region showed that at least one more molecule was present in this fraction. This was

further supported by LC-MS analysis that showed two compounds with molecular ion peaks at 261.13 $[M+H]^+$ and 452.09 $[M+H]^+$.

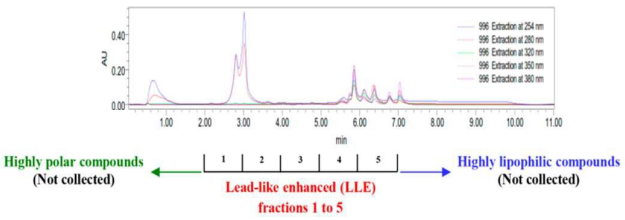

Fig. 3 HPLC chromatogram depicting the drug-like/lead-like region containing the desired constituents of one of the selected crude extracts. Adapted from Camp *et al.*[11]

Similarly, the GYES crude extract from the other microorganism examined, *Streptomyces* sp. USC 593 exhibited distinctive ^{1}H NMR spectral fingerprints in LLE fractions 3 to 5. LLE fraction 4 displayed NMR resonances at δ_H 6.16 (s) and 5.64 (s) which suggested the presence of an exocyclic methylene. In addition, this fraction showed characteristic resonances of fused aromatic rings at δ_H 7.66 (dd, *J* = 8.2, 7.6 Hz), 7.60 (d, *J* = 7.2 Hz), 7.55, (dd, *J* = 8.2, 7.6 Hz), 7.48 (d, *J* = 7.2 Hz), 7.26 (d, *J* = 8.2 Hz) and 7.06 (d, *J* = 8.2 Hz), and trisubstituted benzene rings at δ_H 7.42 (s), 7.33 (s), 7.02 (s) and 6.87 (s) (Figure 5). Likewise, LLE fractions 3 and 5 revealed NMR resonances at δ_H 7.68 (dd, *J* =8.2, 7.6 Hz), 7.58 (dd, *J* = 8.2, 7.6 Hz), 7.21 (dd, *J* = 8.2, 1.0 Hz), 7.50 (d, *J* = 0.8 Hz) and 7.12 (s), and at δ_H 7.69 (dd, *J* = 7.6, 8.2 Hz), 7.61 (dd, *J* = 8.2, 7.6 Hz), 7.29 (dd, *J* = 8.2, 1.0 Hz), 7.55 (s) and 7.12 (s), respectively, thus indicating the occurrence of other analogues (for complete NMR fingerprints, see Supporting Information). LC-MS data of LLE fractions 4 and 5 consisted of molecular ion peaks at 407.10 $[M+H]^+$, 369.09 $[M+H]^+$ and 409.12 $[M+H]^+$ which did not correspond to any of the known compounds published on the DNP from the genus *Streptomyces*.

Due to the occurrence of interesting NMR fingerprint patterns in the LLE fractions generated from the GYES extracts, the producing strains *Streptomyces* sp. USC 592 and *Streptomyces* sp. USC 593 were grown in 60 Petri dishes (100 x 15 mm) containing GYES medium. The plates were incubated for 15 days at 28°C and then extracted overnight with EtOAc to yield 190.0 and 130.0 mg, respectively, of the crude extract. A portion of each of the crude extracts (~43.0 mg) was separated on a reversed-phase C_{18} HPLC column, 60 fractions were collected and analysed by ^{1}H NMR spectroscopy. NMR-guided isolation led to the identification of 3 new natural products, namely, actinoglycosidines A (**1**) and B (**2**) and actinopolymorphol D (**3**) together with 3 known compounds, BE-54017-derivative 4 (**4**), BE-54017 (**5**) and 2-amino-6-methoxy-9H-pyrrolo[2,3-d]pyrimidine-7-carbonitrile (**6**) from *Streptomyces* sp. USC 592 as well as 3 new metabolites, namely, niveamycins A (**7**), B (**8**) and C (**9**) together with two co-occurring known compounds, namely, WS-5995 A (**10**) and B (**11**) from *Streptomyces* sp. USC 593.

Compound **1** was isolated as a stable amorphous solid. The HRESIMS of **1** contained the protonated molecular peak at 393.1513 $[M+H]^+$ consistent with a molecular formula of $C_{16}H_{21}N_6O_6$ (calcd. for $C_{16}H_{21}N_6O_6$, 393.1414) requiring 10 double-bond equivalents (DBEs). The ^{1}H NMR spectrum of **1** (Table 2) in MeOD-d_4 displayed ten resonances attributable to two methyls at δ_H 4.06 (3H, s, H-7) and 1.96 (3H, s, H-9'), seven proton signals at δ_H 5.29 (1H, d, *J* = 10.0 Hz, H-1'), 3.88 (1H, dd, *J* = 10.0, 3.3 Hz, H-2'), 3.56 (1H, ddd, *J* = 10.0, 8.5, 3.3 Hz, H-3'), 3.40 (1H, m, H-4'), 3.41 (1H, m, H-5'), 3.85 (1H, dd, *J* = 12.0, 2.8 Hz, H-6a') and 3.70 (1H, dd, *J* = 12.0, 5.1 Hz, H-6b') and one sp^2-hybridized methine at δ_H 7.58 (1H, s, H-9). The ^{13}C NMR spectrum of **1** (Table 2) exhibited 16 resonances comprised of two methyls at δ_C 54.3 (C-7) and 22.8 (C-9'), six sp^3-hybridized methines at δ_C 83.7 (C-1'), 56.2 (C-2'), 76.6 (C-3'), 72.3 (C-

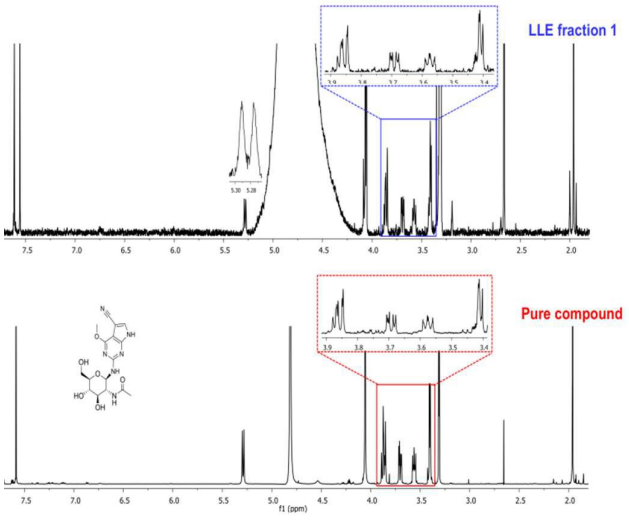

Fig. 4 Top spectrum depicts the ^{1}H NMR fingerprint of LLE fraction 1, which displays characteristic NMR resonances of a sugar moiety. The spectrum at the bottom shows a new natural product identified by large-scale NMR-guided isolation.

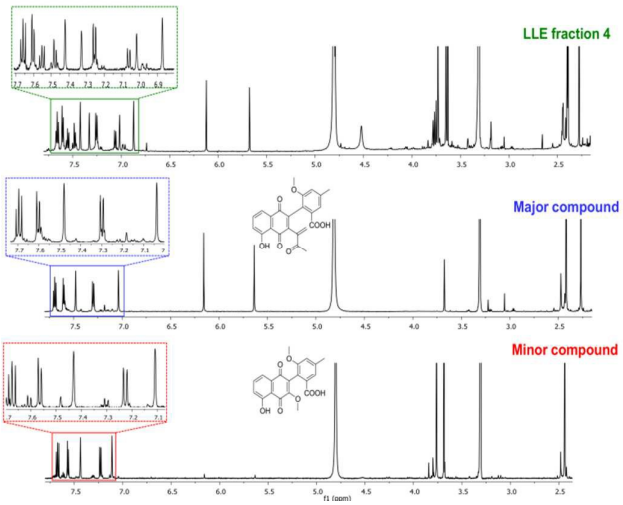

Fig. 5 ^{1}H NMR fingerprint spectra of *Streptomyces* sp. USC 593. Top spectrum shows the ^{1}H NMR fingerprint of LLE fraction 4. Middle spectrum displays NMR resonances of the major compound, named niveamycin A. The spectrum at the bottom exhibits NMR signals of the minor compound, named niveamycin C.

Table 1 Colony characteristics and chemical profiles of the selected actinomycete strains.

Actinomycete species	AM [a]	SM [b]	DP [c]	RT (min) [d]	ESIMS [e]	NMR Fingerprints [f]
Streptomyces sp. USC 592	Lime	Yellow	Light yellow	3.0	393.14	**LLE 1.** 12.25, 7.99, 7.89, 6.80, 5.01, 4.46.
				5.8	401.61	**LLE 4.** 8.54, 4.26, 4.13, 2.62.
Streptomyces sp. USC 593	Yellow	Orange	Orange	5.2 5.6	407.10 369.09	**LLE 4.** 7.66, 7.60, 7.48, 7.55, 7.42, 7.33, 7.25, 7.06, 7.02, 6.87, 6.12, 5.68, 3.73, 3.65, 3.63, 2.39, 2.28

[a] Aerial mycelium. [b] Substrate mycelium. [c] Diffusible pigment. [d] Retention times. [e] Positive ionization mode $[M+H]^+$. [f] Unique/interesting resonances in ppm. All the samples were acquired in [D6] DMSO at 600 MHz.

4'), 79.4 (C-5'), and 62.9 (C-6'), one sp^2-hybridized methine at δ_C 131.2 (C-9), and seven quaternary resonances including one carbonyl at δ_C 174.6 (C-8), one oxygen-bearing aromatic carbon at δ_C 164.7 (C-6), four olefinic carbons at δ_C 160.5 (C-2), 155.4 (C-4), 98.7 (C-5), and 84.8 (C-8), and one unsaturated carbon at δ_C 116.5 (C-10) suggesting that either an acetylene or a nitrile were attached to it.[26]

Interpretation 1D and 2D NMR data allowed for the identification of 2 partial structures, fragments A and B depicted in Figure 6. Fragment A, showed characteristic resonances of an *N*-acetylglucosamine 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 at δ_H 5.29 (1H, d, *J* = 10.2 Hz, H-1'). The absolute configuration of the sugar was determined to be D via hydrolysis (see Supporting Information) and subsequent comparison of its optical rotation value with the commercial *N*-*β*-D-acetylglucosamine (Sigma-Aldrich). The structural assignment of fragment B was challenging due to the absence of proton resonances and a large number of heteroatoms. The only proton peak observed was a singlet at δ_H 7.58 (H-9) showing HMBC correlations to the quaternary carbons at δ_C 155.4 (C-4), 98.7 (C-5), 84.8 (C-8) and 116.5 (C-10). These correlations were consistent with the presence of a purine residue which was further confirmed by NMR data comparison with related natural products containing the same residue such as dapiramicins A and B.[27, 28] Crucial HMBC correlations from the anomeric proton (H-1') to the quaternary carbon (C-2) indicated that fragment A and B are connected through a nitrogen atom. Compound **1** was therefore concluded to be *N*-*β*-D-acetylglucosamine 2-amino-6-methoxy-9H- pyrrolo[2,3-d]pyrimidine-7-carbonitrile and given the trivial name, actinoglycosidine A.

The molecular formula of compound **2** was established to be $C_{16}H_{21}N_6O_6$ by HRESIMS at *m/z* 393.1513 $[M+H]^+$, isomeric to the natural product **1**. The ^{1}H NMR and ^{13}C NMR spectra of **2** (Table 2) resembled those of **1** and in particular the spectral data attributable to the purine residue which were almost superimposable. The most significant NMR spectral differences were observed in the sugar moiety, especially the resonances of the anomeric carbon C-1' and C-5' had shifted upfield to δ_C 78.7 and 72.1, respectively. The stereochemistry of the anomeric proton of the sugar residue was consistent with the presence of an *α* sugar, based on the magnitude of the ^{1}H-^{1}H coupling constant [δ_H 6.02 (1H, d, *J* = 5.0 Hz, H-1')]. This was further confirmed by strong NOESY correlations between H-1' and H-2' which were indicative of the presence of the *α* anomer. Based on the co-occurrence of compounds **1** and **2** in the same organism and in agreement with the NMR spectral data, optical rotation and ECD values, the structure of actinoglycosidine B (**2**) was determined to be *N*-*α*-D-acetylglucosamine 2-amino-6-methoxy-9H-pyrrolo[2,3-d]pyrimidine-7-carbonitrile.

Compound **3** was isolated as a stable amorphous solid and gave a molecular formula of $C_{18}H_{17}N_2$ deduced from the HRESIMS peak at *m/z* 261.1380 $[M+H]^+$ (calcd for $C_{18}H_{17}N_2$, 261.1313), indicating that this molecule required 12 degrees of unsaturation. The ^{1}H NMR spectrum of **3** (Table 3) displayed eight resonances attributable to two sp^3-hybridized methylenes at δ_H 4.12 (2H, s, H-7) and six sp^2-hybridized methines at δ_H 7.31 (2H, dd, *J* = 7.9, 5.6 Hz, H-2), 7.19 (2H, dd, *J* = 7.3, 5.6 Hz, H-3), 7.28 (1H, dd, *J* = 7.9, 5.6 Hz, H-4), 8.46 (1H, s, H-9). The ^{13}C NMR spectrum of **3** (Table 3) exhibited 9 resonances comprised of one sp^3-hybridized methylene at δ_C 41.8 (C-7), six sp^2-hybridized methines at δ_C 129.8 (C-2), 127.2 (C-3), 129.4 (C-4), 127.2 (C-5), 129.8 (C-6) and 144.4 (C-9), and two quaternary resonances at δ_C 140.1 (C-1) and 154.8 (C-8). From the molecular formula of **3** it was observed that the remaining atoms required by the molecular formula $C_{18}H_{16}N_2$ were $C_9H_8N_2$ and consequently six additional degrees of unsaturation were needed to be established. On the basis of the analysis of the proton and carbon resonances, the structure of compound **3** was concluded to be a symmetric dimer. Moreover, interpretation of the proton resonances in the aromatic region allowed for the identification of two monosubstitued benzene rings. Characteristic proton and carbon resonances at δ_H 8.46 (H-9) and δ_C 144.4 (C-9) indicated the presence of a pyrazine molecule substituted at C-8 and C-8'.[29] COSY correlations observed between the methylene at H-7 to the olefinic protons at H-2 and H-9 suggested that the two parts of the molecule were connected through a sp^3-hybridized methylene.

Table 2 ^{1}H NMR and ^{13}C NMR spectroscopic data for actinoglycosidine A and B (**1**-**2**) in MeOD-d_4.

	1[a]			**2**[a]		
Position	δ_C [ppm]	δ_H [ppm] (*J* in Hz)	HMBC	δ_C [ppm]	δ_H [ppm] (*J* in Hz)	HMBC
2	160.5			160.3		
4	155.4			155.0		
5	98.7			98.8		
6	164.7			164.7		
7	54.3	4.06 (s)	C-6	54.6	4.08 (s)	C-6
8	84.8			84.3		
9	131.2	7.58 (s)	C-4, C-5, C-8, C-10	131.7	7.60 (s)	C-4, C-5, C-8, C-10
10	116.5			116.6		
1'	83.7	5.29 (d, 10.0)	C-2, C-2', C-3', C-5'	78.7	6.02 (d, 5.1)	C-2, C-2', C-3'
2'	56.2	3.88 (dd, 10.0, 3.3)	C-1', C-3', C-8'	54.4	4.11 (dd, 11.2, 5.1)	C-1', C-3', C-8'
3'	76.6	3.56 (ddd, 10.0, 8.5, 3.3)	C-2', C-4'	73.8	3.62 (ddd, 9.6, 5.1, 2.6)	C-6'
4'	72.3	3.40 (m)[b]	C-3', C-5', C-6'	71.9	3.79 (dd, 8.8, 6.7)	C-2', C-3', C-5'
5'	79.4	3.41 (m)[b]	C-3', C-4', C-6'	72.1	3.44 (dd, 9.6, 8.8)	C-3', C-4', C-6'
6'	62.9	3.85 (dd, 12.0, 2.8)	C-4', C-5'	62.3	3.76 (dd, 9.6, 2.6)	C-2', C-3'
		3.70 (dd, 12.0, 5.1)			3.72 (dd, 12.0, 5.1)	
8'	174.6			174.3		
9' 22.8	1.96 (s)	C-8'	22.9	1.93 (s)	C-8'	

[a] Proton and carbon resonances were acquired at 600 MHz and 150 MHz, respectively. [b] Coupling constants of these resonances are unclear due to overlapping.

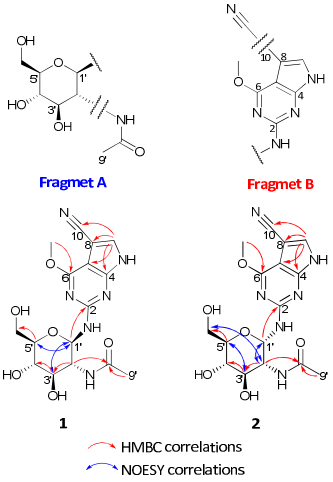

Fig. 6 Fragments found during the elucidation process and crucial HMBC and NOESY correlations for actinoglycosidines A and B.

This was further confirmed by HMBC correlations from H-7 to the quaternary carbons at C-1 and C-8 and to the olefinic carbons at C-2 and C-9 (Figure 7). Thus, the structure of actinopolymorphol D (**3**) was assigned to be 2,5-dibenzyl pyrazine. While the synthesis of **3** has been reported elsewhere,[30, 31] this is the first report of the structure as a naturally-occurring 2,5-dibenzyl pyrazine.

In addition to the three new natural products, the GYES extract from *Streptomyces* sp. USC 592 also yielded the co-occurring known natural products, namely, BE-54017-derivative 4 (**4**), BE-54017 (**5**) and 7*H*-Pyrrolo[2,3-*d*]pyrimidine-5-carbonitrile, 2-amino-4-methoxy (**6**).[32, 33]

NMR guided isolation work on the GYES extract obtained from *Streptomyces* sp. USC 593 afforded the new natural products **7**-**9**. Compound **7** was isolated as a yellow amorphous solid. The molecular formula of **7** was established as $C_{23}H_{19}O_7$ by HRESIMS at *m/z* 407.1115 $[M+H]^+$ (calcd. for $C_{23}H_{19}O_7$, 407.1053). The ^{1}H NMR spectrum of **7** (Table 4) in MeOD-d_4 displayed 10 resonances attributable to three methyls at δ_H 2.27 (3H, s, H-12), 3.68 (3H, s, H-

7') and 2.42 (3H, s, H-8'), five sp^2-hybridized methines at δ_H 7.30 (1H, dd, *J* = 8.3, 1.1 Hz, H-6), 7.69 (1H, dd, *J* = 8.3, 7.6 Hz, H-7), 7.61 (1H, dd, *J* = 7.6, 1.1 Hz, H-8), 7.04 (1H, s, H-3') and 7.48 (1H, s, H-5'), and one sp^2-hybridized methylene pair at δ_H 6.16 (1H, s, H-10a) and 5.64 (1H, s, H-10b). The ^{13}C NMR spectrum of **7** exhibited 23 resonances comprised of three methyls at δ_C 26.4 (C-12), 56.0 (C-7'), and 21.6 (C-8'), five sp^2-hybridized methines at δ_C 124.7 (C-6), 137.4 (C-7), 120.0 (C-8), 116.3 (C-3'), and 123.9 (H-5'), one sp^2-hybridized methylene at δ_C 130.1 (C-10), and fourteen quaternary resonances including four carbonyls at δ_C 184.5 (C-1), 190.4 (C-4), 199.3 (C-11) and 170.9 (C-9'), two oxygen-bearing aromatic carbons δ_C 162.5 (C-5) and 157.4 (C-2'), eight olefinic carbons at δ_C 149.5 (C-2), 143.4 (C-3), 116.1 (C-4a), 134.1 (C-8a), 144.5 (C-9), 122.1 (C-1'), 141.5 (C-4') and 128.5 (C-6'). Detailed analysis of the COSY and HMBC spectra allowed for the identification of three partial fragments, A, B, C (Figure 8). In fragment A, COSY correlations between the olefinic protons at H-6, H-7 and H-8 to each other indicated the presence of an ortho, meta-substituted aromatic spin system. Moreover, crucial HMBC correlations from the aromatic protons at H-6 to C-4a, C-5 and C-8; H-7 to C-4a, C-5 and C-8a, and H-8 to C-1, C-4a, C-5 and C-6 were consistent with the presence of a hydroxyl naphthoquinone moiety. In fragment B, COSY correlations from H-8' to the singlets at δ_H 7.48 (H-5') and 7.04 (H-3') suggested that a methyl group was attached to the olefinic carbon at δ_C 141.5 (C-4'). This was further confirmed by HMBC correlations from H-8' to C-3', C-4' and C-5'. The position of the methoxy substituent was established based on HMBC correlations from H-7' to the carbon at δ_C 157.4 (C-2') and ROESY correlations from the singlet at H-3' to H-7'. HMBC correlations from the aromatic proton at H-5' to C-6' and C-9' indicated that a carboxylic acid was attached to C-6'. The methylene pair at δ_H 6.16 and 5.64 from fragment C showed HMBC correlations to the quaternary carbons at C-3 (fragment A), C-9 and C-11. Furthermore, HMBC correlations from H-12 to C-11, C-10, and C-9 indicated the presence of a terminal acetyl group. These correlations clearly indicated that fragment A substituent is connected to C-3. Based on the degrees of unsaturation calculated from the molecular formula ($C_{23}H_{19}O_7$) and comparison of the spectroscopic data of **7** with those of the known natural products WS 5995 A, B, and C,[34-37] fragments A-C were connected to complete the structure of **7** as 2-(5-hydroxy-1,4-dioxo-3-(3-oxobut1-en-2-yl)-1,4-dihydronaphthalen-2-yl)-3-methoxy-5-methyl benzoic acid. The new natural product **7** was given the trivial name, niveamycin A.

Table 3. ^{1}H NMR and ^{13}C NMR spectroscopic data for actinopolymorphol D (**3**) in Acetone-d_5.

		3[a]	
Position	δ_C [ppm]	δ_H [ppm] (*J* in Hz)	HMBC
1; 1'	140.1		
2; 2'	129.8	7.31 (dd, 7.9, 5.6)	C-3, C-4, C-7
3; 3'	127.2	7.19 (dd, 7.3, 5.6)	C-2
4; 4'	129.4	7.28 (dd, 7.9, 5.6)	C-1
5; 5'	127.2	7.19 (dd, 7.3, 5.6)	C-2
6; 6'	129.8	7.31 (dd, 7.9, 5.6)	C-3, C-4, C-7
7; 7'	41.8	4.12 (s)	C-1, C-2, C-8, C-9
8; 8'	154.8		
9; 9'	144.4	8.46 (s)	

[a] Proton and carbon resonances were acquired at 600 MHz and 150 MHz, respectively.

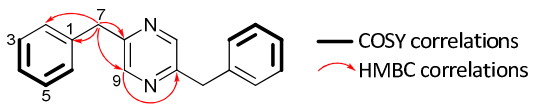

Fig. 7 Crucial COSY and HMBC correlations for actinopolymorphol D.

Niveamycin B (**8**) was isolated as a yellow amorphous solid, the molecular formula of **8** was established as $C_{23}H_{21}O_7$ by HRESIMS measurements at *m/z* 409.1279 $[M+H]^+$ (calcd. for $C_{23}H_{21}O_7$, 409.1209), indicating the presence of two additional protons compared to **7**. The ^{1}H and ^{13}C NMR spectroscopic data for **8** were superimposable with those of **7**, except that a 3-methylbut-3-en-2-one group of **7** at C-9 was replaced by a 3-methylbutan-2-one group in **8**. Consequently, the C-11 resonance was observed to shift downfield from δ_C 199.3 to δ_C 203.1. The planar structure of **8** was established on the basis of the ^{1}H-^{1}H COSY, HSQC, and HMBC spectral analysis. The absolute configuration of the side chain chiral carbon at C-9 was determined by electronic circular dichroism (ECD) calculations. The ECD spectra of the most stable conformers for **8** were calculated at the B3LYP/6-311 + G(d,p)// B3LYP/6-311 + G(2d,p) level. The ECD spectra for the conformers were Boltzman-averaged, the resulting ECD spectrum agreed well with that of the experimental and led to the conclusion that the absolute configuration at C-9 was *S*. The calculated and experimental ECD spectra for niveamycin B (**8**) are depicted in Figure 9.

Table 4. ^{1}H NMR and ^{13}C NMR spectroscopic data for niveamycins A-C (**7**-**9**) in MeOD-d_4

	7[a]			**8**[a]			**9**[a]		
Position	δ_C [ppm]	δ_H [ppm] (*J* in Hz)	HMBC	δ_C [ppm]	δ_H [ppm] (*J* in Hz)	HMBC	δ_C [ppm]	δ_H [ppm] (*J* in Hz)	HMBC
1	184.5			184.4			182.7		
2	149.5			150.7			[b]		
3	143.4			145.6			168.0		
4	190.4			191.0			[b]		
4a	116.1			116.2			114.1		
5	162.5			162.4			162.5		
6	124.7	7.30 (dd, 8.3, 1.1)	C-4a, C-5, C-8	124.7	7.30 (dd, 8.3, 1.0)	C-4a, C-5, C-6, C-8	122.8	7.23 (dd, 8.3, 1.0)	C-4a, C-8
7	137.4	7.69 (dd, 8.3, 7.6)	C-4a, C-5, C-8a	137.5	7.70 (t, 8.3, 7.6)	C-4a, C-5, C-8a	137.6	7.67 (dd, 8.3, 7.6)	C-5, C-8a
8	120.0	7.61 (dd, 7.6, 1.1)	C-1, C-4a, C-5, C-6	120.0	7.61 (dd, 7.6, 0.9)	C-1, C-3, C-4a, C6, C-7	119.2	7.56 (dd, 7.6, 1.0)	C-1, C-4a, C-6, C-8a
8a	134.1			133.8			134.0		
9	144.5			49.9	3.03 (q, 6.8)	C-2, C-3, C-4, C-10, C-11	52.0	3.68 (s)	C-3
10	130.1	6.16 (s)	C-3, C-9, C-11	14.3	1.27 (d, 6.8)	C-3, C-9, C-11			
		5.64 (s)	C-3, C-9, C-11						
11	199.3			208.0					
12	26.4	2.27(s)	C-9, C-11	28.5	1.99 (s)	C-11			
1'	122.1			121.1			121.9		
2'	157.4			157.9			157.7		
3'	116.3	7.04 (s)	C-2', C-5', C-8'	116.3	7.15 (s)	C-1', C-2', C-4', C-5', C-8'	116.6	7.11 (s)	C-2', C-5', C-8'
4'	141.5			142.0			139.1		
5'	123.9	7.48 (s)	C-1', C-3', C-6', C-8', C-9'	124.3	7.58 (s)	C-1', C-3', C-5', C-8', C-9'	123.3	7.43 (s)	C-1', C-3', C-6', C-8', C-9'
6'	128.5			[b]			128.3		
7'	56.0	3.68 (s)	C-2'	56.2	3.78 (s)	C-2'	56.2	3.76 (s)	C-2'
8'	21.6	2.42 (s)	C-3', C-4', C-5'	21.6	2.47 (s)	C-3', C-4', C-5'	21.2	2.44 (s)	C-3', C-4', C-5'
9'	170.9			169.8			167.8		

[a] Proton and carbon resonances were acquired at 600 MHz and 125 MHz, respectively. [b] Not determined

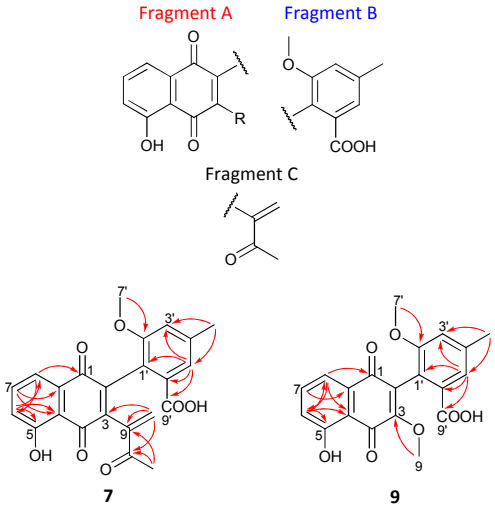

Fig. 8 Fragments found during the elucidation process and crucial HMBC correlations for niveamycins A and C.

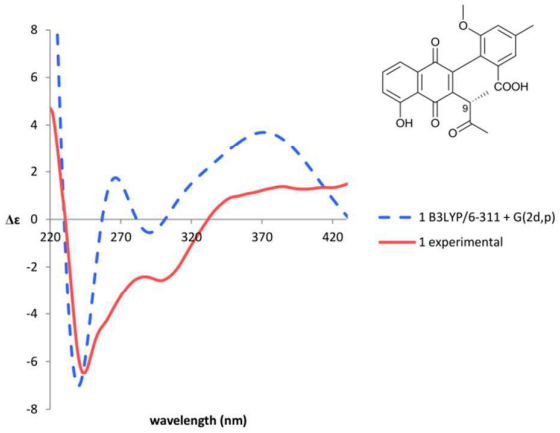

Fig. 9 Calculated (9*S*) and experimental ECD spectra of niveamycin B

Compound **9** was isolated as a yellow amorphous solid with a molecular formula of $C_{20}H_{17}O_7$, m/z 369.0980 $[M+H]^+$, (calcd. for $C_{20}H_{17}O_7$, 369.0896) (Table 4) established on the basis of the spectroscopic and spectrometric data. Comparison of 1D and 2D NMR spectroscopic data of **7** with those of **9**, revealed that these compounds were virtually identical, except that a 3-methylbut-3-en-2-one group of **7** was substituted by a methoxy group in **9** at C-3. As a consequence, the oxygen-bearing aromatic carbon resonance has significantly shifted downfield to δ_C 168.0

This side chain substitution was supported by a HMBC correlation from the methyl at δ_H 3.68 (H-8) to C-3. Thus, the structure of **9** was elucidated as 2-(5-hydroxy-3-methoxy-1,4-dioxo-1,4-dihydronaph thalen-2-yl)-3--methoxy-5-methylbenzoic acid.

Based upon comparison of the ^{1}H NMR and LC-MS spectral data of the co-occurring compounds **10** and **11** (obtained from the crude -extract of *Streptomyces* sp. USC 593) with those reported on the literature, it was determined that compound **10** corresponded to WS 5995 C and **11** to WS 5995 A.[34-37]

In order to assess the biological activities of the isolated natural products, compounds **1**-**11** were tested for their antitubercular activity against *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) 1173P2 strain transformed with green fluorescent protein (GFP) constitutive expression plasmid pUV3583c with direct readout of fluorescence as a measure of bacterial growth.[38] Almost all tested compounds exhibited weak antitubercular activity with a MIC value of 100 µg/mL. Compound **7** showed moderate activity with a MIC value of 50 µg/mL.

Conclusions

The remarkable chemical diversity encompassed by marine and terrestrial microorganisms continues to be of relevance to drug discovery programs. Efforts to identify new and novel natural products from under-explored environments include approaches that are based on the premise that adaptation to unique environments will include the production of new microbial metabolites and that those environments will contain novel microbial strains. However, the identification of phylogenetic uniqueness does not guarantee that the organism has the necessary biosynthetic machinery to produce new or novel metabolites or that the pathway is expressed rather than being cryptic. NMR metabolomic fingerprints offer the advantage that it detects, in a quantitative fashion, all drug-like natural products produced by a microorganism. The developed approach consisted of the analysis by high resolution ^{1}H NMR spectroscopy of HPLC fractions containing constituents with drug-like properties, using this method we were able to isolate and identify six new natural products, namely, actinoglycosidines A (**1**) and B (**2**), actinopolymorphol D (**3**), and niveamycins A (**7**), B (**8**) and C (**9**) together with five co-occurring known compounds.

Experimental Section

Culture conditions. Twenty one actinomycete species isolated from the gut of the wood-feeding termite *Coptotermes lacteus* (Froggatt).[15,16] were grown in solid media (four Petri dishes, 100 x 15 mm) using four different solid culture conditions, OMA (Oatmeal 20.0 g, yeast extract 3.0 g, agar bacteriological 20.0 g, dH_2O 1L), LFA (Lupin flour 5.0 g, peptone 100.0 mg, glucose 1.0 g, agar bacteriological 20.0 g, dH_2O 1L), RFA (Rye flour 5.0 g, peptone 100.0 mg, glucose 1.0 g, agar bacteriological 20.0 g, dH_2O 1L) and GYES (glucose 10.0 g, yeast extract 2.50 g, corn starch 2.50 g, sodium chloride 1.25 g, calcium carbonate 0.75 g, agar bacteriological 20.0 g, dH_2O 1L), media was adjusted to a pH of 7.2 before autoclaving. The actinomycete cultures were incubated at 28°C for 15 days, and then the agar containing the cells and mycelium was cut into small squares and soaked overnight in EtOAc. The EtOAc extracts were dried under reduced pressure to yield between 10 to 15 mg for each culture condition. The extracts were afterward subjected to chemical investigations where based on the NMR-guided fingerprinting approach, five strains and three culture conditions were selected to perform solid fermentations in larger amounts (sixty Petri dishes, 100 x 15 mm).

Lead-like enhanced (LEE) fractions. A portion of the microbial extracts (1 mg) was reconstituted in DMSO (150 µL). HPLC separations were performed on a Phenomenex C_{18} Monolithic HPLC column (100 x 4.6 mm) using conditions that consisted of a linear gradient from 90% H_2O/10% MeOH to 50 H_2O/50% MeOH in 3.0 min at a flow rate of 4mL/min; a convex gradient to MeOH in 3.50 min at a flow rate of 3 mL/min, held at MeOH for 0.50 min at a flow rate of 3 mL/min, held at MeOH for a further 1.0 min at a flow rate of 4 mL/min; then a linear gradient back to 90% H_2O/10% MeOH in 1.0 min at a flow rate, then held at 90% H_2O/10% MeOH for 2.0 min at a flow rate of 4 mL/min. The total run time for each crude extract was 11 min and 5 fractions were collected between 2.0 and 7.0 min (5 x 1 min). Because the intent of this separation step was to collect fractions most likely to contain compounds with drug-like properties, the early eluting material consisting of media components and highly polar compounds was not collected nor was the late-eluting lipophilic portion.

Metabolic fingerprinting approach. Five LLE fractions replicates were combined accordingly and further analyzed by ^{1}H NMR spectroscopy. The fractions were dried down under reduced pressure and subsequently dissolved in 220 µL of MeOD-d_4, the samples were run under the following parameters: pw = 45°, pl = 0 µs, d_2 = 0 s, d_1 = 1 s, at = 1.7 s, sw = 9615Hz, nt = 256 scans.

Scale-up solid cultures growth and isolation. The producing strains *Streptomyces* sp. USC 592 and *Streptomyces* sp. USC 593 were grown for 15 days at 28°C in 60 agar plates (100 x 15 mm) containing GYES medium. A similar methodology as described above was followed to obtain the crude extract. The EtOAc extracts were dried down to yield a dark brown solid extract (190.0 mg) and a red dark solid extract (130.0 mg), respectively. A portion of the crude extract (~43.0 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 9mL/min. 60 fractions were collected from 0 to 60 min (60 x 1 min), and then analysed by (+)-LR-ESIMS. Fraction 14 yielded 2.4 mg of compound **1** (5.5% dry wt), fraction 16 yielded 1.1 mg of compound **2** (2.5% dry wt). Combined fractions 37 and 38 were further purified by semi-preparative HPLC using a BDS Hypersil C_8 column [250 x 10 mm] eluting with a gradient from 60% MeOH/40% H_2O to 90% MeOH/10 H_2O over 30 min to yield 1.1 mg of compound **3** (2.5% dry wt) and 2.0 mg of compound **4** (4.6% dry wt). Fractions 32 to 34 were combined and subsequently purified using a BDS Hypersil C_8 column [250 x 10 mm] eluting with a gradient from 40% MeOH/60 % H_2O to 90% MeOH/10 H_2O over 30 min to yield 1.0 mg of compound **5** (2.3% dry wt). Combined fractions 17 to 21 yielded 2.0 mg of compound **6** (4.6% dry wt). Fractions 29 to 31 were combined to afford 1.7 mg of compound **7** (3.9% dry wt). Combined fractions 32 and 33 yield 0.9 mg of compound **8** (2.0% dry wt). Compound **9** was isolated from fraction 28 in very slow yields, 0.7 mg (1.6% dry wt). Fractions 24 and 25 contained, compounds **10** 0.8 mg (1.8% dry wet) and **11** 1.9 mg (4.4% dry wet).

*Actinoglycosidine A (**1**):* amorphous colourless solid; HRESIMS m/z $[M+H]^+$ 393.1513 (calcd for $C_{16}H_{21}N_6O_6$, 393.1414); $[\alpha]^{25}_D$ = +40 (*c* 0.1, MeOH); UV (MeOH) λ_{max} nm (log ε): 207 (3.65), 228 (4.29), 261 sh (3.59), 284 (3.55), 307 (3.13); ^{1}H NMR (MeOD-d_4, 600 MHz) and ^{13}C NMR (MeOD-d_4, 125 MHz) data, see Table 2.

*Actinoglycosidine B (**2**):* amorphous colourless solid; HRESIMS m/z $[M+H]^+$ 393.1513 (calcd for $C_{16}H_{21}N_6O_6$, 393.1414); $[\alpha]^{25}_D$ = +39 (*c* 0.07, MeOH); UV (MeOH) λ_{max} nm (log ε): 203 (3.93), 225 (4.20), 253 sh (3.68), 269 (3.35), 2.92 (3.57); ^{1}H NMR (MeOD-d_4, 600 MHz) and ^{13}C NMR (MeOD-d_4, 125 MHz) data, see Table 2.

*Actinopolymorphol D (**3**):* amorphous colourless solid; HRESIMS m/z $[M+H]^+$ 261.1380 (calcd for $C_{18}H_{16}N_2$, 261.1313); UV (MeOH) λ_{max} nm (log ε): 205 (4.06), 250 sh (3.63), 281 (3.65), 3.03 sh (3.44); ^{1}H NMR (Acetone-d_6, 600 MHz) and ^{13}C NMR (Acetone-d_6, 125 MHz) data, see Table 3.

*Niveamycin A (**7**):* yellow amorphous solid; HRESIMS at *m/z* 407.1115 $[M+H]^+$ (calcd. for $C_{23}H_{19}O_7$, 407.1053); UV (MeOH) λ_{max} (log ε): 208 (4.65), 249 sh (4.10), 287 (4.00), 4.12 (3.61); ^{1}H NMR (MeOD-d_4, 600 MHz) and ^{13}C NMR (MeOD-d_4, 125 MHz) data, see Table 4.

*Niveamycin B (**2**):* yellow amorphous solid; $[\alpha]^{25}_D$ = -100 (*c* 0.05, MeOH); HRESIMS m/z 409.1279 $[M+H]^+$ (calcd for $C_{23}H_{21}O_7$, 409.1209); UV (MeOH) λ_{max} (log ε): 208 (4.61), 248 (3.96), 290 (3.83), 407 (3.53); ^{1}H NMR (MeOD-d_4, 600 MHz) and ^{13}C NMR (MeOD-d_4, 125 MHz) data, see Table 4.

*Niveamycin C (**3**):* yellow amorphous solid; HRESIMS m/z 369.1509 $[M+H]^+$ (calcd for $C_{20}H_{17}O_7$, 369.0896); UV (MeOH) λ_{max} (log ε): 206 (4.37), 283 (3.84), 398 (3.30); ^{1}H NMR (MeOD-d_4, 600 MHz) and ^{13}C NMR (MeOD-d_4, 125 MHz) data, see Table 4.

Chemical investigations. The UV spectra were recorded on a JASCO Varian-650 UV/Vis spectrophotometer and analysed using SDAR v3.2.[39] NMR spectra were recorded at 30°C on either a Varian 500 or 600 MHz Unity INOVA spectrometer. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peak at δ_H 2.50 and δ_C 39.52 for DMSO-d_6. (+)-LR-ESIMS were recorded on a Waters ZQ single quadropole ESI spectrometer or on an Agilent 6120 quadruple LCMS system. All HR-ESIMS were recorded on an Agilent Q-TOF 6520 mass spectrometer. RP-HPLC prefractionation was performed on a Waters 600 pump equipped with Waters 996 PDA detector and Gilson 717 liquid handler. 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). All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade (RCI Lab-Scan, Bangkok, Thailand), and the H_2O was Millipore Milli-Q PF filtered.

Acid hydrolysis of actinoglycosidines A. A solution of actinoglycosidine A (2.4 mg) in MeOH (2.0 mL) was treated with 5% HCl (200 µL) and the reaction was kept stirring at 60°C for 81 hrs. After hydrolysis, the solution was evaporated and subjected to semi-preparative HPLC using a Thermo Scientific BDS Hypersil C_8 column (250 X 10 mm). Isocratic HPLC conditions of 90% H_2O/10% MeOH were initially employed for 10 min, then a linear gradient to 21% MeOH was run over 15 min, followed by a gradient to 100% MeOH in 10 min and then isocratic conditions of 100% MeOH were held for 5 min, all at a flow rate of 4 mL/min. Sixty fractions (60 x 30 sec) were collected from time = 0 min, and then analyzed by high resolution NMR spectroscopy and (+)-LR-ESIMS. Fraction 10 yielded 0.3 mg of *N-δ*-D-acetylglucosamine.

Anti-BCG assay. Identification of inhibitors was performed in an aerobic, logarithmic growth screen of BCG as previously reported.[40] The BCG used was a *M. bovis* BCG 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 OD600=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 twofold 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.[38]

MIC determination. Antimicrobial assays were performed according to the Antimicrobial Susceptibility Testing Standards outlined by the Clinical and Laboratory Standards Institute (CLSI) (NCCLS 1999) using the bacteria Staphylococcus aureus (ATCC 6538), Bacillus subtilis (ATCC 6633), and methicillin-resistant S. aureus (MRSA). For each organism, a loopful of glycerol stock was streaked on an LB agar plate and incubated overnight at 37°C. A single bacterial colony was picked and suspended in Mueller-Hinton Broth to approximately 1×104 CFU/mL. A twofold serial dilution of each compound to be tested (4,000 to 31.25 µg/mL in DMSO) was prepared, and an aliquot of each dilution (2 µL) was added to a 96-well flat-bottom microtiter plate (Greiner). Vancomycin and ciprofloxacin were used as positive controls and DMSO as the negative control. An aliquot (78 µL) of bacterial suspension was then added to each well (to give final compound concentrations of 100 to 0.78 µg/mL in 2.5% DMSO), and the plate was incubated at 37°C aerobically for 16 h. Finally, the optical density of each well at 600 nm was measured with an EnVision 2103 Multi-label Plate Reader (Perkin-Elmer Life Sciences). MIC values were defined as the minimum concentration of compound that inhibited visible bacterial growth. All the experiments were performed in triplicate.[38]

Computational chemistry. The conformation search for **8** was performed in MacroModel[41] in the gas phase, including all rotatable single bonds using the Monte Carlo (MCMM) method with the Polac-Ribieri Conjugate Gradient (PRCG), the MMFF force field, dielectric constant-dependent electronics (ε = 1), and normal cut-off points to model the non-bonded interactions. All heavy atoms were included in the test for redundant conformers, using the default cut-off (maximum atom deviation) of 0.5 Å. The energy window for saving new structures was 5 kcal mol^{-1}, relative to the current global minimum with a maximum number of steps of 30,000 and 1,000 per rotatable bond. Each search continued until the global energy minima were found at least 10-20 times, thus ensuring that all of the relevant conformers had been found.[42] All conformers identified by MacroModel[41] were subjected to further methanol geometry optimization in Gaussian 09[43] using the level B3LYP functional and 6-31G(d,p) basis set.[44] A fine integration grid with accurate SCF convergence criteria was employed and all geometry-optimised structures were characterised as true minima by the absence of imaginary vibrational frequencies at the stationary point. The ECD spectra of the most stable conformers for **8** were calculated at the B3LYP/6-311 + G(d,p)// B3LYP/6-311 + G(2d,p) level in MeOH using the SCRF method, with the CPCM model.[44] The ECD spectra for the conformers were Boltzman-weighted averaged to obtain the ECD spectrum of the isomers using SpecDis v1.62.[45]

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