Discovery of ianthelliformisamines D–G from the sponge *Suberea ianthelliformis* and the total synthesis of ianthelliformisamine D

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Abstract

The marine sponge *Suberea ianthelliformis* was investigated for new chemistry after the recent discovery that polyamines ianthelliformisamines A–C (**1**–**3**) – originally sourced from this Australian sponge – act as *Pseudomonas aeruginosa* biofilm inhibitors and antibiotic enhancers. Large-scale extraction and isolation studies resulted in the discovery of four new and minor natural products, ianthelliformisamines D–G (**4**–**7**) and the known steroid, aplysterol (**8**). Compounds **4**–**7** were fully characterised following 1D/2D NMR, MS and UV data analyses. All compounds were assessed for their inhibition on planktonic growth of *P. aeruginosa* PAO1 in addition to their ability to inhibit the formation of biofilms. None of the tested natural products inhibited planktonic growth or biofilm formation of PAO1 when screened at 50 µM. Ianthelliformisamine D (**4**) contains a rare *N*-(3-aminopropyl)-2-pyrrolidone moiety only found in <30 natural products. Owing to the novelty of compound **4**, we undertook the first total synthesis of this natural product, which was achieved in three steps.

Introduction

The marine environment covers over two thirds of the earth's surface and it encompasses a wide range of complex ecosystems that are highly variable in their physical attributes including pressure, salinity, temperature, and light availability. Both flora and fauna have evolved over billions of years to survive within this unique environment [1,2], which has led to the production and diversification of unique and novel metabolites with specialised biological functions [3,4]. The richest diversity of marine metabolites are derived from invertebrates; most of which are sessile and lack the ability to physically defend themselves from both predators and competitors and thus rely on chemical mechanisms for their defense [1]. Metabolites derived

from marine sponges contribute more than half of all compounds identified from marine invertebrates [1,5], therefore it is no surprise that sponges are highly sought after for novel bioactive metabolites and have been a major focus of marine natural product drug discovery for over 70 years.

The identification of ianthelliformisamines A–C from the Australian marine sponge *Suberea ianthelliformis*, which displayed activity against both *Pseudomonas aeruginosa* and *Staphylococcus aureus,* has contributed to a surge in the interest of polyamines as new antibacterial leads [6]. To date the total synthesis of ianthelliformisamines A–C (**1**–**3**) has been described [7] and numerous synthetically related analogues have been published with their antibiotic assessment against numerous bacterial species reported [8-11]. Our recent publication showed that of the naturally occurring metabolites, compound **3** inhibits the formation of *P. aeruginosa* PAO1 biofilms (MIC 53.1 µg/mL), whilst **1** and **2** enhanced the antibiotic effect of ciprofloxacin when used in combination [12]. In efforts to further examine the chemistry of *S. ianthelliformis* and potentially discover new antibiotic leads, we undertook a scaled-up chemical investigation on the original specimen from which ianthelliformisamines A–C were isolated.

Herein, we describe the large-scale extraction, isolation, and structure elucidation of four new metabolites, ianthelliformisamines D–G (**4**–**7**). Additionally, we report the isolation of the known natural products, aplysterol (**8**) and ianthelliformisamines A–C (**1**–**3**). Owing to the novelty of ianthelliformisamine D (**4**) we undertook the first total synthesis of this natural product, which was successfully achieved in only three steps and respectable yield. All newly identified metabolites were subsequently assessed for their ability to inhibit the growth of planktonic *P. aeruginosa* PAO1 and formation of biofilms.

Results and Discussion

For a more comprehensive chemical investigation into the chemistry of *Suberea ianthelliformis*, a new aliquot of the freeze-dried and ground sample was extracted exhaustively with *n*-hexane, CH_2Cl_2, and MeOH. The CH_2Cl_2 and MeOH extracts were combined then subjected to phenyl-bonded reversed-phase HPLC (RP-HPLC), which led to the purification of the known metabolites, ianthelliformisamines A–C (**1**–**3**) [7] and aplysterol (**8**) [13], and four new natural products, ianthelliformisamines D–G (**4**–**7**) (Figure 1). This extraction and isolation process was repeated twice to obtain sufficient
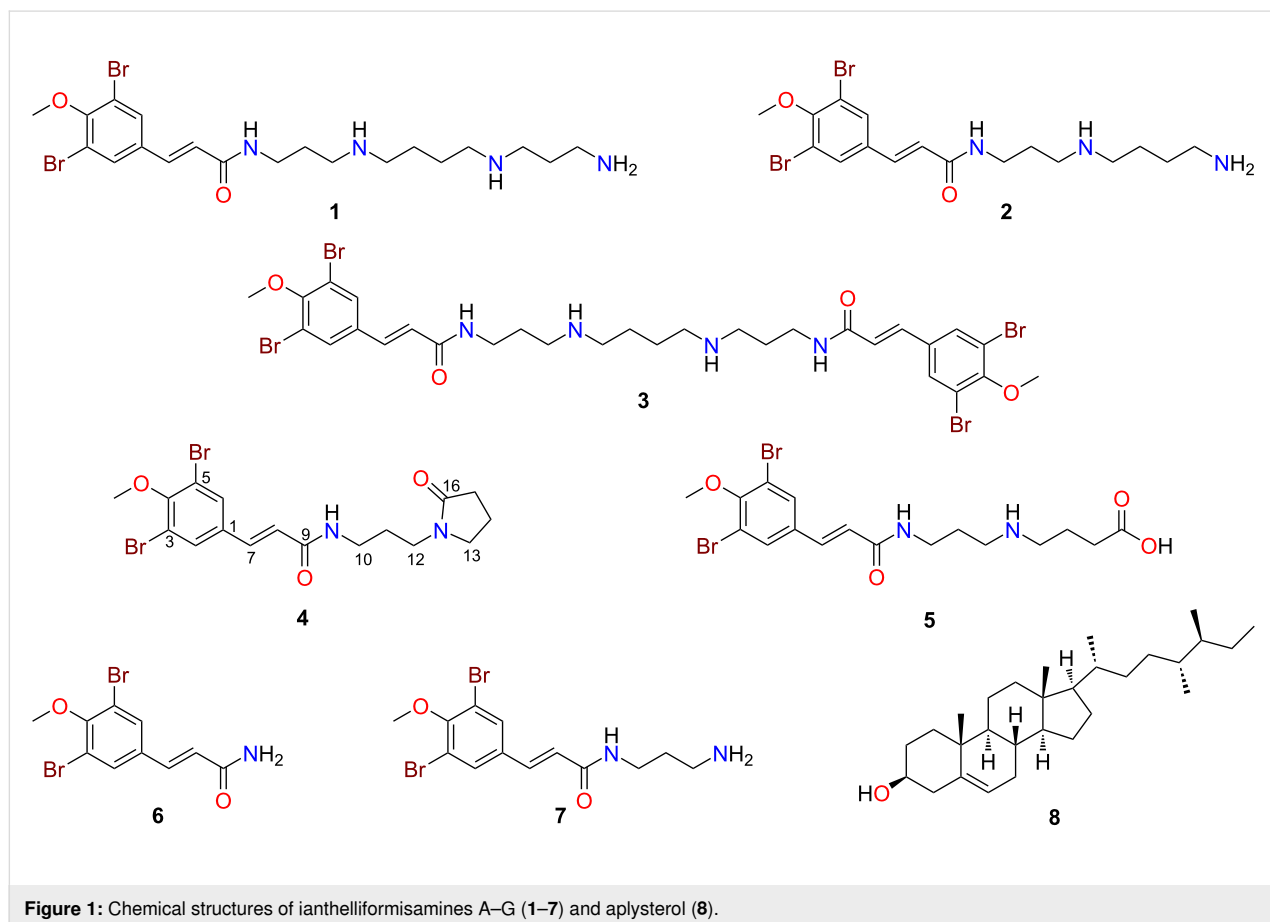

Figure 1: Chemical structures of ianthelliformisamines A–G (**1**–**7**) and aplysterol (**8**).

quantities of the minor and previously undescribed natural products for full characterisation studies and biological assessment. The spectroscopic and spectrometric data of all known compounds isolated during our studies matched well with the literature values [7,14].
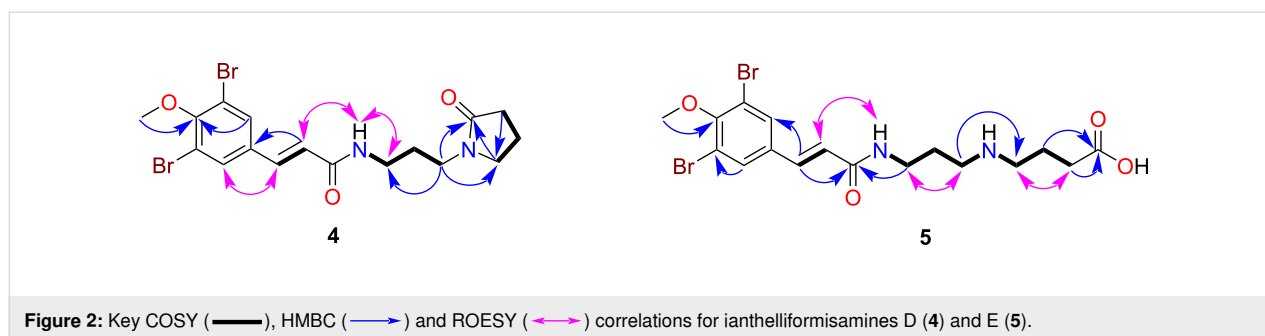
Ianthelliformisamine D (**4**) was isolated as a stable brown gum. The LRESIMS of **4** indicated the presence of two bromine atoms due to a 1:2:1 ion cluster at *m/z* 459/461/463 [M + H]$^+$, whilst the HRESIMS data allowed a molecular formula of $C_{17}H_{20}Br_2N_2O_3$ to be assigned. The ^{1}H NMR (Table 1) and edited HSQC spectra of **4** in DMSO-d_6 indicated the presence of one methoxy group (δ_H 3.81), six methylene signals (δ_H 3.33, 3.20, 3.14, 2.21, 1.91, 1.63) and one exchangeable proton triplet (δ_H 8.04) that was indicative of a secondary amide [7]. Additionally, signals for one isolated *trans* olefin (δ_H 7.33, 6.66) and one aromatic signal (δ_H 7.89) that integrated for two protons indicating the presence of a symmetrical aromatic moiety, were observed. The ^{13}C NMR (Table 1) spectrum of **4** showed two carbonyls (δ_C 164.4, 173.9), with the carbonyl at δ_C 164.4 readily assigned to an acrylamide group, which is present in all previously published ianthelliformisamine molecules [7]. Methylenes resonating at δ_H 3.14, 1.64 and 3.20 were assigned to a three-carbon alkyl spin system that was flanked by

nitrogen atoms [NH–$(CH_2)_3$–N] based on COSY correlations from the amide proton at δ_H 8.04. A terminal pyrrolidone was assigned based on COSY data for the three remaining methylene protons (δ_H 3.33, 1.91, 2.21), and a three-bond HMBC correlation from δ_H 3.33, and a two-bond correlation from δ_H 2.20 to the carbonyl resonating at δ_C 173.9 [15]. The observation of a $^3J_{CH}$ correlation from δ_H 3.20 to the nitrogen-bearing carbon at δ_C 46.4 and to δ_C 173.9 supported the assignment of the pyrrolidone moiety. A 1,3,4,5-tetrasubstituted aromatic ring was assigned based on $^3J_{CH}$ correlations from the isolated aromatic signal (δ_H 7.89) to its symmetrically placed carbon (δ_C 131.6) and to an oxygen-bearing carbon (δ_C 153.9) along with $^2J_{CH}$ correlations to a brominated (δ_C 118.0) and sp^2 phenyl carbons (δ_C 134.5) (see Figure 2). Observation of three-bond correlations in the HMBC spectrum between the olefin protons and aromatic ring carbons (δ_H 7.33 to δ_C 131.6 and δ_H 6.66 to δ_C 134.5) linked the olefin moiety to the symmetrical 1,3,4,5-tetrasubstituted phenyl system. ROESY and HMBC correlations from the amide proton linked the propyl-2-pyrrolidine moiety to the brominated acrylamide fragment and thus the full chemical structure of **4** was elucidated and assigned to ianthelliformisamine D. A refined literature search using Scifinder Scholar [16] revealed that compound **4** contains a novel scaffold due to the *N*-(3-aminopropyl)-2-pyrrolidone

Table 1: NMR data of ianthelliformisamines D (**4**) and E (**5**) in DMSO-d_6.^{a}

| position | Ianthelliformisamine D (**4**) | | Ianthelliformisamine E (**5**)^b | |
	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)
1	134.5, C		134.3, C	
2	131.6, CH	7.89, s	131.6, CH	7.89, s
3	118.0, C		118.0, C	
4	153.9, C		153.9, C	
4-OCH$_3$	60.6, CH$_3$	3.81, s	60.6, CH$_3$	3.82, s
5	118.0, C		118.0, C	
6	131.6, CH	7.89, s	131.6, CH	7.89, s
7	135.2, CH	7.33, d (15.8)	135.5, CH	7.36, d (15.8)
8	124.5, CH	6.66, d (15.8)	124.1, CH	6.65, d (15.8)
9	164.4, C		164.9, C	
9-NH		8.04, t (5.7)		8.23, t (5.7)
10	36.5, CH$_2$	3.14 dt (5.7, 6.5)	35.9, CH$_2$	3.25 dt (5.7, 6.5)
11	27.0, CH$_2$	1.63, m	26.1, CH$_2$	1.77, m
12	39.7, CH$_2$	3.20, t (7.2)	44.8, CH$_2$	2.92, m
12-NH				8.35, brs
13	46.4, CH$_2$	3.33, m	46.3, CH$_2$	2.92, m
14	17.5, CH$_2$	1.91, m	21.1, CH$_2$	1.79, m
15	30.5, CH$_2$	2.21, t (8.0)	30.4, CH$_2$	2.36, t (7.3)
16	173.9, C		173.6, C	
16-OH				^{c}

^{a}Spectra recorded at 25 °C (800 MHz for ^{1}H NMR and 200 MHz for ^{13}C NMR); ^{b}isolated as a TFA salt; ^{c}not observed.

Figure 2: Key COSY (▬▬), HMBC (⟶) and ROESY (⟷) correlations for ianthelliformisamines D (**4**) and E (**5**).

moiety, which is rarely found in natural products with <30 metabolites reported to date.

The TFA salt of ianthelliformisamine E (**5**) was obtained as a stable brown gum. The LRESIMS of **5** indicated the presence of two bromine atoms, displaying a 1:2:1 ion cluster at *m/z* 477/479/481 [M + H]$^+$. Similarly to compound **4**, the ^{1}H NMR (Table 1) and edited HSQC spectra of **5** displayed six methylene signals (δ_H 3.25, 2.92, 2.36, 1.79, 1.77). Additionally, a broad exchangeable proton (δ_H 8.35) was observed that was indicative of a protonated dialkylated amino group [7]. The ^{13}C NMR (Table 1) data of **5** displayed six aliphatic carbons (δ_C 35.9, 26.1, 44.8, 46.3, 21.1, 30.4) and two carbonyl signals (δ_C 164.9, 173.6). Similarly to the other ianthelliformisamines, the aromatic (δ_C 131.6) and olefin (δ_C 124.5, 135.2) carbons were observed [7]. COSY correlations associated with the methylene signals of **5** enabled the assignment of an NH–(CH$_2$)$_3$–NH–(CH$_2$)$_3$ spin system, which was confirmed by HMBC and ROESY correlations. The carboxyl group at δ_C 173.6 was positioned at the end of the alkyl chain based on HMBC correlations from the methylene protons (δ_H 2.36, 1.79) to this carbon signal. Although a downfield exchangeable CO$_2$H proton was not observed in the ^{1}H NMR spectrum of **5**, a carboxylic acid moiety was assigned based on the ^{13}C NMR shift value (δ_C 173.6) [17], and analysis of the HRESIMS ion at *m/z* 477.0022 [M + H]$^+$, which confirmed the molecular formula to be C$_{17}$H$_{22}$Br$_2$N$_2$O$_4$.

Ianthelliformisamine F (**6**) was isolated as a stable brown gum. The LRESIMS of **6** indicated the presence of two bromine atoms due to a 1:2:1 ion cluster at *m/z* 334/336/338 [M + H]$^+$, whilst the HRESIMS data enabled a molecular formula of C$_{10}$H$_9$Br$_2$NO$_2$ to be assigned. Comparison of the 1D NMR data of **6** to metabolites **4** and **5** and the previously characterised ianthelliformisamines A–C readily allowed the assignment of dibromo-4-methoxyphenyl and propenamide moieties. However, the ^{1}H NMR spectrum of **6** displayed two broad exchangeable signals (δ_H 7.19, 7.46) indicating the presence of a primary amide [18], thus enabling the full chemical structure of ianthelliformisamine F to be determined.

The TFA salt of ianthelliformisamine G (**7**) was also purified as a stable brown gum. In a similar manner to compound **6**, the LRESIMS indicated the presence of two bromine atoms; these data also indicated that **7** had an additional 57 amu (atomic mass units) compared to **6**. Comparison of the ^{1}H NMR (Table 2) and edited HSQC spectra with **7** showed that **6** contained three additional methylene signals (δ_H 3.25, 2.81, 1.73). Two exchangeable protons were observed in **7**, including a broad singlet (δ_H 7.73) that integrated for three protons and a triplet (δ_H 8.23) that were indicative of a protonated terminal amino group and a secondary amide functionality, respectively [7]. ^{13}C NMR shifts and COSY correlations associated with the methylene signals of **7** enabled the assignment of an NH–(CH$_2$)$_3$–NH$_2$ moiety, which was confirmed by HMBC correlations (Figure 3). This fragment was connected to the previously assigned moiety through a HMBC correlation from δ_H 3.25 to the amide carbonyl (δ_C 164.9), thus enabling the full chemical structure of ianthelliformisamine G (**7**) to be assigned.

Compounds **6** and **7** are both commercially available [16] but neither of these compounds has been fully characterised with only the total synthesis of **7** being reported in the literature [8]. Our study reports the first identification of **6** and **7** from a natural origin and the first full characterisation of these molecules using NMR, UV, and MS data.

Owing to the novelty of ianthelliformisamine D (**4**), we attempted the first total synthesis of this natural product (Scheme 1). In a similar, but modified manner to that reported by Gan et al. [19] we firstly methylated the commercially sourced 3,5-dibromo-4-hydroxybenzaldehyde using TMSCHN$_2$ in MeOH/CH$_2$Cl$_2$ at room temperature (17% yield). Subjecting the methoxylated benzaldehyde intermediate **9** to a Doebner–Knoevenagel condensation with malonic acid and pyridine afforded the brominated cinnamic acid analogue **10** in 54% yield [19]. Amidation chemistry using carbonyldiimidazole (CDI) [18] and the commercially available primary amine, 1-(3-aminopropyl)pyrrolidin-2-one completed the total synthesis of the natural product in an overall yield of 1.5%. The NMR

Table 2: NMR data of ianthelliformisamines F (**6**) and G (**7**) in DMSO-d_6.[a]

	Ianthelliformisamine F (**6**)		Ianthelliformisamine G (**7**)[b]	
position	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)
1	134.4, C		134.3, C	
2	131.6, CH	7.88, s	131.6, CH	7.89, s
3	118.0, C		118.0, C	
4	153.9, C		153.9, C	
4-OCH$_3$	60.6, CH$_3$	3.81, s	60.6, CH$_3$	3.82, s
5	118.0, C		118.0, C	
6	131.6, CH	7.88, s	131.6, CH	7.89, s
7	135.7, CH	7.32, d (15.8)	135.4, CH	7.35, d (15.8)
8	124.6, CH	6.65, d (15.8)	124.2, CH	6.65, d (15.8)
9	166.1, C		164.9, C	
9-NH$_2$		7.19, brs		
		7.46, brs		
9-NH				8.23, t (5.9)
10			35.9, CH$_2$	3.25, dt (5.9, 6.8)
11			27.5, CH$_2$	1.73, tt (6.8, 7.5)
12			36.9, CH$_2$	2.81, m
12-NH$_2$				7.73, brs

[a]Spectra recorded at 25 °C (800 MHz for ^{1}H NMR and 200 MHz for ^{13}C NMR); [b]isolated as a TFA salt.
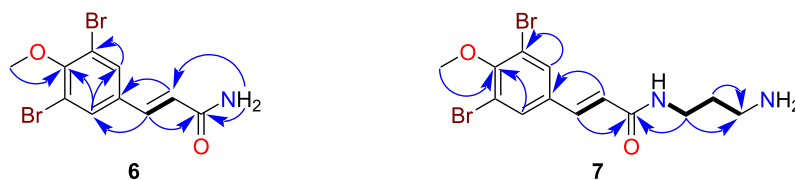
Figure 3: Key COSY (━━) and HMBC (⟶) correlations for ianthelliformisamines F (**6**) and G (**7**).
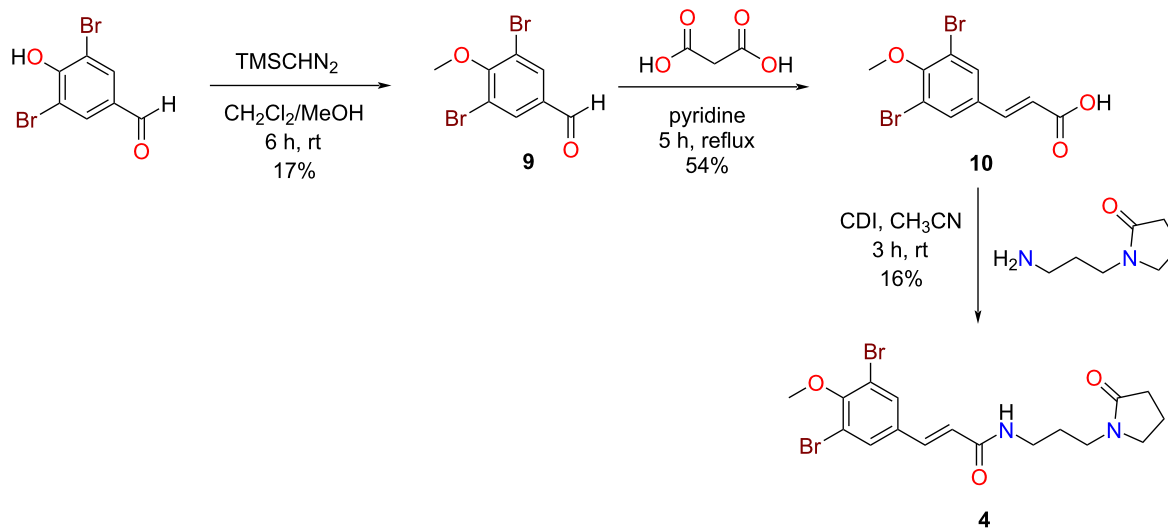
Scheme 1: Total synthesis of ianthelliformisamine D (**4**).

data comparison of the natural product and our synthetic compound was essentially identical.

Due to our interest in the identification of potential new leads against *Pseudomonas aeruginosa* and supported by numerous reports of favourable activity for ianthelliformisamines A–C [7,12] and their synthetic analogues [8-10], we investigated the planktonic and biofilm activity of the new natural products **4–7** in addition to the known metabolite, aplysterol (**8**) [13]. Our biological assessment of compounds **4–8** showed no inhibition of planktonic growth or biofilm formation for *P. aeruginosa* when screened at 50 µM. Previously reported antibacterial assessment of ianthelliformisamines A–C (**1–3**) and their synthetic analogues has generated structure–activity relationship data, leading to speculation over the moieties responsible for their antibiotic effects. A reduction in the number of amines in the polymeric chain and the absence of a primary amine was noted to decrease bioactivity by Xu et al. [7]. Research reported by Khan et al. in 2014 [9] also supported the important role that the polyamine chain length plays in antibacterial activity. Of note, increasing research providing structure–activity relationship analyses shows that polyamines (including spermine) are bacterial membrane disruptors and are beneficial in enhancing activity of known antibacterial agents [20,21]. The absence of polyamine chains in ianthelliformisamines D–G (**4–7**) could explain the loss of activity seen in our assessment against *P. aeruginosa*. The synthetic molecule of **7** has been tested by other researchers against Gram-negative bacteria including *P. aeruginosa* with reported MIC values of >200 µg/mL [8], which is consistent with the data we report in this paper.

Finally, and of note, our studies described here report the first isolation of aplysterol (**8**) from the genus *Suberea*. To date, there has been no evaluation of this compound's antibiotic potential towards *P. aeruginosa*, including biofilm inhibition. Furthermore, this is the first report of any biological assessment of aplysterol (**8**) as a pure compound, since prior studies have only tested a mixture of **8** with 24,28-didehydroaplysterol, where it was found to inhibit DNA topoisomerase II-α (MIC = 50 µM) [22].

Conclusion

In summary, we report here the discovery and full characterisation of four new natural products, ianthelliformisamines D–G from the marine sponge *Suberea ianthelliformis*. Furthermore, we describe the first total synthesis of ianthelliformisamine D which contains a rare *N*-(3-aminopropyl)-2-pyrrolidone moiety. Whilst testing of these natural products against *Pseudomonas aeruginosa* showed that none of them inhibited planktonic growth or biofilm formation at 50 µM, synthetic efforts has

generated sufficient quantities of the novel compound ianthelliformisamine D that will enable additional biological profiling.

Experimental
General experimental procedures

Specific rotations were recorded using a JASCO P-1020 polarimeter. UV data was recorded on a JASCO V-650 UV–vis spectrophotometer. NMR spectra were recorded at 25 °C on a Bruker Avance III HD 800 MHz NMR spectrometer equipped with a cryoprobe. The ^{1}H and ^{13}C chemical shifts were referenced to solvent peaks for DMSO-d_6 at δ_H 2.50 and δ_C 39.52, respectively. LRESIMS data was recorded on an Ultimate 3000 RS UHPLC coupled to a Thermo Fisher Scientific ISQEC single quadruple ESI mass spectrometer. HRESIMS data was acquired on a Bruker maXis II ETD ESI-qTOF. Alltech Davisil (30–40 µm, 60 Å) C_8-bonded silica and Alltech Davisil (30–40 µm, 60 Å) diol-bonded silica were used for pre-adsorption work before RP- or NP-HPLC, respectively. The pre-adsorbed material was subsequently packed into an Alltech stainless steel guard cartridge (10 × 30 mm) then attached to a HPLC column prior to fractionation. A Waters 600 pump fitted with a Waters 996 photodiode array detector fitted with a Gilson 717-plus autosampler were used for RP-HPLC separations. A Thermo Fisher Scientific Dionex Ultimate 3000 UHPLC was used for NP-HPLC separations. Thermo Betasil phenyl-bonded silica (5 µm, 100 Å, 150 × 21.2 mm) and Phenomenex Luna C_{18} column (5 µm, 90–110 Å, 10 mm × 250 mm) were used for RP-HPLC separations. For NP-HPLC, a YMC diol-bonded silica (5 µm, 120 Å, 150 × 20 mm) column was used. The frozen marine sponge was dried using a Dynamic FD12 freeze dryer and ground using a Fritsch Universal Cutting Mill Pulverisette 19. The ground marine sponge was extracted at room temperature using an Edwards Instrument Company Bioline orbital shaker set to 200 rpm. Solvents were removed from extracts with a Büchi R-144 rotary evaporator and from HPLC fractions using a GeneVac XL4 centrifugal evaporator. All solvents used for chromatography, UV, MS, and [α]$_D$ were Honeywell Burdick & Jackson or Lab-Scan HPLC grade. H_2O was filtered using a Sartorius Stedium Arium Pro VF ultrapure water system. Reagents used for the total synthesis of Ianthelliformisamine D were purchased from either Sigma or Aaron Chemicals.

Sponge material

The sponge sample was obtained from the NatureBank [23] biota library housed at the Institute for Biomedicine and Glycomics, Griffith University, Australia. A voucher specimen of *Suberea ianthelliformis* (NB6014998; phylum Porifera, class Demospongiae, order Verongida, family Aplysinellidae) has been previously lodged (G322255) at the Queensland Museum, South Brisbane, Queensland, Australia [7].

Extraction and isolation

In a manner similar to that reported by Xu et al. [7], the freeze-dried and ground specimen of *S. ianthelliformis* (10 g) was extracted sequentially with *n*-hexane (250 mL, 2 h), CH_2Cl_2 (250 mL, 2 h), and MeOH (250 mL, 2 h; 250 mL, 16 h). The *n*-hexane extract was discarded (as it contained only highly lipophilic material) and the CH_2Cl_2 and MeOH extracts were combined to produce a brown gum (173.3 mg) that was pre-adsorbed to C_8-bonded silica (≈1 g), packed into a stainless-steel guard cartridge, and subjected to phenyl semipreparative RP-HPLC separation. Isocratic conditions of 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) were initially employed for the first 10 min, then a linear gradient to 100% MeOH (0.1% TFA) was run over 40 min, followed by isocratic conditions of 100% MeOH (0.1% TFA) for a further 10 min, all at a flowrate of 9 mL/min; 60 fractions (60 × 1 min) were collected. This first HPLC fractionation afforded ianthelliformisamine B (**2**, 10.4 mg, t_R 36–37 min, 0.104% dry wt) and several other UV-active fractions that contained mixtures of polyamine-type alkaloids, which were combined for further isolation work. The following describes the purification of these fractions. Fractions 34–35 (5.0 mg) were purified by semipreparative phenyl RP-HPLC using isocratic conditions of 70% H_2O (0.1% TFA)/30% MeOH (0.1% TFA) for the first 10 min, then a linear gradient to 10% H_2O (0.1% TFA)/90% MeOH (0.1% TFA) was run over 50 min at a flowrate of 9 mL/min; 60 fractions (60 × 1 min) were collected and resulted in the purification of ianthelliformisamine A (**1**, 1.0 mg, t_R 16 min, 0.010% dry wt). Fractions 38–40 were combined (45.0 mg) and purified by semipreparative phenyl RP-HPLC using the same solvent gradient as above and afforded the new natural products, ianthelliformisamines G (**7**, 1.2 mg, t_R 19 min, 0.012% dry wt) and E (**5**, 1.5 mg, t_R 23 min, 0.015% dry wt). Fractions 41–42 were combined (22.4 mg) and purified by semipreparative RP-HPLC using the same solvent gradient and afforded the new natural products, ianthelliformisamines F (**6**, 1.2 mg, t_R 19 min, 0.012% dry wt) and D (**4**, 1.3 mg, t_R 41 min, 0.013% dry wt). Fractions 43–45 were combined (28.7 mg) and purified by semipreparative RP-HPLC using the same solvent gradient and afforded the known metabolite, ianthelliformisamine C (**3**, 1.8 mg, t_R 38 min, 0.018% dry wt). Fractions 46–60 (20.1 mg) was purified by semipreparative NP-HPLC. Isocratic conditions of 100% *n*-hexane were initially employed for the first 10 min, then a linear gradient to 80% *n*-hexane/20% iPrOH was run over 50 min at a flow rate of 9 mL/min; 60 fractions (60 × 1 min) were collected and resulted in the purification of the known sterol, aplysterol (**8**, 0.9 mg, t_R 12–13 min, 0.009% dry wt). The extraction and isolation process described above was repeated twice more (identical scale) to obtain sufficient quantities of the minor natural products for characterisation and biological assessment with similar recoveries obtained.

Ianthelliformisamine D (4): Stable brown gum; UV (MeOH) λ_{max}, nm (log ε): 226 (4.01), 278 (3.86); ^1H and ^{13}C NMR data (DMSO-d_6), see Table 1; LRESIMS (*m/z*): 459/461/463 [M + H]$^+$; HRESIMS (*m/z*): [M + H]$^+$ calcd for $C_{17}H_{21}{}^{79}Br_2N_2O_3$, 458.9913; found, 458.9917.

Ianthelliformisamine E TFA salt (5): Stable brown gum; UV (MeOH) λ_{max}, nm (log ε): 229 (4.25), 279 (4.17); ^1H and ^{13}C NMR data (DMSO-d_6), see Table 1; LRESIMS (*m/z*): 477/479/481 [M + H]$^+$; HRESIMS (*m/z*): [M + H]$^+$ calcd for $C_{17}H_{23}{}^{79}Br_2N_2O_4$, 477.0019; found, 477.0022.

Ianthelliformisamine F (6): Stable brown gum; UV (MeOH) λ_{max}, nm (log ε): 229 (3.83), 280 (3.71); ^1H and ^{13}C NMR data (DMSO-d_6), see Table 2; LRESIMS (*m/z*): 334/336/338 [M + H]$^+$; HRESIMS (*m/z*): [M + Na]$^+$ calcd for $C_{10}H_9{}^{79}Br_2NO_2Na$, 355.8892; found, 355.8896.

Ianthelliformisamine G TFA salt (7): Stable brown gum; UV (MeOH) λ_{max}, nm (log ε): 229 (3.63), 280 (3.57); ^1H and ^{13}C NMR data (DMSO-d_6), see Table 2; LRESIMS (*m/z*): 391/393/395 [M + H]$^+$; HRESIMS (*m/z*): [M + H]$^+$ calcd for $C_{13}H_{17}{}^{79}Br_2N_2O_2$, 390.9651; found, 390.9647.

Aplysterol (8): Stable white powder; $[\alpha]_D^{20}$ −46.7 (*c* 0.025, CHCl$_3$), lit. $[\alpha]_D$ −25 (*c* not specified, CHCl$_3$) [24]; HRESIMS (*m/z*): [M + Na]$^+$ calcd for $C_{29}H_{50}ONa$, 437.3754; found, 437.3741.

Methylation of 3,5-dibromo-4-hydroxybenzaldehyde

3,5-Dibromo-4-hydroxybenzaldehyde (279.0 mg, 1.0 mmol) was dissolved in CH_2Cl_2/MeOH 1:1 (1 mL) then a solution of TMSCHN$_2$ in hexanes (2.0 M, 1.5 mL, 11 mmol) was slowly added and the mixture stirred at room temperature for 6 h. The reaction crude was pre-adsorbed to C_8-bonded silica, packed into a stainless steel guard cartridge then purified by Luna C_{18} semipreparative RP-HPLC using isocratic conditions of 50% H_2O (0.1% TFA)/50% MeOH (0.1% TFA) for the first 5 min, then a linear gradient to 100% MeOH (0.1% TFA) was run over 20 min and held at 100% MeOH (0.1% TFA) for a further 5 min at a flowrate of 4 mL/min; 30 fractions (30 × 1 min) were collected and resulted in the purification of 3,5-dibromo-4-methoxybenzaldehyde (**9**, 50.1 mg, t_R 18–22 min, 17% yield) [19].

3,5-Dibromo-4-methoxybenzaldehyde (9): White powder; ^1H NMR (DMSO-d_6, 800 MHz) δ_H 9.89 (s, 1H, H-7), 8.17 (s, 2H, H-2, H-6), 3.88 (s, 3H, 4-OCH$_3$); ^{13}C NMR (DMSO-d_6, 200 MHz) δ_C 190.1 (C-7), 158.1 (C-4), 134.5 (C-1), 133.8 (2C, C-2, C-6), 118.6 (2C, C-3, C-5), 60.8 (4-OCH$_3$); LRESIMS (*m/z*): 293/295/297 [M + H]$^+$.

Doebner–Knoevenagel condensation of 3,5-dibromo-4-methoxybenzaldehyde with malonic acid

3,5-Dibromo-4-methoxybenzaldehyde (**9**, 80.0 mg, 0.27 mmol), malonic acid (56.0 mg, 0.54 mmol) and dry pyridine (1 mL) were refluxed at 100 °C for 5 h. The reaction crude was pre-adsorbed to C_8-bonded silica, packed into a stainless steel guard cartridge then purified by Betasil semipreparative RP-HPLC using isocratic conditions of 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) that were initially employed for the first 10 min, then a linear gradient to 100% MeOH (0.1% TFA) was run over 40 min, followed by isocratic conditions of 100% MeOH (0.1% TFA) for a further 10 min, all at a flowrate of 9 mL/min; 60 fractions (60 × 1 min) were collected resulting in the purification of (*E*)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (**10**, 49.0 mg, t_R 46–48 min, 54% yield) [19].

(*E*)-3-(3,5-Dibromo-4-methoxyphenyl)acrylic acid (10): White powder; ^{1}H NMR (DMSO-d_6, 800 MHz) δ_H 8.06 (s, 2H, H-2, H-6), 7.50 (d, *J* = 16.0 Hz, 1H, H-7), 6.62 (d, *J* = 16.0 Hz, 1H, H-8), 3.82 (s, 3H, 4-OCH$_3$); ^{13}C NMR (DMSO-d_6, 200 MHz) δ_C 167.3 (C-9), 154.4 (C-4), 140.5 (C-7), 133.7 (C-1), 132.4 (2C, C-2, C-6), 121.5 (C-8), 118.0 (2C, C-3, C-5) 60.6 (4-OCH$_3$) ; LRESIMS (*m/z*): 333/335/337 [M − H]$^-$.

Synthesis of Ianthelliformisamine D

(*E*)-3-(3,5-Dibromo-4-methoxyphenyl)acrylic acid (**10**, 49.0 mg, 0.15 mmol) and 1,1′-carbonyldiimadazole (24.0 mg, 0.15 mmol) was added to a reaction vial with dry CH$_3$CN (400 µL) and stirred at room temperature for 10 min. 1-(3-Aminopropyl)pyrrolidin-2-one (100 µL, 0.60 mmol) was then added dropwise and the resulting solution was stirred at room temperature for 3 h. The reaction crude was pre-adsorbed to C_8-bonded silica, packed into a stainless steel guard cartridge then purified by Luna semipreparative RP-HPLC using isocratic conditions of 50% H_2O (0.1% TFA)/50% MeOH (0.1% TFA) for the first 5 min, then a linear gradient to 100% MeOH (0.1% TFA) was run over 20 min and held at 100% MeOH (0.1% TFA) for a further 5 minutes at a flowrate of 4 mL/min; 30 fractions (30 × 1 min) were collected and resulted in the purification of synthetic Ianthelliformisamine D (**4**, 11.1 mg, t_R 21 min, 16% yield). The ^{1}H and ^{13}C NMR spectra of the synthetic compound matched that of the natural product.

Synthetic Ianthelliformisamine D (4): Clear film: ^{1}H NMR (DMSO-d_6, 800 MHz) δ_H 8.04 (t, *J* = 5.7 Hz, 1H, 9-NH), 7.88 (s, 2H, H-2, H-6), 7.33 (d, *J* = 15.7 Hz, 1H, H-7), 6.66 (d, *J* = 15.7 Hz, 1H, H-8), 3.81 (s, 3H, 4-OCH$_3$), 3.33 (t, *J* = 7.1 Hz, 2H, H-13), 3.20 (t, *J* = 7.2 Hz, 2H, H-12), 3.14 (dt, *J* = 5.7, 6.8 Hz, 2H, H-10), 2.21 (t, *J* = 8.1 Hz, 2H, H-15), 1.91 (tt, *J* = 7.1, 8.1 Hz, 2H, H-14), 1.63 (tt, *J* = 6.8, 7.2 Hz, 2H, H-11);

^{13}C NMR (DMSO-d_6, 200 MHz) δ_C 173.9 (C-16), 164.4 (C-9), 153.9 (C-4), 135.2 (C-7), 134.5 (C-1), 131.6 (2C, C-2, C-6), 124.5 (C-8), 118.0 (2C, C-3, C-5) 60.5 (4-OCH$_3$), 46.4 (C-13), 39.6 (C-12), 36.5 (C-10), 30.5 (C-15), 26.9 (C-11), 17.5 (C-14); LRESIMS (*m/z*): 459/461/463 [M + H]$^+$.

Bacterial strains and media

Wild-type *Pseudomonas aeruginosa* strain PAO1 (prototrophic wild-type) [25] was used in this study. Bacteria were grown in Luria–Bertani (LB) medium (10 g/L tryptone, 10 g/L sodium chloride and 5 g/L yeast extract).

Biofilm inhibition assay

The overnight cultures at 37 °C in LB medium were washed once with sterile saline 0.9% (w/v) and adjusted to an OD_{600} of 0.05. Then, 1% inoculum was transferred into fresh LB medium. Following the incubation at 37 °C, 200 rpm for 5–6 h to reach the mid-log phase, the cells were washed once with sterile saline 0.9% (w/v) and diluted to an OD_{600} of 0.01. Test compounds (15 µL) were loaded prior to the addition of bacteria (135 µL) into 96-well plates. The plates were incubated for 24 h at 37 °C in static conditions. The effects of compounds on bacterial growth and the viability of biofilm bacteria was determined by the OD_{600} and resazurin (RSZ) metabolic assay, respectively. The final concentration of DMSO in the assays was 1% (v/v). The negative controls consisted of inoculum and 1% DMSO. The antibiotic tobramycin (Sigma-Aldrich; 16 µg/mL) was used as a positive control. The initial OD_{600} and final OD_{600} were read before incubation at 37 °C and after 24 h of incubation, respectively, followed by the assessment of biofilm viability by resazurin metabolic assay. The experiments were carried out with three technical replicates and three biological replicates. The assay was performed as previously described [26]. The cultures were withdrawn, and the plates were washed twice with sterile water. Then, 50 µL of diluted RSZ solution (0.2% w/v) in LB medium was added into each well followed by incubation at 37 °C for 5 h. A microplate reader (iD5 Multi-Mode) was used to measure the fluorescence intensity (excitation 530 nm, emission 590 nm).

Supporting Information

Supporting Information File 1

NMR data tables for compounds **4–7**, 1D and 2D NMR spectra of compounds **4–7**, ^{1}H NMR spectra of natural products **1–3** and **8** and ^{1}H and ^{13}C NMR spectra of synthetic compounds **4, 9**, and **10**.
[https://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-20-266-S1.pdf]

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Data Availability Statement

Data generated and analyzed during this study is available from the corresponding author upon reasonable request.

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