Heterofibrins: inhibitors of lipid droplet formation from a deep-water southern Australian marine sponge, *Spongia* (*Heterofibria*) sp.†

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A bioassay-guided search for inhibitors of lipid droplet formation in a deep-water southern Australian marine sponge, *Spongia* (*Heterofibria*) sp., yielded six new compounds, fatty acids heterofibrins A1 (**1**) and B1 (**4**), along with related monolactyl and dilactyl esters, heterofibrins A2 (**2**), B2 (**5**), A3 (**3**) and B3 (**6**). Heterofibrin structures were assigned on the basis of detailed spectroscopic analysis, with comparison to chiral synthetic model compounds. All heterofibrins possess a diyne-ene moiety, while the monolactyl and dilactyl moiety featured in selected heterofibrins is unprecedented in the natural products literature. SAR by co-metabolite studies on the heterofibrins confirmed them to be non-cytotoxic, with the carboxylic acids **1** and **4** inhibiting lipid droplet formation in A431 fibroblast cell lines. Such inhibitors have potential application in the management of obesity, diabetes and atherosclerosis.

Introduction

Lipid droplets (LDs) are cytoplasmic organelles containing neutral lipid found in eukaryotic cells. In the past, LDs were thought to be inert and only act as a lipid storage depot. More recent data has revealed LDs as highly dynamic organelles that play a crucial role in maintaining the cellular levels of lipids and cholesterol by regulating the interplay between storage, hydrolysis and trafficking.[1,2] Malfunction in lipid regulation has been associated with a wide range of human diseases, including obesity, atherosclerosis, and type II diabetes. The identification of small bioactive molecules that can manipulate the level of LDs in cells would be invaluable to probe the mechanisms underlying LD formation as well as to develop potential therapeutic agents for the related diseases.

Two mouse macrophage cell-based assays (morphological and biochemical) of lipid droplet synthesis have been described.[3,4] The morphological assay involves microscopic evaluation of cytosolic lipid droplets, while the biochemical assay involves measuring incorporation of oleic acid into cholesteryl ester and triacylglycerols in lipid droplets. Japanese researchers have used these assays to screen about 20000 microbial samples, identifying a diverse array of lipid droplet inhibitors.[5] These inhibitors include the fungal beauveriolides (cyclodepsipeptides), isobisvertinol (hexahydrodibenzofuran), phenochalasin A (cytochalasan skeleton), sespendole (indolosesquiterpene), and spylidone (a tetracyclic containing a 2,4-pyrrolidinedione spiro ring).[5] They also include the actinomycete metabolites K97-0239A and B (cyclic lipopeptides).[5] Some of the above compounds (beauveriolides, spylidone, and sespendole) have been shown to inhibit the Acyl-CoA:cholesterol acyltransferase (ACAT) enzyme in macrophages.[5]

To the best of our knowledge, marine extracts have not been explored as a possible source of lipid droplet inhibitors. To pursue this opportunity we recently developed a semi-automated quantitative cell-based confocal microscopy assay to screen for the presence of lipid droplet inhibitors (Fig. 1), and used this to screen >2000 extracts derived from southern Australian and Antarctic marine invertebrates. One particularly promising extract detected during this survey was that of a deep-water specimen of *Spongia* (*Heterofibria*) sp., obtained in 2001 at a depth of 125 m during scientific trawling operations in the Great Australian Bight. This report describes the results of our investigation into this specimen.

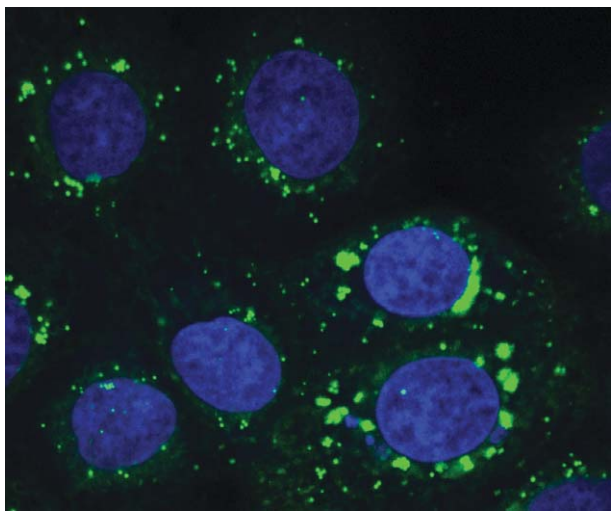

Fig. 1 A431 cells at 40× magnification with DAPI nuclei staining (blue) and Bodipy493/503 lipid droplets staining (green).

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Results and discussion

A portion of the aqueous EtOH extract of *Spongia* (*Heterofibria*) sp. was decanted, concentrated *in vacuo* and partitioned between *n*-BuOH and water. The *n*-BuOH portion was dried *in vacuo* and then sequentially triturated with hexane, DCM and MeOH, to yield a set of fractions that were subsequently screened for their ability to inhibit lipid droplet formation. These assays confirmed that inhibitory activity was localized in the hexane soluble fraction, which following reverse phase HPLC fractionation (Fig. 2) yielded two diyne-ene fatty acids, heterofibrins A1 (**1**) and B1 (**4**), together with two monolactyl and two dilactyl esters, heterofibrins A2/B2 (**2**/**5**) and A3/B3 (**3**/**6**) respectively (Fig. 3). The characterization, structure elucidation and lipid droplet inhibitory properties of the heterofibrins are described below.

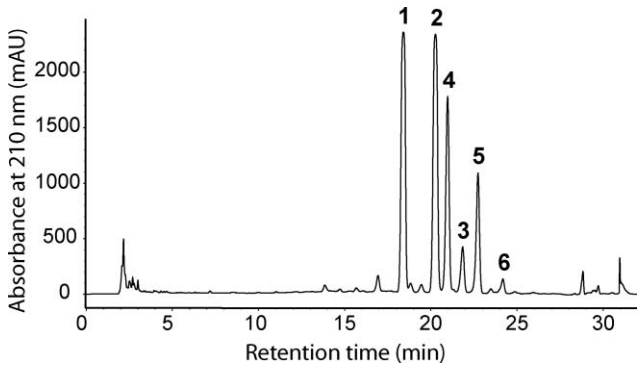

Fig. 2 HPLC trace (210 nm) of heterofibrins in the hexane fraction (Agilent Zorbax SB C_{18} 5 μm, 250 × 9.4 mm, 4 mL min^{-1}, 65–90% H_2O–MeCN over 25 min, isocratic 0.01% TFA modifier).

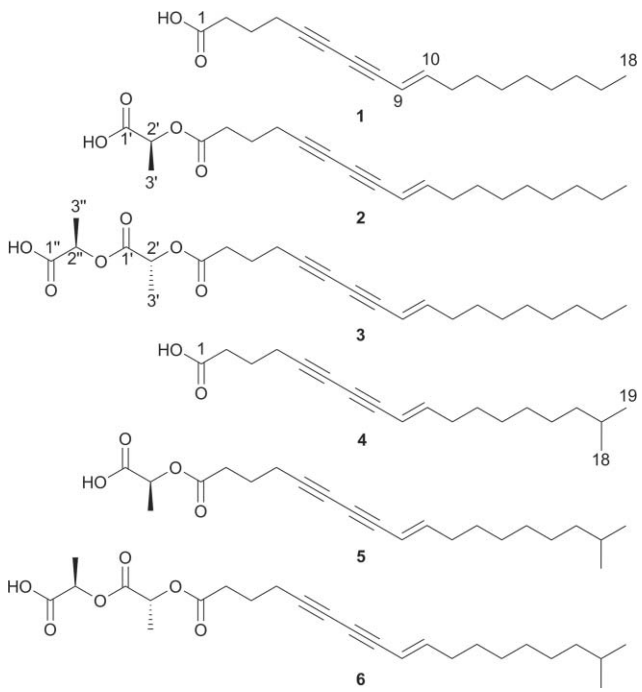

Fig. 3 Heterofibrins isolated from *Spongia* (*Heterofibria*) sp.

Heterofibrin A1 (**1**) was the major metabolite isolated from *Spongia* (*Heterofibria*) sp., and its HRESI(+)MS revealed a pseudomolecular ion $(M + Na)^+$ consistent with a molecular formula ($C_{18}H_{26}O_2$, Δmmu +0.8) requiring six double-bond equivalents (DBE). The UV spectrum of **1** was particularly diagnostic, with highly distinctive absorption bands (213, 230, 241, 254, 268, 284 nm) consistent with a diyne-ene moiety,[6,7] while the NMR ($CDCl_3$) data for **1** (Supplementary Information†) revealed a carboxylic acid (δ_C 177.8, C-1); these two functionalities together account for all six DBE. Further analysis of the NMR data confirmed the presence of two fully substituted acetylene moieties (δ_C 81.9, C-5; 66.5, C-6; 72.7, C-7; 74.8, C-8) and an *E* ($J_{9,10}$ 15.9 Hz) 1,2-disubstituted olefin (δ_H 5.47, H-9; 6.29, H-10; δ_C 108.6, H-9; 148.9, C-10), consistent with the proposed diyne-ene, as well as a primary methyl (δ_H 0.88, H_3-18; δ_C 14.3, C-18) and ten methylene carbons, suggestive of an acyclic unbranched fatty acid. Analysis of the 2D NMR data (Fig. 4) revealed a diagnostic correlation sequence from the carboxylic acid terminus (C-1) through H_2-2 (δ_H 2.51), H_2-3 (δ_H 1.87) and H_2-4 (δ_H 2.42) methylenes, to the four acetylenic carbons, and from there to the olefinic methines H-9 and H-10, and on through the H_2-11 (δ_H 2.11), H_2-12 (δ_H 1.38) and H_2-13 (δ_H 1.26) methylenes. These observations unambiguously positioned the diyne-ene moiety and established the structure for heterofibrin A1 (**1**) as (*E*)-9-octadecen-5,7-diynoic acid.

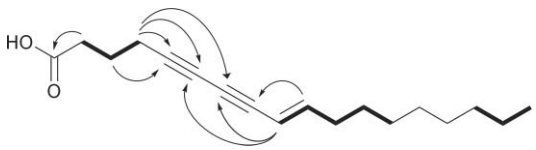

Fig. 4 ^1H–^{13}C HMBC (arrows) and ^1H–^1H COSY (bold) correlations for heterofibrin A1(**1**).

The HRESI(+)MS data for heterofibrin B1 (**4**) revealed a pseudomolecular ion $(M + Na)^+$ consistent with a molecular formula ($C_{19}H_{28}O_2$, Δmmu +1.2) indicative of a CH_2 higher homologue of **1**. Indeed, the NMR and UV data for **4** (see Supplementary Information†) were similar with that for **1**, with the only significant difference being conversion of the terminal primary methyl (C-18) in **1** into an isopropyl moiety (δ_H 1.51, tq, J = 6.7 and 6.7 Hz, H-17; 0.86, d, J = 6.7 Hz, H_3-18 and H_3-19; δ_C 28.2, C-17; 22.9, C-18 and C-19) in **4**. Thus the structure of heterofibrin B1 (**4**) can be assigned as (*E*)-17-methyl-9-octadecen-5,7-diynoic acid.

The HRESI(+)MS data for the chiral co-metabolite heterofibrin A2 (**2**) revealed a pseudomolecular ion $(M + Na)^+$ consistent with a molecular formula ($C_{21}H_{30}O_4$, Δmmu +1.1) indicative of a $C_3H_4O_2$ adduct of heterofibrin A1 (**1**). The NMR and UV data for **2** (see Supplementary Information†) were very similar to **1**, with the principal difference being a shielding of C-1 (Δ −5.2 ppm) consistent with conversion of the carboxylic acid terminus to an ester, the appearance of resonances for a lactyl ester (δ_H 5.14, H-2; 1.54, H_3-3′; δ_C 173.6, C-1′; 68.7, C-2′; 17.0, C-3′), and imposition of a diastereotopic character on H_2-2′ (δ_H 2.56, m; 2.51, m) due to proximity to the chiral centre C-2′. Full analysis of the 2D NMR data for **2** (see Fig. 5 and Supplementary Information†) confirmed these structure assignments. A 2′*S* absolute configuration was assigned to heterofibrin A2 on the basis of a comparison of the optical rotation for **2** ($[\alpha]_D$ −9.2) with that of the synthetic model compound (2′*S*)-lactyl linoleoate (**7a**) ($[\alpha]_D$ of −13) (Fig. 6),

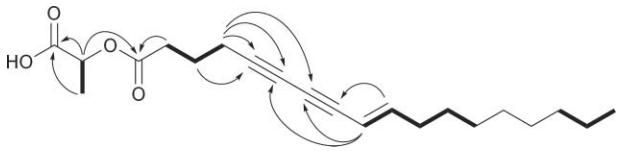

Fig. 5 ^{1}H–^{13}C HMBC (arrows) and ^{1}H–^{1}H COSY (bold) correlations for heterofibrin A2 (**2**).

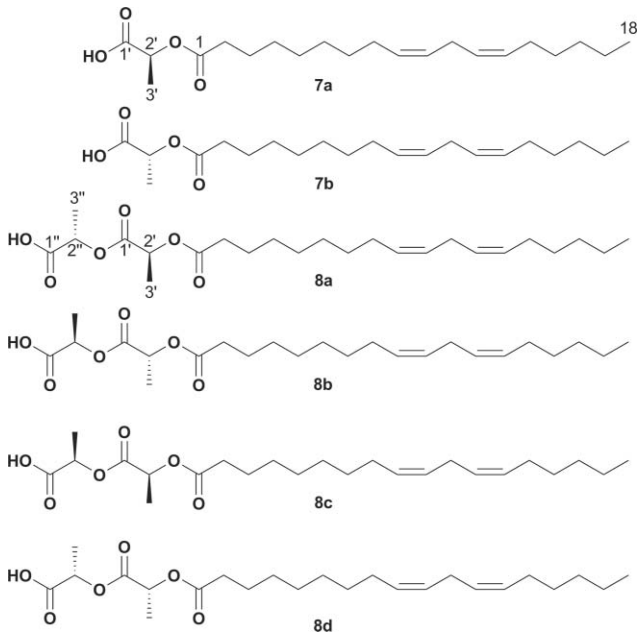

Fig. 6 Synthetic model compounds.

prepared from esterification of linoleoyl chloride with (*S*)-lactic acid. The synthetic compound (2′*R*)-lactyl linoleoate (**7b**) ([α]$_D$ of +14) was also prepared from the corresponding (*R*)-lactic acid. Thus the structure of heterofibrin A2 (**2**) can be assigned as (2′*S*)-lactyl (*E*)-9-octadecen-5,7-diynoate.

The HRESI(+)MS data for the chiral co-metabolite heterofibrin B2 (**5**) displayed a pseudomolecular ion (M + Na)$^+$ consistent with a molecular formula ($C_{22}H_{32}O_4$, Δmmu +1.1) indicative of a $C_3H_4O_2$ adduct of heterofibrin B1 (**4**). As with the comparison between the acid **1** and the lactyl ester **2**, the NMR and UV data for **5** (see Supplementary Information†) were very similar to **4**, with the principal difference being a shielding of C-1 (Δ –2.6 ppm) consistent with conversion of the carboxylic acid terminus to an ester and the appearance of resonances for a lactyl ester (δ_H 5.13, H-2′; 1.54, H$_3$-3′; δ_C 174.0, C-1′; 68.3, C-2′; 17.0, C-3′). A 2′*S* absolute configuration was assigned to **5** on the basis of a comparison of its optical rotation ([α]$_D$ –10) with that of the co-metabolite **2** and the synthetic model compound **7a** (see above). Thus the structure of heterofibrin B2 (**5**) can be assigned as (2′*S*)-lactyl (*E*)-17-methyl-9-octadecen-5,7-diynoate.

Two very minor co-metabolite heterofibrins were also isolated, each as an inseparable mixture with an achiral saturated fatty acid. Repeated HPLC attempts to purify heterofibrins A3 (**3**) and B3 (**6**) proved unsuccessful. Heterofibrin A3 (**3**) was isolated as a mixture with 13-methylmyristic acid, while heterofibrin B3 (**6**) was isolated as a mixture with palmitic acid. The ratios and the structure elucidation of each heterofibrin and associated fatty acid were confirmed by spectroscopic analysis and comparisons with model compounds, as detailed below.

HRESI(–)MS and NMR analysis identified 13-methylmyristic acid as the 4 : 1 (mole ratio) co-eluant with heterofibrin A3 (**3**), confirmed by comparison with an authentic sample (Supplementary Information†). The HRESI(+)MS data for heterofibrin A3 (**3**) revealed a pseudomolecular ion (M + Na)$^+$ consistent with a molecular formula ($C_{24}H_{34}O_6$, Δmmu –0.2) indicative of a $C_3H_4O_2$ (lactyl) adduct of heterofibrin A2 (**2**). The NMR and UV data for **3** (see Supplementary Information†) were very similar to **2**, with the principal difference being the appearance of resonances for a second lactyl ester residue (δ_H 5.20, H-2″; 1.56, H$_3$-3″; δ_C 173.6, C-1″; 68.6, C-2″; 16.9, C-3′). Thus heterofibrin A3 (**3**) was assigned as the lactyl ester adduct of heterofibrin A2 (**2**). Full analysis of the 2D NMR data for **3** (see Fig. 7 and Supplementary Information†) confirmed these structure assignments.

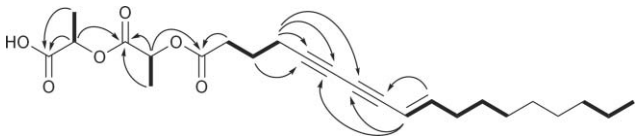

Fig. 7 ^{1}H–^{13}C HMBC (arrows) and ^{1}H–^{1}H COSY (bold) correlations for heterofibrin A3 (**3**).

To establish the absolute configuration we compared the chiroptical data for **3** (measured [α]$_D$ +11, corrected for 70% mass content of 13-methylmyristic acid [α]$_D$ +37) with four synthetic model compounds; (2′*S*,2′*S*)-dilactyl linoleoate (**8a**) ([α]$_D$ –33), (2′*R*,2″*R*)-dilactyl linoleoate (**8b**) ([α]$_D$ +34), (2′*S*,2″*R*)-dilactyl linoleoate (**8c**) ([α]$_D$ –4.8), and (2′*R*,2″*S*)-dilactyl linoleoate (**8d**) ([α]$_D$ +4.2). The model compounds **8a–8d** (Fig. 6) were prepared by esterification of the acyl chloride derived from the mono (*S*)-lactyl ester **7a** and mono (*R*)-lactyl ester **7b** with (*S*)- or (*R*)-lactic acid respectively. These comparisons clearly discount the 2′*S*,2′*S* (**8a**) and 2′*S*,2′*R* (**8c**) configurations, due to their negative optical rotations, and require that **3** possess either a 2′*R*,2′*R* (**8b**) or 2′*R*,2′*S* (**8d**) configuration (with the former being favored due to the magnitude of the rotation). Fortunately the ^{1}H NMR spectra of the synthetic diastereomeric dilactyl linoleoates 2′*R*,2′*R* (**8b**) and 2′*R*,2′*S* (**8d**) provide diagnostic shift differences (Fig. 8 and Supplementary Information†) that unambiguously resolve this stereochemical assignment. More specifically, comparison of the dilactyl resonances in **3** (δ_H 5.20, q, H-2″; 5.13, q, H-2′; 1.57, d, H$_3$-3″; 1.56, d, H$_3$-3′) with **8b** (δ_H 5.20, q, H-2″; 5.11, q, H-2′; 1.57, d, H$_3$-3″; 1.55, d, H$_3$-3′) and **8d** (δ_H 5.16, q, H-2″; 5.14, q, H-2′; 1.53, d, H$_3$-3″; 1.52, d, H$_3$-3′) required that **3** possess the 2′*R*,2′*R* configuration (as predicted from the optical rotations). Thus the structure for heterofibrin A3 (**3**) can be assigned as (2′*R*,2′*R*)-dilactyl (*E*)-9-octadecen-5,7-diynoate.

HRESI(–)MS and NMR analysis identified palmitic acid as the 1 : 1 (mole ratio) co-eluant with heterofibrin B3 (**6**), confirmed by comparison with an authentic sample (Supplementary Information†). The HRESI(+)MS data for **6** revealed a pseudomolecular ion (M + Na)$^+$ consistent with a molecular formula ($C_{25}H_{36}O_6$, Δmmu –1.1) indicative of a $C_3H_4O_2$ (lactyl) adduct of heterofibrin B2 (**5**) and a CH_2 higher homologue of heterofibrin A3 (**3**). Analysis of the NMR ($CDCl_3$) data (see Supplementary Information†) suggested that heterofibrin B3 (**6**) was dilactyl

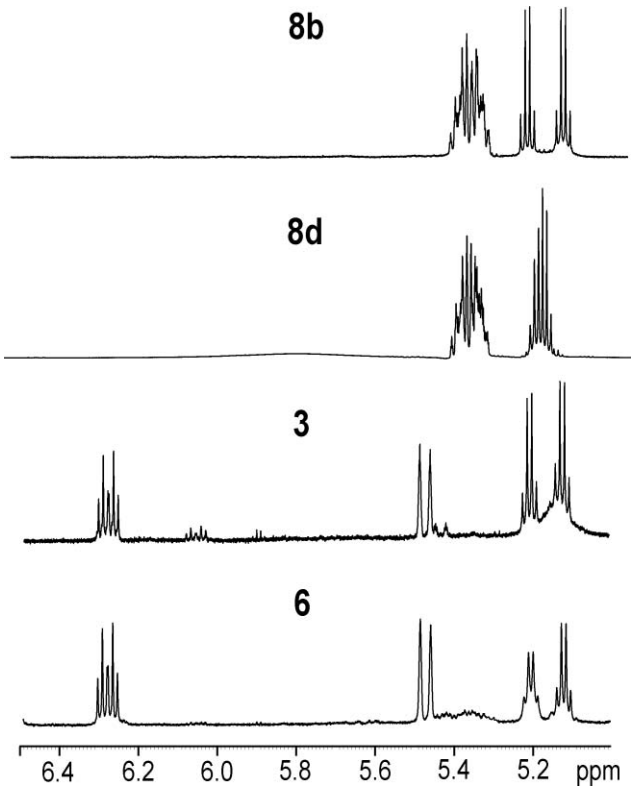

Fig. 8 ^{1}H NMR ($CDCl_3$, 600 MHz) spectral comparison of synthetic diastereomeric dilactyl linoleoate model compounds (**8b** and **8d**) with the natural dilactyl esters, heterofibrins A3 (**3**) and B3 (**6**).

(*E*)-17-methyl-9-octadecen-5,7-diynoate. The chiroptical properties for **6** (measured $[\alpha]_D$ +16, corrected for 40% mass content of palmitic acid $[\alpha]_D$ +27) together with diagnostic ^{1}H NMR shifts **6** (δ_H 5.21, H-2″; 5.12, H-2′; 1.57, H_3-3″; 1.56, H_3-3′) (see Fig. 8 and Supplementary Information†) were supportive of a 2′*R*,2″*R* configuration. Thus the structure for heterofibrin B3 (**6**) can be assigned as (2′*R*,2′*R*)-dilactyl (*E*)-17-methyl-9-octadecen-5,7-diynoate.

The heterofibrins incorporate a number of unusual structural features. Firstly, conjugated diyne-ene lipids are uncommon among marine metabolites, with examples possessing an *E* configuration having been reported from sponges of the families Axinellidae[7] and Petrosiidae,[8–12] and examples with a *Z* configuration being described from Callispongiidae,[13–16] Chalinidae[17] and Petrosiidae.[10,12,18] Diyne-ene lipids are more common in the terrestrial natural products literature, with exocarpic acid [(*E*)-13-octadecen-9,11-diynoic acid] isolated from several plants of the family Santalaceae and Olacaceae,[19] being a regioisomer of heterofibrin A1 (**1**). While we are unaware of any accounts attributing biological activity to exocarpic acid, it is noteworthy (in the context of our study) that the related plant metabolite ximenynic or santalbic acid [(*E*)-11-octadecen-9-ynoic acid] has been reported to inhibit lipoxygenase and prostaglandin synthetase,[20] induce cytochrome P450,[21] and modify the fatty acid composition of mouse adipose tissue.[22] Secondly, of particular structural novelty is the natural occurrence of the lactyl ester moiety. While synthetic monolactyl fatty acid esters (stearoyl-2-lactylates) are known as emulsifying agents in bakery products,[23] **2** and **5** represent the first reported account of monolactyl lipid esters from natural sources. Even more intriguing, the dilactyl ester moieties found in **3** and **6** are unprecedented in both the synthetic and natural products literature. We also note an unexpected stereochemical dimension to heterofibrin biosynthesis, with the monolactyl esters possessing a 2′*S* configuration and the co-metabolite dilactyl esters possessing a 2′*R*,2′*R* configuration. This observation suggests that the dilactyl esters **3** and **6** are not simply extended esters of the monolactyl esters **2** and **5**.

On subjecting the co-metabolites **1–6** to the high content LD assay, only the carboxylic acid heterofibrins A1 (**1**) and B1 (**4**) inhibited LD formation in A431 fibroblast cells (up to 60% at 10 μM). Given possible interest in heterofibrins as probes to explore the mechanisms underlying LD formation, and as potential leads for new therapeutic agents, we were keen to establish a cytotoxicity profile. The co-metabolites **1–6** were not cytotoxic to the A431 fibroblast cells in the LD bioassay, or mammalian HeLa (cervical) and MDA-MB-231 (mammary epithelium) cancer cell lines (up to 30 μM). Likewise, **1–6** were not cytotoxic to *Candida albicans*, *Pseudomonas aeruginosa* or *Escherichia coli* (IC_{90} values >50 μM). The heterofibrins **1–6** did display weak antibacterial activity against the Gram positive bacterium *Bacillus subtilis* ($10 < IC_{90} < 60$ μM), while **1** and **4** displayed weak activity against *Staphylococcus aureus* ($4 < IC_{90} < 45$ μM) – possibly indicative of a chemical ecology role as antibacterials. The biological activities of **1–6** are summarized in Table 1.

Experimental

General experimental procedure

Chiroptical measurements were measured on a Jasco P-1010 polarimeter with a 10 cm quartz cell. UV-Vis spectra were measured on a Cary 50 spectrophotometer in a 1 cm quartz cell. NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer. Chemical shifts are reported in ppm using residual solvent signal as an internal reference. Electrospray ionization mass spectra (ESIMS) were acquired using an Agilent 1100 series separations module equipped with an Agilent 1100 Series LC/MSD mass detector in both +ve and −ve ion modes. High resolution (HR) ESIMS measurements were obtained on a Bruker micrOTOF high resolution mass spectrometer equipped with an ESI probe by direct infusion in MeCN at 3 μL min^{-1} using sodium formate clusters as an internal calibrant. HPLC was performed using an Agilent 1100 series separations module equipped with Agilent 1100 series diode array detector, and Agilent 1100 series fraction collector, controlled using ChemStation Rev.9.03A and Purify version A.1.2 software.

Animal material

The sponge sample (UQ code: CMB-03399) was collected in 2001 at a depth of 125 m during scientific trawling operations in the Great Australian Bight (33°12′ S, 128°15′ E). The freshly collected sponge sample was frozen (–4 °C) for shipping to the laboratory, where it was thawed, catalogued, diced and steeped in aqueous EtOH at –20 °C for prolonged storage. The specimen was identified as Class: Demospongiae, Order: Dictyoceratida, Family: Spongiidae, Genus: *Spongia* (*Heterofibria*) and a voucher sample was deposited with Museum Victoria (Reg No. MVF166231). A

Table 1 Biological activities of heterofibrins (**1–6**)[a]

Compound	LD inhibition at 10 μM (%)	Cytotoxicity at 30 μM			Antimicrobial IC_{90}/μM				
	A431	A431	HeLa	MDA-MB-231	*B. subtilis*	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
1	52	—	—	—	22	45	—	—	—
2	0	—	—	—	26	—	—	—	—
3	14	—	—	—	29	—	—	—	—
4	60	—	—	—	10	21	—	—	—
5	24	—	—	—	17	—	—	—	—
6	24	—	—	—	27	—	—	—	—

[a] Dash (—) indicates that the compound does not show any activity.

description of the specimen is provided in the Supplementary Information.†

Extraction and isolation

The aqueous EtOH extract was decanted and concentrated *in vacuo* (1.9 g) and then partitioned into *n*-BuOH (223 mg) and water (1.78 g) soluble materials. The *n*-BuOH partition was triturated into hexane (212 mg), DCM (1.3 mg) and H_2O (9.5 mg) soluble fractions, and a portion of the hexane solubles (90 mg) was subjected to HPLC fractionation (Agilent Zorbax SB C_{18} 5 μm, 250 × 9.4 mm column, 4 mL min^{-1} gradient elution from 65% to 90% H_2O–MeCN over 25 min, with isocratic 0.01% TFA modifier) to yield in order of elution heterofibrin A1 (**1**) (10 mg, t_R 18.2 min, 4.5%), heterofibrin A2 (**2**) (11 mg, t_R 20.0 min, impure), heterofibrin B1 (**4**) (4.6 mg, t_R 20.9 min, 2.1%), a 4 : 1 mixture of 13-methylmyristic acid and heterofibrin A3 (**3**) (3.1 mg, t_R 21.7 min, 1.4%), heterofibrin B2 (**5**) (4.1 mg, t_R 22.6 min, impure), and a 1 : 1 mixture of palmitic acid and heterofibrin B3 (**6**) (1.5 mg, t_R 24.0 min, 0.7%). Heterofibrin A2 (**2**) and B2 (**5**) were further purified by semi-prep HPLC (Agilent Zorbax SB C_3 5 μm, 250 × 9.4 mm column, 4 mL min^{-1} gradient elution from 65% to 80% H_2O–MeCN over 15 min, with isocratic 0.01% TFA modifier) to yield pure heterofibrins A2 (**2**) (4 mg, 1.8%) and B2 (**5**) (2 mg, 0.9%). [Note: % yields are expressed as a weight-to-weight estimate of the composition of the *n*-BuOH solubles].

Heterofibrin A1.(1). A pale yellow oil. UV (MeOH) λ_{max} (log ε) 230 (3.44), 241 (3.73), 254 (4.02), 268 (4.18), 284 (4.08) nm; NMR ($CDCl_3$, 600 MHz) data see Supplementary Information;† ESI(+)MS *m/z* 275 $[M + H]^+$; ESI(–)MS *m/z* 273 $[M - H]^-$; HRESI(+)MS *m/z* 297.1817 $[M + Na]^+$ (calc. for $C_{18}H_{26}O_2Na^+$, 297.1825).

Heterofibrin A2 (2). A pale yellow oil. $[\alpha]_D^{20}$ –9.2 (*c* 0.25, $CHCl_3$), UV (MeOH) λ_{max} (log ε) 230 (3.37), 241 (3.48), 254 (3.67), 268 (3.79), 284 (3.69) nm; NMR ($CDCl_3$, 600 MHz) data see Supplementary Information;† ESI(+)MS *m/z* 347 $[M + H]^+$; ESI(–)MS *m/z* 345 $[M - H]^-$; HRESI(+)MS *m/z* 369.2025 $[M + Na]^+$ (calc. for $C_{21}H_{30}O_4Na^+$, 369.2036).

Heterofibrin A3 (3). A pale yellow oil. $[\alpha]_D^{20}$ +11 (*c* 0.05, $CHCl_3$, measured on a sample comprising 70% (mass content) 13-methylmyristic acid); NMR ($CDCl_3$, 600 MHz) data see Supplementary Information;† ESI(+)MS *m/z* 419 $[M + H]^+$; ESI(–)MS *m/z* 417 $[M - H]^-$; HRESI(+)MS *m/z* 441.2250 $[M + Na]^+$ (calc. for $C_{24}H_{34}O_6Na^+$, 441.2248).

13-Methylmyristic acid. Analyzed as a mixture with **3**. NMR ($CDCl_3$, 600 MHz) δ_H 2.35 (t, J = 7.5 Hz), 1.63 (tt, J = 7.5, 7.5 Hz), 1.51 (tq, J = 6.6, 6.6 Hz), 1.26 (methylenes), 1.15 (dt, J = 6.6, 6.6 Hz), 0.86 (d, J = 6.6 Hz); HRESI(–)MS *m/z* 241.2168 $[M - H]^-$ (calc. for $C_{15}H_{29}O_2$, 241.2171). Authentic standard see Supplementary Information.†

Heterofibrin B1 (4). A pale yellow oil. UV (MeOH) λ_{max} (log ε) 230 (3.36), 241 (3.62), 254 (3.88), 268 (4.03), 284 (3.93) nm; NMR ($CDCl_3$, 600 MHz) data see Supplementary Information;† ESI(+)MS *m/z* 289 $[M + H]^+$; ESI(–)MS *m/z* 287 $[M - H]^-$; HRESI(+)MS *m/z* 311.1970 $[M + Na]^+$ (calc. for $C_{19}H_{28}O_2Na^+$, 311.1982).

Heterofibrin B2 (5). A pale yellow oil. $[\alpha]_D^{20}$ –10 (*c* 0.13, $CHCl_3$); NMR ($CDCl_3$, 600 MHz) data see Supplementary Information;† ESI(+)MS *m/z* 361 $[M + H]^+$; ESI(–)MS *m/z* 359 $[M - H]^-$; HRESI(+)MS *m/z* 383.2182 $[M + Na]^+$ (calc. for $C_{22}H_{32}O_4Na^+$, 383.2193).

Heterofibrin B3 (3). A pale yellow oil. $[\alpha]_D^{20}$ +16 (*c* 0.13, $CHCl_3$, measured on a sample comprising 40% (mass content) palmitic acid); NMR ($CDCl_3$, 600 MHz) data see Supplementary Information;† ESI(+)MS *m/z* 433 $[M + H]^+$; ESI(–)MS *m/z* 431 $[M - H]^-$; HRESI(+)MS *m/z* 455.2415 $[M + Na]^+$ (calc. for $C_{25}H_{36}O_6Na^+$, 455.2404).

Palmitic acid. Analyzed as a mixture with **6**. ^1H NMR ($CDCl_3$, 600 MHz) δ_H 2.35 (t, J = 7.4 Hz), 1.64 (tt, J = 7.4, 7.4 Hz), 1.26 (methylenes), 0.88 (t, J = 6.6 Hz); HRESI(–)MS *m/z* 255.2330 $[M - H]^-$ (calc. for $C_{16}H_{31}O_2$, 255.2330). Authentic standard see Supplementary Information.†

Synthesis and identification of model compounds

2′*S*-Lactyl linoleoate (7a). Oxalyl chloride (7.3 g, 0.060 mol) was added dropwise into linoleic acid (8.3 g, 0.030 mol) under N_2 and the mixture was refluxed (65–70 °C) for 4 h, after which excess oxalyl chloride was distilled off. The resulting linoleoyl chloride was cooled to room temperature, added into *S*-lactic acid (2.7 g, 0.030 mol) in dry pyridine (10 mL) under N_2 and stirred at room temperature for 1 h before being filtered and dried *in vacuo* to yield a crude product (10 g). A portion of the crude product (5.0 g) was used to synthesize (2′*S*,2′*S*)-dilactyl linoleoate without further purification. A small portion (60 mg) was purified by HPLC (Agilent Zorbax SB C_3 5 μm, 250 × 9.4 mm column, 4 mL min^{-1} gradient elution from 60% to 90% H_2O–MeCN over 15 min, with isocratic 0.01% TFA modifier) to give (2′*S*)-lactyl

linoleoate (**7a**) (29 mg, 48%) as a colourless oil ($[\alpha]_D^{20}$ −13, *c* 0.78, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.38 (2H, m, H-10 and H-12), 5.33 (2H, m, H-9 and H-13), 5.10 (1H, q, J = 7.0, H-2′), 2.77 (2H, dd, J = 6.9, 6.9 Hz, H_2-11), 2.40 (1H, m, H-2a), 2.35 (1H, m, H-2b), 2.04 (4H, dt, J = 7.1, 7.1 Hz, H_2-8 and H_2-14), 1.65 (2H, tt, J = 7.5, 7.5 Hz, H_2-3), 1.52 (3H, d, J = 7.0 Hz, H_3-3′), 1.38–1.24 (14 H, m), 0.89 (3H, t, J = 6.9 Hz, H_3-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.3 (C-1′), 173.5 (C-1), 130.4 (C-13), 130.2 (C-9), 128.2 (C-10), 128.1 (C-12), 68.4 (C-2′), 34.1 (C-2), 31.7 (C-16), 29.8-29.2 (methylenes), 27.4 (2C, C-8 and C-14), 25.8 (C-11), 24.9 (C-3), 22.8 (C-17), 17.1 (C-3′), 14.3 (C-18); HRESI(+)MS m/z 375.2502 $[M + Na]^+$ (calc. for $C_{21}H_{36}O_4Na^+$, 375.2506).

2′*R*-Lactyl linoleoate (7b). Oxalyl chloride (17 μL, 0.21 mmol) was added into linoleic acid (60 mg, 0.21 mmol) under N_2 and the mixture was refluxed (65–70 °C) for 4 h. The resulting linoleoyl chloride was cooled to room temperature, added into *R*-lactic acid (15 mg, 0.16 mol) in dry pyridine (0.5 mL) under N_2 and stirred at room temperature overnight before being dried under N_2 at 40 °C to yield a crude product (60 mg), a portion of which was used to synthesize (2′*R*,2′*R*)-dilactyl linoleoate without further purification. 30 mg or the crude product was purified by the same HPLC method as above to give (2*R*)-lactyl linoleoate (**7b**) (11.9 mg, 42%) as a colourless oil ($[\alpha]_D^{20}$ +14, *c* 0.60, $CHCl_3$); NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see Supplementary Information† (the values are identical to those of compound (**7a**); HRESI(+)MS m/z 375.2506 $[M + Na]^+$ (calc. for $C_{21}H_{36}O_4Na^+$, 375.2506).

(2′*S*,2′*S*)-Dilactyl linoleoate (8a). Compound **8a** was synthesized following the same procedure as the synthesis of compound **7a**, using oxalyl chloride (3.4 g, 0.028 mol), crude (2′*S*)-lactyl linoleoate (**7a**) (4.9 g, 0.014 mol), and *S*-lactic acid (1.3 g, 0.014 mol). A portion (100 mg) of the crude product was purified by HPLC as above to yield unreacted (2′*S*)-lactyl linoleoate (**7a**) (12.0 mg, 12%) and (2′*S*,2″*S*)-dilactyl linoleoate (**8a**) (23.0 mg, 23%) as a colourless oil ($[\alpha]_D^{20}$ −33, *c* 0.40, $CHCl_3$); NMR (600 MHz, $CDCl_3$) δ 5.38 (2H, m, H-10 and H-12), 5.33 (2H, m, H-9 and H-13), 5.20 (1H, q, J = 7.0 Hz, H-2″), 5.11 (1H, q, J = 7.0 Hz, H-2′), 2.77 (2H, t, J = 6.9 Hz, H_2-11), 2.40 (1H, m, H-2a), 2.35 (1H, m, H-2b), 2.04 (4H, dt, J = 7.1, 7.1 Hz, H_2-8 and H_2-14), 1.65 (2H, tt, J = 7.5, 7.5 Hz, H_2-3), 1.57 (3H, d, J = 7.0 Hz, H_3-3″), 1.55 (3H, d, J = 7.0 Hz, H_3-3′), 1.38–1.24 (14 H, m), 0.89 (3H, t, J = 6.9 Hz, H_3-18);^{13}C NMR (150 MHz, $CDCl_3$) δ 173.7 (C-1″), 173.6 (C-1), 170.6 (C-1′), 130.4 (C-13), 130.3 (C-9), 128.2 (C-10), 128.1 (C-12), 68.7 (C-2″), 68.4 (C-2′), 34.1 (C-2), 31.7 (C-16), 29.8-29.2 (methylenes), 27.4 (2C, C-8 and C-14), 25.8 (C-11), 24.9 (C-3), 22.8 (C-17), 16.9 (2C, C-3′ and C-3″), 14.3 (C-18); HRESI(+)MS m/z 447.2724 $[M + Na]^+$ (calc. for $C_{24}H_{40}O_6Na^+$, 447.2717).

(2′*R*,2″*R*)-Dilactyl linoleoate (8b). Compound **8b** was synthesized following the same procedure as the synthesis of compound **7b**, using oxalyl chloride (6.6 μL, 0.080 mmol), crude (2′*R*)-lactyl linoleoate (**7b**) (28 mg, 0.080 mmol), and *R*-lactic acid (9.0 mg, 0.080 mmol). The crude product was purified by HPLC using the same method as above to yield unreacted (2′*R*)-lactyl linoleoate (**7b**) (17.7 mg) and (2′*R*,2′*R*)-dilactyl linoleoate (**8b**) (1.7 mg, 5.0%) as a colourless oil ($[\alpha]_D^{20}$ +34, *c* 0.085, $CHCl_3$); NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see Supplementary Information† (the values are identical to those of compound **8a**); HRESI(+)MS m/z 447.2715 $[M + Na]^+$ (calc. for $C_{24}H_{40}O_6Na^+$, 447.2717).

(2′*S*,2″*R*)-Dilactyl linoleoate (8c). Compound **8c** was synthesized following the same procedure as the synthesis of compound **7b**, using oxalyl chloride (7 μL, 0.09 mmol), pure (2′*S*)-lactyl linoleoate (**7a**) (30 mg, 0.09 mmol), and *R*-lactic acid (7.6 mg, 0.09 mmol). The crude product was purified by HPLC using the same method as above to yield unreacted (2′*S*)-lactyl linoleoate (**7a**) (15.6 mg) and (2′*S*,2″*R*)-dilactyl linoleoate (**8c**) (11 mg, 22%) as a colourless oil ($[\alpha]_D^{20}$ −4.8, *c* 0.55, $CHCl_3$); NMR (600 MHz, $CDCl_3$) δ 5.38 (2H, m, H-10 and H-12), 5.32 (2H, m, H-9 and H-13), 5.16 (1H, m, H-2″), 5.14 (1H, m, H-2′), 2.77 (2H, t, J = 6.9 Hz, H_2-11), 2.40 (1H, m, H-2a), 2.35 (1H, m, H-2b), 2.04 (4H, dt, J = 7.1, 7.1 Hz, H_2-8 and H_2-14), 1.64 (2H, tt, J = 7.5, 7.5 Hz, H_2-3), 1.53 (3H, d, J = 7.0 Hz, H_3-3″), 1.52 (3H, d, J = 7.0 Hz, H_3-3′), 1.38-1.24 (14 H, m), 0.89 (3H, t, J = 6.9 Hz, H_3-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ 175.0 (C-1″), 173.5 C-1), 170.4 (C-1′), 130.4 (C-13), 130.3 (C-9), 128.2 (C-10), 128.1 (C-12), 69.0 (C-2″), 68.6 (C-2′), 34.1 (C-2), 31.7 (C-16), 29.8-29.2 (methylenes), 27.4 (2C, C-8 and C-14), 25.8 (C-11), 24.9 (C-3), 22.8 (C-17), 17.0 (C-3′ or C-3″), 16.9 (C-3′ or C-3″), 14.3 (C-18); HRESI(+)MS m/z 447.2717 $[M + Na]^+$ (calc. for $C_{24}H_{40}O_6Na^+$, 447.2717).

(2′*R*,2″*S*)-Dilactyl linoleoate (8d). Compound **8d** was synthesized following the same procedure as the synthesis of compound **7b**, using oxalyl chloride (6 μL, 0.08 mmol), pure (2′*R*)-lactyl linoleoate (**7b**) (27 mg, 0.08 mmol), and *S*-lactic acid (7.2 mg, 0.08 mmol). The crude product was purified by HPLC using the same method as above to yield unreacted (2′*R*)-lactyl linoleoate ester (**7b**) (15 mg) and (2′*R*,2″*S*)-dilactyl linoleoate ester (**8d**) (8 mg, 15%) as a colourless oil ($[\alpha]_D^{20}$ +4.2, *c* 0.40, $CHCl_3$); NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see Supplementary information† (the values are identical to those of compound **8c**); HRESI(+)MS m/z 447.2714 $[M + Na]^+$ (calc. for $C_{24}H_{40}O_6Na^+$, 447.2717).

Bioassays

Lipid droplet assay. A431 cells were plated onto 96 well imaging plates (BD Falcon) in Dulbecco's modified Eagles high glucose medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, penicillin (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}) and allowed to adhere. Cells were subsequently incubated overnight with compounds to be tested at 50 μg mL^{-1} (extracts) or 10–20 μM (heterofibrins) prior to supplementation with 50 μM oleic acid for 8 h. Following fixation cells were labelled using Bodipy493/503 (Invitrogen, Australia) and DAPI (Sigma, USA) to label LDs and nuclei respectively. Cells were imaged using a BD Pathway 855 microscope (BD Biosciences) running Attovision 1.5/1.6 software. The average number of LDs per cell was quantified using CellProfiler© software.[24] Details of the development, optimisation and performance of the lipid droplet assay will be published elsewhere.

MTT cytotoxicity assay. The following protocol was adapted from published method.[25] Cells [HeLa (ATCC-CCL2) and MDA-MB-231 (ATCC HTB-26)] were seeded in a 96-well microtitre plate at 2×10^5 cells mL^{-1} in 100 μL of DMEM supplemented

with 10% FBS, and the plate incubated for 3–5 h (37 °C; 5% CO_2). Compounds to be tested were dissolved in DMSO (3 mM) and diluted with H_2O to 300 μM. Aliquots (10 μL) of each dilution (or of 10% aqueous DMSO for control wells) were added to the assay plate in duplicate. The assay plate was incubated for 24 h (37 °C; 5% CO_2) after which a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) in PBS was added to each well to a final concentration of 0.5 mg mL^{-1} and the plate incubated for a further 2 h (37 °C; 5% CO_2). The medium was then carefully aspirated from each well and precipitated formazan crystals were dissolved in DMSO (100 μL), and the absorbance of each well measured spectrophotometrically at 600 nm.

Antimicrobial testing. The microorganism to be tested was streaked onto a Tryptic Soy Agar plate and incubated at 37 °C for 24 h [*Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 9144) or *Bacillus subtilis* (ATCC 6051)] or 48 h [*Candida albicans* (ATCC 10231)]. One colony was then transferred to fresh Tryptic Soy broth (15 mL) and the cell density adjusted to 10^4–10^5 cfu mL^{-1}. A solution of the test compound in 10% aqueous DMSO (333 μg mL^{-1}) was serially diluted to 0.130 μg mL^{-1} with 1% aqueous DMSO. An aliquot of each dilution (20 μL) was added to a 96-well microtitre plate and freshly prepared microbial broth (180 μL) was added to each well. The plate was incubated at 37 °C for 24 h and the optical density of each well was measured spectrophotometrically at 600 nm.

Conclusions

The heterofibrins represent new marine natural products incorporating both rare and unprecedented structural features. Full chemical structures have been assigned using a combination of spectroscopic analysis and chemical synthesis. The heterofibrins display no cytotoxicity in a variety of bioassays, with the carboxylic acid heterofibrins A1 (**1**) and B1 (**4**) being solely responsible for the LD inhibitory activity detected in the *Spongia* (*Heterofibria*) sp. extract. Access to related diyne-ene fatty acids from various plant species, as well as anticipated ease of access to this achiral pharmacophore through total synthesis, makes the pharmacophore an attractive target for further SAR investigation. A detailed account of the high content LD assay, and the *in vitro* and *in vivo* LD inhibitory pharmacology of the heterofibrins, will be reported elsewhere.[26]

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