

# Isolation and structural characterisation of two antibacterial free fatty acids from the marine diatom, *Phaeodactylum tricornutum*

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**Abstract** One solution to the global crisis of antibiotic resistance is the discovery of novel antimicrobial compounds for clinical application. Marine organisms are an attractive and, as yet, relatively untapped resource of new natural products. Cell extracts from the marine diatom, *Phaeodactylum tricornutum*, have antibacterial activity and the fatty acid, eicosapentaenoic acid (EPA), has been identified as one compound responsible for this activity. During the isolation of EPA, it became apparent that the extracts contained further antibacterial compounds. The present study was undertaken to isolate these additional antibacterial factors using silica column chromatography and reverse-phase high-performance liquid chromatography. Two antibacterial fractions, each containing a pure compound, were isolated and their chemical structures were investigated by mass spectrometry and nuclear magnetic resonance spectroscopy. The antibacterial compounds were

identified as the monounsaturated fatty acid (9Z)-hexadecenoic acid (palmitoleic acid; C16:1 n-7) and the relatively unusual polyunsaturated fatty acid (6Z, 9Z, 12Z)-hexadecatrienoic acid (HTA; C16:3 n-4). Both are active against Gram-positive bacteria with HTA further inhibitory to the growth of the Gram-negative marine pathogen, *Listonella anguillarum*. Palmitoleic acid is active at micro-molar concentrations, kills bacteria rapidly, and is highly active against multidrug-resistant *Staphylococcus aureus*. These free fatty acids warrant further investigation as a new potential therapy for drug-resistant infections.

**Keywords** Antimicrobial · (6Z·9Z·12Z)-hexadecatrienoic acid · Palmitoleic acid · Micro-alga · *Phaeodactylum tricornutum* · MRSA.

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## Introduction

The discovery of novel antibacterial compounds that can be incorporated into new medicines is one solution to the global problem posed by antibiotic-resistant bacteria, such as multidrug-resistant *Staphylococcus aureus* (MRSA). The recent emergence of MRSA strains resistant to vancomycin, the drug of choice for such infections, has increased the urgency for new antibiotics (Centers for Disease Control and Prevention 2002; Srinivasan et al. 2002). Terrestrial micro-organisms have been widely investigated for natural products with antibacterial properties and many have been successfully exploited in medicinal treatments (Cragg et al. 1997) although so far marine organisms have attracted less attention (Jensen and Fenical 1994; Harvey 2000). Marine eukaryotic micro-algae show huge genetic and chemical diversity (Norton et al. 1996; Shimizu 1996; Faulkner

2002) and are known to produce many compounds of potential biotechnological interest, including pigments, hydrocarbons and vitamins (Borowitzka 1995). They are increasingly attracting interest in the search for new drugs and have been shown to be a primary source of bioactive natural products including antivirals, antifungals, enzyme inhibitors and toxins (Borowitzka 1995). Importantly, they have been found to produce antibacterial compounds (reviewed by Pesando 1990), either to defend against microbial pathogens and opportunists or to provide a competitive advantage over surrounding micro-organisms.

Cell extracts from the model marine diatom, *Phaeodactylum tricornerutum* Bohlin, have been shown to be antibacterial against numerous bacterial species (Duff et al. 1966; Cooper et al. 1983, 1985; Kellam and Walker 1989) and we have recently demonstrated that the polyunsaturated fatty acid, eicosapentaenoic acid (EPA), is one such antibacterial component (Desbois et al. 2008). However, during its isolation from methanol cell extracts, it became clear that additional bactericidal compounds are present in the extracts (Desbois et al. 2008). Accordingly, the present study was undertaken to isolate these other antibacterial compounds from methanol cell extracts of *P. tricornerutum* using a different separation strategy to our previous study.

## Materials and methods

### Reagents

Unless stated otherwise high-performance liquid chromatography (HPLC)-grade reagents (or the highest grade available) and culture media were purchased from Sigma Aldrich Ltd. (Poole, Dorset, UK) and all solutions were made with ultra pure de-ionised water (Option 3; Elga, High Wycombe, Bucks, UK).

### Micro-organisms

The marine diatom, *P. tricornerutum* SAG 1090-6, was purchased from the Experimental Phycology and Culture Collection of Algae at the University of Göttingen (this strain is held elsewhere as CCAP 1052/6 and UTEX 646). Seven marine bacterial strains were used, namely, *Alteromonas haloplanktis* (NCIMB 19), *Aeromonas hydrophila* (NCIMB 1108), *Micrococcus luteus* (NCIMB 9278), *Photobacterium phosphoreum* (NCIMB 64), *Planococcus citreus* (NCIMB 1493), *Psychrobacter immobilis* (NCIMB 308) and the fish and shellfish pathogen, *Listonella anguillarum* (MT1637; isolated from a skate gill). Six human and animal pathogenic bacteria were used and these were laboratory strains of *Bacillus cereus* (883-00), *Escherichia coli* B, *Pseudomonas aeruginosa* (NCIMB 10775), *S. aureus*

(SH1000) and clinical isolates of *Bacillus weihenstephanensis* (10390), MRSA252, MRSA16a and *Staphylococcus epidermidis*. Four clinical isolates of human pathogenic fungi were used: *Candida glabrata*, *Candida neoformis*, *Candida* sp. and *Saccharomyces cerevisiae* BY4741a. All strains were routinely maintained at +4°C on either 2216E (Difco, Sparks, MD, USA; the marine bacteria), Luria Bertani (LB; the human and animal pathogenic bacteria), nutrient (Bacilli) or yeast extract peptone dextrose (fungi) agar.

### Disc diffusion assay for antimicrobial activity

A disc diffusion assay was used to assess the antimicrobial activity of cell extracts, fractions and purified compounds. Briefly, test samples (prepared according to the procedures detailed below) were pipetted onto sterile paper discs (AA; Whatman International Ltd., Maidstone, Kent, UK), air-dried at room temperature for 4 h and laid onto the surface of agar plates that had been seeded with a uniform covering of the test bacteria or fungi. Discs impregnated with an equivalent volume of carrier solvent were used as controls. The plates bearing the impregnated paper discs were incubated for 24 h at 25, 30 or 37°C (as appropriate for the microbe) and then inspected for the appearance of clearing around each disc. The clear zones were measured and the area of microbial growth inhibition was calculated as total area of clear zone minus the area of the disc.

### Algal culture and preparation of cell extracts

*P. tricornerutum* was cultured axenically in 20 L polypropylene carboys to late-exponential phase. The culture medium was Miquel seawater (Allen and Nelson 1910). This was made by adding sterile nutrient solutions to 1- $\mu$ m filtered seawater that had itself been sterilised with 0.1% sodium hypochlorite for 24 h followed by neutralisation with sodium thiosulphate. Cultures were kept at 15 $\pm$ 5°C, aerated with sterile air at 0.3 v/v per minute and illuminated with white light at 200–450  $\mu$ mol s<sup>-1</sup> m<sup>-2</sup> operating a 14:10 h light–dark cycle. Algal cells were harvested by centrifugation (1,400 $\times$ g, 5 min, 18°C) and antibacterial compounds were extracted with a methanol–water (5:1) mix for 16 h on ice. The extracts were centrifuged (5,525 $\times$ g, 1 h, 4°C) to remove cellular debris and dried in a GL11 Gyrovap vacuum centrifuge (Philip Harris Scientific, Lichfield, Staffs, UK) for ~4 h at 30°C.

### Solvent extraction and fractionation of dried cell extracts by silica column chromatography

Dried cell extract (3 g) was initially extracted into ethyl acetate, methanol and water. The cell extract was resus-

pended in 33 mL ethyl acetate and centrifuged (13,000×g, 10 min, 20°C) to remove the insoluble compounds. The supernatant was designated the ethyl acetate extract. The insoluble pellet was resuspended in 12 mL methanol and re-centrifuged (as above). This supernatant was aspirated and designated the methanol extract. The remaining insoluble pellet was dissolved in 9-mL water and designated the water extract. Thirty micro-litres of each extract was tested for antibacterial activity against *S. aureus* as above.

The ethyl acetate extract was mixed with ~5-g silica gel (Matrex Silica 60; Fisher Scientific, Loughborough, Leics, UK) and left to dry overnight at 20°C before drying to completion with a rotary evaporator at 40°C (Rotovapor R-134; Büchi Ltd., Oldham, Lancs, UK). The dried mix was applied to a Sep Pak (SP) silica cartridge (10 g; Waters Ltd., Elstree, Herts, UK) and eluted with 100 mL hexane followed by 100-mL volumes of 10% step increases of hexane–ethyl acetate mix until pure ethyl acetate. One hundred millilitres of ethyl acetate–methanol mixes were then added in stepwise increases of 20% methanol until pure methanol. Sixteen fractions of 100 mL were collected (fractions SP 1–16). The methanol extract was also mixed with ~5-g silica gel and dried as above. The dried mix was applied to a silica Sep Pak cartridge that was eluted with 100 mL ethyl acetate followed by 100-mL volumes of 10% step increases of ethyl acetate–methanol mix until pure methanol. Fractions of 100 mL were collected (fractions SP 17–27) corresponding to each change of solvent. Each fraction was dried to completion on the rotary evaporator as above. The dried residues were each resuspended in 2 mL of appropriate solvent and 30-µL volumes used to test for antibacterial activity against *S. aureus* as above. The remainder of each fraction was stored at –20°C until further analysis.

#### Reverse-phase high-performance liquid chromatography of antibacterial Sep Pak fractions

SP fractions showing antibacterial activity were dried as before and resuspended in ~1.6 mL methanol. After centrifugation (13,000×g, 10 min, 25°C), the supernatant was passed through a 0.2-µm nylon filter (Gelman Sciences Ltd., Northampton, Northants, UK) and 1 mL was applied to a semi-prep C<sub>18</sub> column (250×10 mm; Phenomenex, Macclesfield, Cheshire, UK) on an HPLC machine (Finnigan Surveyor; Thermo Fisher Scientific Inc., Waltham, MA, USA) consisting of an autosampler, pumps, UV-VIS detector and a separate fraction collector (Foxy Jr.; Teledyne Isco, Inc., Lincoln, NE, USA). Fractions SP 1–11, 17 and 18 were eluted at 2 mL min<sup>-1</sup> with a linear gradient: 0–50 min 0–100% v/v B; 50–80 min isocratic B (buffer A was 50% methanol, 49.93% water, 0.07% trifluoroacetic acid (TFA); buffer B was 99.93% methanol, 0.07% TFA) whilst

fractions SP 12–16 and SP 19–27 were eluted at 2 mL min<sup>-1</sup> with a linear gradient: 0–100 min 0–100% v/v B; 100–120 min isocratic B (buffer A was 99.93% water, 0.07% TFA; buffer B was 99.93% methanol, 0.07% TFA). The eluate was monitored at 214 nm and 2-mL fractions were collected (i.e. one fraction per minute). Of this, 75 µL of each fraction was tested for antibacterial activity against *S. aureus* as above. Active fractions were freeze-dried (Lyolab 3000; Heto-Holten A/S, Allerød, Denmark) at –55°C to completion in pre-massed sterile bottles and stored at 4°C.

#### Nuclear magnetic resonance spectroscopy and mass spectrometry

Each HPLC fraction showing antibacterial activity was resuspended in ~0.7 mL methanol-d<sub>4</sub>, filtered and <sup>1</sup>H-nuclear magnetic resonance (NMR) and 2D <sup>1</sup>H,<sup>13</sup>C-heteronuclear single quantum coherence (HSQC), <sup>1</sup>H,<sup>13</sup>C-heteronuclear multiple bond correlation (HMBC) and <sup>1</sup>H,<sup>1</sup>H-COSY (correlation spectroscopy) spectra were recorded at 500 MHz (Avance 500; Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts were measured in parts per million (ppm) with respect to tetramethylsilane (δ <sup>1</sup>H for CD<sub>3</sub>OD=3.31 ppm, δ <sup>13</sup>C for CD<sub>3</sub>OD=49.15 ppm). Each fraction was dried on the speed vac at 30°C and resuspended in 0.4–1 mL methanol. Electrospray ionisation mass spectrometry (ESI-MS) was performed for each fraction (negative mode; 3 kV; injection volume 10–20 µL; LCT; Micromass, Manchester, Lancs, UK). Where appropriate, high-resolution ESI-MS was also performed to generate empirical elemental composition data.

#### Synthesis of dimethyl disulphide adducts

The position of the double bond in monounsaturated fatty acids was determined by electron impact mass spectrometry (EI-MS) of dimethyl disulphide (DMDS) adduct derivatives (Moss and Daneshvar 1992). Briefly, 300 µL of sample in methanol was converted to methyl esters as in Walton et al. (2000) and then dissolved in 200 µL DMDS. To this was added 50 µL of iodine in diethyl ether (60 mg mL<sup>-1</sup>). This was left to mix on an orbital shaker (60 rpm, 24 h, 25°C) before the addition of 5 mL hexane and three washes with 2% aqueous sodium thiosulphate solution. Residual water was removed by mixing with anhydrous sodium sulphate and, after filtering through non-absorbent cotton wool (pre-washed with diethyl ether), the hexane was removed by gentle heating under a stream of nitrogen. The final product was finally resuspended in 50 µL hexane for EI-MS performed on a time of flight mass spectrometer (GCT; Micromass, Manchester, Lancs, UK) with an ionisation energy of 70 eV.

## Antibacterial potency

The antibacterial potency of purified factors were determined by measurement of the 50% inhibitory concentration ( $IC_{50}$ ), defined here as the lowest molar concentration required to inhibit bacterial growth by 50% compared to the control, and the minimum bactericidal concentration (MBC), the lowest molar concentration that completely killed the inoculum. *S. aureus* was used as the test bacterium and the assay was carried out in 96-well plates with round-bottomed wells (Corning Inc., Corning, NY, USA). Serial twofold dilutions of the test compounds (0.625–80  $\mu$ M) were made in LB medium and experimental wells were inoculated with  $1 \times 10^5$  cfu  $mL^{-1}$  of log-phase *S. aureus*. The final well volumes were 100  $\mu$ L. For negative controls, the test compound was replaced with carrier solvent only. Each treatment was performed in quadruplicate. The plates was covered with an AirPore™ tape sheet (Qiagen Ltd., Crawley, West Sussex, UK) and incubated at  $\sim 20^\circ C$ . After 24 h, the mean  $A_{570}$  of each well was determined from triplicate measurements using a plate reader (MRX II; Dynex Technologies Ltd., Worthing, West Sussex, UK) against a blank comprising LB medium only. The  $A_{570}$  of each experimental well was then compared to their respective negative control wells to generate values of percent growth compared to control. To determine the MBC, the contents of each well showing no obvious growth were spread across LB agar plates. To control for plate contamination, the contents of the blank (medium only) wells were similarly plated. The number of colony-forming units for each plate was determined after incubation at  $37^\circ C$  for 48 h.

## Killing kinetics

The time required to kill log-phase *S. aureus* was determined for each of the purified antibacterial compounds. Briefly, 198  $\mu$ L volumes of  $1 \times 10^6$  cfu  $mL^{-1}$  bacterial suspension in phosphate-buffered saline (PBS) were dispensed into eight wells of a 96-well plate. To four wells was added 2  $\mu$ L of purified antibacterial compound in methanol to give a concentration equal to the upper MBC value whilst the remaining four wells (controls) received 2  $\mu$ L methanol only. At time 0, 15, 30 and 60 min, the number of colony-forming units per millilitre was determined for each well by serial dilution in PBS and plating on LB agar.

## Results

### Fractionation of antibacterial cell extracts

Antibacterial activity against *S. aureus* was found mainly in the ethyl acetate and methanol extracts of the initial *P. tricorutum* cell extracts (data not shown).

Sep Pak separation of the ethyl acetate extract produced very strong antibacterial activity against *S. aureus* (area of growth inhibition on disc diffusion assay  $>120$   $mm^2$ ) in fractions SP 2, 3, 4, 5 and 12, with weak activity (area of growth inhibition  $\leq 60$   $mm^2$ ; Table 1) in fractions SP 1, 6, 7, 8, 9, 10 and 11 (Table 1). Sep Pak separation of the methanol extract produced very strong activity in fractions SP 17 and 18 with weak activity in fractions SP 19 and 20 (Table 1). Every Sep Pak fraction found to have very strong antibacterial activity was further fractionated by reverse-phase (RP)-HPLC (fractions SP 2, 3, 4 and 5 were pooled for this step and are referred to as SP 2–5 herein).

RP-HPLC of pooled fractions SP 2–5 yielded four active fractions (nos. 52, 55, 56 and 57) while RP-HPLC of SP 12 yielded two active fractions (nos. 105 and 106). RP-HPLC of SP 17 gave four active fractions (nos. 52, 55, 56 and 57)

**Table 1** Antibacterial activity of fractions from Sep Pak separation of ethyl acetate and methanol extracts of initial *P. tricorutum* cell extracts as determined from the clear zone area on the *S. aureus* disc diffusion assay

Fraction	Antibacterial activity (clear zone area in square millimetres)	
Ethyl acetate extract		
SP 1	+	(50)
SP 2	+++	(173)
SP 3	+++	(424)
SP 4	+++	(255)
SP 5	+++	(173)
SP 6	+	(22)
SP 7	+	(10)
SP 8	+	(10)
SP 9	+	(22)
SP 10	+	(35)
SP 11	+	(35)
SP 12	+++	(173)
SP 13	–	(0)
SP 14	–	(0)
SP 15	–	(0)
SP 16	–	(0)
Methanol extract		
SP 17	+++	(352)
SP 18	+++	(286)
SP 19	+	(22)
SP 20	+	(22)
SP 21	–	(0)
SP 22	–	(0)
SP 23	–	(0)
SP 24	–	(0)
SP 25	–	(0)
SP 26	–	(0)
SP 27	–	(0)

– no activity, + weak activity (area of growth inhibition  $\leq 60$   $mm^2$ ), ++ strong activity (area of growth inhibition  $>60$  but  $\leq 120$   $mm^2$ ); +++ very strong activity (area of growth inhibition  $>120$   $mm^2$ ). Actual areas of growth inhibition are given in brackets in  $mm^2$ .



and, finally, RP-HPLC of SP 18 gave three active fractions (nos. 55, 56 and 57).

#### Identification of compounds in antibacterial fractions

Two antibacterial HPLC fractions were deemed sufficiently pure by  $^1\text{H-NMR}$  to enable a full structural characterisation. These were HPLC fraction 57 (dried mass=2.4 mg) from SP 2–5 (Fig. 1a) and HPLC fraction 52 (dried mass=0.8 mg) from SP 17 (Fig. 1b). The remaining active HPLC fractions contained different mixtures of the compounds found in the two pure fractions.

The NMR data for HPLC fraction 57 from SP 2–5 are shown in Tables 2 and 3. Mass spectrometry of this fraction gives a prominent  $m/z$  ion at 253.19  $m/z$  corresponding to  $[\text{M-H}]^-$  with the minor  $m/z$  ion at 267.17  $m/z$  probably being the methylated product  $[\text{M+Me}]^-$  (Fig. 2a). High-resolution mass spectrometry predicts an empirical formula of  $\text{C}_{16}\text{H}_{27}\text{O}_3$  for  $[\text{M-H}]^-$  (data not shown). The  $^1\text{H-}^{13}\text{C}$  HMBC spectrum shows correlation of the triplet in  $^1\text{H-NMR}$  ( $\delta=2.27$  ppm) with the  $^{13}\text{C}$  resonance at 176.6 ppm, which clearly confirms that the compound is a fatty acid. Furthermore, the relative integral intensities of  $^1\text{H}$  resonances indicate a monounsaturated 16-carbon chain. However, in this case, the NMR data do not allow an assignment for the position of the double bond. Thus, the location of the double bond was investigated by synthesising DMDS adduct derivatives. The EI-MS of these DMDS adducts reveals two substantial fragment ions where the molecule was cleaved between the carbons that originally constituted the double bond. These are the  $\omega$  fragment (aliphatic end of the molecule) at 145.10  $m/z$  and the  $\Delta$  fragment (carboxyl end of the molecule) at 217.12  $m/z$  (Fig. 2c). The other

diagnostic mass fragment peaks corresponding to the molecular ion  $[\text{M-H}]^-$  and the  $\Delta$ -32 fragment (the loss of methanol from the  $\Delta$  fragment) found at 362.23 and 185.09  $m/z$ , respectively (Fig. 2c). These data position the double bond in the fatty acid chain between carbons 9 and 10 from the carboxyl end (or the n-7 position) meaning the fatty acid is (9Z)-hexadecenoic acid or C16:1 n-7 (Fig. 3a), which is more commonly referred to as palmitoleic acid.

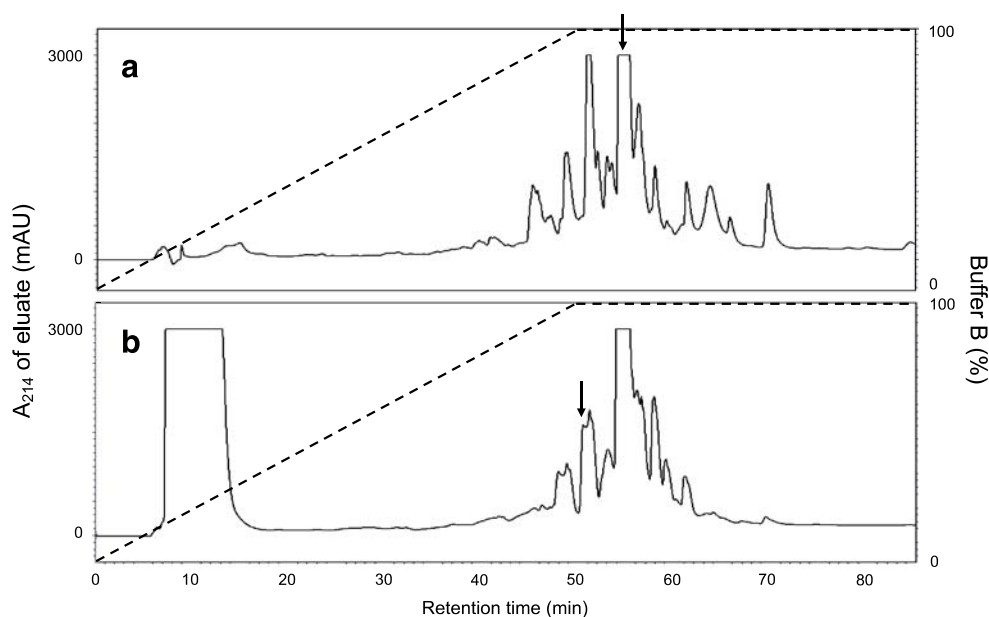
NMR data for HPLC fraction 52 from SP 17 are shown in Tables 2 and 3. Mass spectrometry of the fraction gives a prominent ion at 249.39  $m/z$  corresponding to  $[\text{M}]^-$  (Fig. 3b). In this case,  $^1\text{H-}^{13}\text{C}$  HMBC spectrum also clearly proves the presence of carboxylic moiety ( $\delta=177.5$ ). The  $^1\text{H-NMR}$  and  $^1\text{H,}^1\text{H-COSY}$  spectrum indicates three double bonds ( $\delta=5.32$ – $5.38$  ppm, multiplet, 6H) separated by methylene groups ( $\delta=2.82$  ppm, apparent triplet, 4H,  $J=5.4$  Hz; Fig. 4). Furthermore,  $^1\text{H,}^1\text{H-COSY}$  shows two and four methylene groups separating the double bonds from terminal methyl and carboxylic acid, respectively (Fig. 4). These data enable the compound to be identified as the unsaturated fatty acid (6Z, 9Z, 12Z)-hexadecatrienoic acid (HTA) or C16:3 n-4 (Fig. 3b).

#### Antibacterial potency and killing kinetics

As only very small quantities of the antibacterial compounds were isolated, palmitoleic acid (>99%) was purchased from Sigma Aldrich Ltd. HTA is not commercially available so the 0.8 mg isolated above was used in the following experimentation.

The  $\text{IC}_{50}$  values of palmitoleic acid and HTA against *S. aureus* were determined to be in the ranges 10–20 and 20–40  $\mu\text{M}$ , respectively (Table 4). The MBC value for

**Fig. 1** Absorbance at 214 nm of eluate during reverse-phase high-performance liquid chromatography  $\text{C}_{18}$  separation of **a** pooled Sep Pak fractions 2–5 (SP 2–5) showing the peak corresponding to active HPLC fraction 57 (marked with arrow); and **b** SP 17 showing the peak corresponding to active HPLC fraction 52 (marked with arrow). Dashed lines show the elution gradients (buffer A 50% methanol, 49.93% water, 0.07% trifluoroacetic acid (TFA); buffer B 99.93% methanol, 0.07% TFA)



**Table 2**  $^1\text{H}$ -NMR data obtained in methanol- $\text{d}_4$  at 500 MHz for antibacterial fractions

$\delta$ (ppm)	Integral intensity	Multiplicity	$J$ (Hz)	Assignment
HPLC fraction 57 from SP 2–5				
0.91	3 H	t	6.8	H16
1.24–1.39	16 H	–	–	H4, H5, H6, H7, H12, H13, H14, H15
1.60	2 H	a.q	7.1	H3
1.98–2.14	4 H	–	–	H8, H11
2.27	2H	t	7.4	H2
5.30–5.41	2 H	–	–	H9, H10
HPLC fraction 52 from SP 17				
0.92	3 H	t	7.4	H16
1.35–1.46	4 H	–	–	H4, H15
1.59–1.66	2 H	–	–	H3
1.99–2.15	4 H	–	–	H5, H14
2.26	2 H	a.q	7.6	H2
2.80–2.84	4 H	a.t	5.4	H8, H11
5.32–5.38	6 H	–	–	H6, H7, H9, H10, H12, H13

$\delta$  chemical shift,  $J$  coupling constant,  $t$  triplet,  $a.q$  apparent quintet,  $a.t$  apparent triplet

palmitoleic acid is 40–80  $\mu\text{M}$  (Table 4). At 80  $\mu\text{M}$ , palmitoleic acid completely killed a *S. aureus* inoculum ( $1 \times 10^6$  cfu  $\text{mL}^{-1}$ ) within 60 min (Fig. 5). Unfortunately, it was not possible to determine the MBC for HTA but it is unlikely that it would be much greater than 640  $\mu\text{M}$ , as these plates showed only very few colonies. For comparison, ampicillin has an  $\text{IC}_{50}$  value between 0.25 and 0.5  $\mu\text{M}$  and a MBC between 320 and 640  $\mu\text{M}$  (Table 4).

#### Spectrum of activity

The spectrum of activity was determined for palmitoleic acid and HTA against a variety of bacteria and fungi by disc

diffusion. Palmitoleic acid potently inhibits the growth of the Gram-positive pathogens *B. cereus*, *S. aureus*, *S. epidermidis* and two clinical isolates of MRSA (Table 5). Palmitoleic acid also inhibits the growth of the food-borne pathogen, *B. weihenstephanensis* (Table 5). However, this fatty acid is not active against any of the Gram-negative bacteria or fungi tested (Table 5). HTA inhibits the growth of *S. aureus* and *S. epidermidis* and also the two marine bacteria, *P. citreus* and *L. anguillarum* (Table 5). HTA has no killing activity against any of the fungal species tested (Table 5) and insufficient material was available to assess its effect upon the Bacilli or MRSA.

**Table 3**  $^{13}\text{C}$ -NMR data obtained in methanol- $\text{d}_4$  at 500 MHz for antibacterial fractions

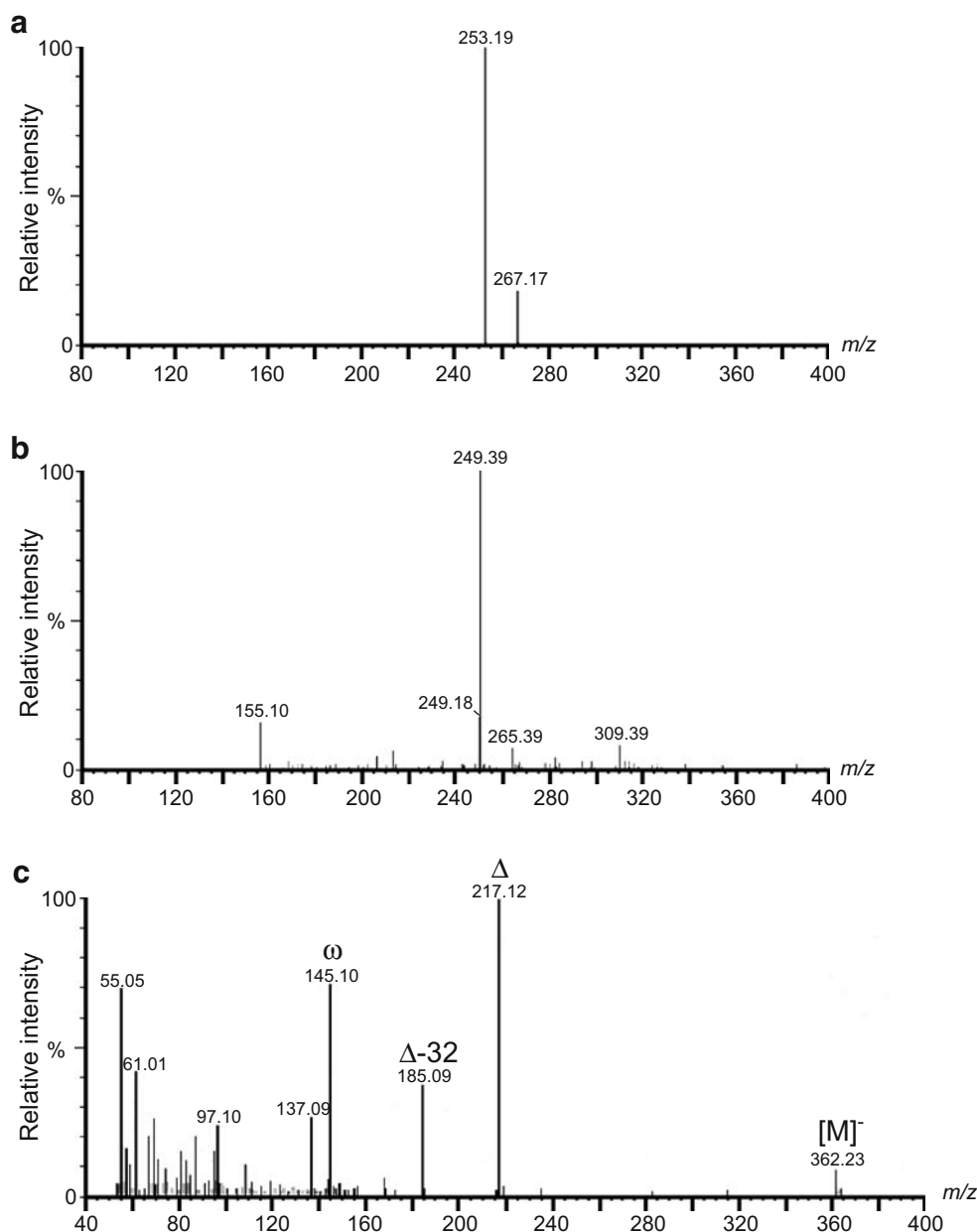
$\delta$ (ppm)	Assignment
HPLC fraction 57 from SP 2–5	
13.2	C16
22.0–31.5	C4, C5, C6, C7, C12, C13, C14, C15
24.5	C3
26.5	C8, C11
33.8	C2
129.4	C9, C10
176.6	C1
HPLC fraction 52 from SP 17	
12.8	C16
24.6	C3
25.1	C8, C11
26.4	C5, C14
28.9	C4, C15
34.6	C2
127.6	C6, C7, C9, C10, C12, C13
177.5	C1

C1 data from 2D  $^1\text{H}$ - $^{13}\text{C}$  HMBC; C2–C16 data from 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC.

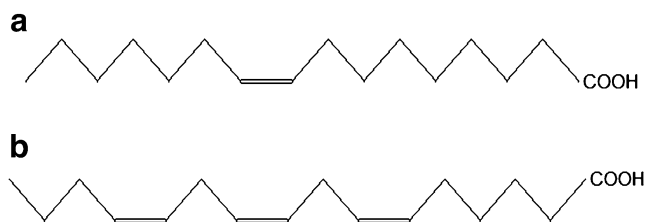
#### Discussion

It is well known that eukaryotic micro-algae are of growing biotechnological significance with applications in, for example, wastewater treatment, discovery of new nutraceuticals and pharmaceuticals and, more recently, nanotechnology. The present study shows that two unsaturated free fatty acids, palmitoleic acid and HTA, present in aqueous methanol cell extracts of *P. tricorutum* have antibacterial properties in vitro. Both fatty acids are active against *S. aureus* at micro-molar concentrations and are able to inhibit the growth of Gram-positive bacterial species. HTA also inhibits the growth of the Gram-negative bacterium, *L. anguillarum*. Moreover, palmitoleic acid is able to kill *S. aureus* cells within 60 min. That *P. tricorutum* cell extracts have antibacterial activity has been demonstrated previously although the compounds responsible were not fully defined (Duff et al. 1966; Cooper et al. 1983, 1985; Kellam and Walker 1989; Desbois et al. 2008).

**Fig. 2** Electrospray ionisation mass spectrometry of antibacterial fractions **a** HPLC fraction 57 from Sep Pak fractions 2–5 (SP 2–5) showing a prominent ion at 253.19  $m/z$  corresponding to  $[M-H]^-$  and a minor ion at 267.17  $m/z$  probably corresponding to the methylated product  $[M+Me]^-$ ; and **b** HPLC fraction 52 from SP 17 showing the prominent ion at 249.39  $m/z$  corresponding to  $[M-H]^-$ . **c** Electron impact mass spectrometry of dimethyl disulphide adducts of the monounsaturated fatty acid in HPLC fraction 57 from SP 2–5. This shows the two substantial fragment ions where the molecule was cleaved between the carbons that originally constituted the double bond:  $\omega$  and  $\Delta$  fragments at 145.10 and 217.12  $m/z$ , respectively. The other diagnostic mass fragment peaks correspond to  $[M-H]^-$  (362.23  $m/z$ ) and the  $\Delta$ -32 fragment (185.09  $m/z$ )

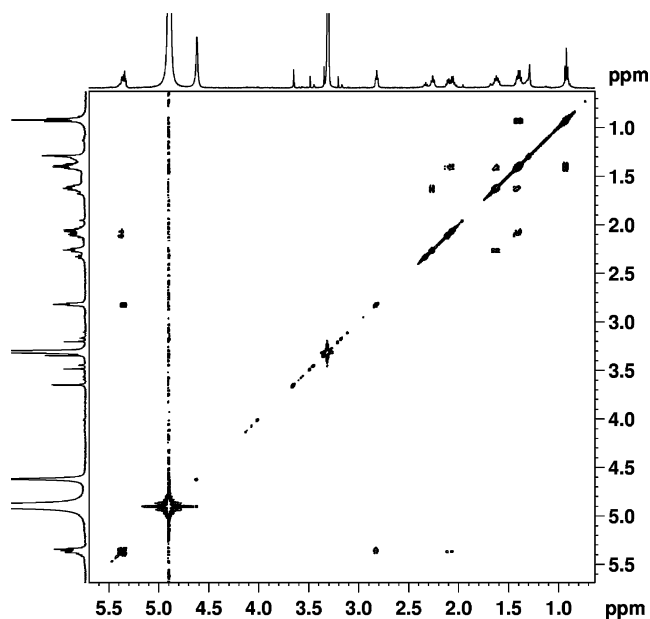


We have already reported that EPA derived from *P. tricornutum* also has antibacterial effects (Desbois et al. 2008). The present study shows that this is not the only free fatty acid with bactericidal properties that is synthesised by



**Fig. 3** Structures of **a** palmitoleic acid and **b** (6Z, 9Z, 12Z)-hexadecatrienoic acid

diatoms. The antibacterial nature of free fatty acids has already been recognised (reviewed by Kabara et al. 1972). Our study confirms the reports by Kabara et al. (1972), Miller et al. (1977), Bergsson et al. (2001), Sun et al. (2003) and Zheng et al. (2005) that palmitoleic acid is active against Gram-positive species but to the best of our knowledge this is the first time that this fatty acid has been shown to inhibit the growth of the important human pathogen, MRSA. Moreover, the present study is the first to report that palmitoleic acid inhibits the growth of two food-borne pathogens, *B. cereus* and *B. weihenstephanensis*, indicating that this fatty acid could find application in reducing or controlling numbers of these disease-causing bacteria in food products, such as ready meals.



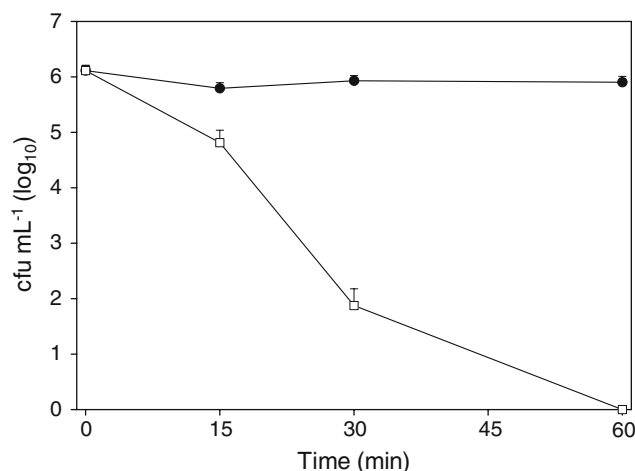
**Fig. 4**  $^1\text{H}$ ,  $^1\text{H}$ -correlation spectroscopy for HPLC fraction 52 from Sep Pak fraction 17 showing cross-peaks which unambiguously enable the identification of the compound as (6Z, 9Z, 12Z)-hexadecatrienoic acid

The present study is also the first to demonstrate that the relatively unusual fatty acid, HTA (C16:3 n-4), has bactericidal properties. Most photosynthetic organisms produce the more common C16:3 n-3 isomer (see for example Jamieson and Reid 1971; Li et al. 2002). Previously, Findlay and Patil (1984) claimed to have demonstrated the antibacterial activity of HTA that had been isolated from the marine diatom, *Navicula delognei*, but in their study HTA was only a minor contaminant of a fraction containing the antibacterial fatty acid (6Z, 9Z, 12Z, 15Z)-octadecatetraenoic acid (C18:4 n-3). Thus, the bactericidal properties of HTA were not unequivocally demonstrated. Similarly, Cooper et al. (1985) isolated an antibacterial fraction from *P. tricorutum* cell extracts that contained a mixture of six fatty acids, one of which was HTA. However, Cooper et al. (1985) concluded that HTA was not responsible for the activity in this fraction because the closest commercially available structural homologue, palmitoleic acid, was found to be inactive in their

**Table 4**  $\text{IC}_{50}$  and MBC values against *S. aureus* at  $1 \times 10^5$  cfu  $\text{mL}^{-1}$  for palmitoleic acid and HTA showing that palmitoleic acid is more active than HTA against *S. aureus* by both measures of potency

Test compound	$\text{IC}_{50}$ ( $\mu\text{M}$ )	MBC ( $\mu\text{M}$ )
Palmitoleic acid	10–20	40–80
HTA	20–40	>640
Ampicillin	0.25–0.5	320–640

Values for ampicillin are given for comparative purposes and in each case  $n=4$ .



**Fig. 5** Determination of killing kinetics for palmitoleic acid showing that, at a concentration of 80  $\mu\text{M}$ , *S. aureus* (at  $1 \times 10^6$  cfu  $\text{mL}^{-1}$ ) was completely killed within 60 min (open squares) whilst the controls (black circles) showed no decrease in colony-forming units per millilitre over the same time period.  $n=4$ ; error bars are +1 SE

**Table 5** Susceptibility of microbial strains to the growth inhibitory effect of palmitoleic acid and HTA

Species and Gram's stain	Growth inhibition by fatty acid	
	Palmitoleic acid	HTA
<b>Marine strains</b>		
<i>Aeromonas hydrophila</i> (–)	–	(0) n/d
<i>Listonella anguillarum</i> (–)	–	(0) + (vs)
<i>Micrococcus luteus</i> (+)	–	(0) n/d
<i>Photobacterium</i> sp. (–)	–	(0) n/d
<i>Planococcus citreus</i> (+)	–	(0) + (vs)
<i>Pseudoalteromonas haloplanktis</i> (–)	–	(0) n/d
<i>Psychrobacter immobilis</i> (–)	–	(0) – (0)
<b>Potential human and animal pathogens</b>		
<i>Bacillus cereus</i> (+)	++	(85) n/d
<i>Bacillus weihenstephanensis</i> (+)	+	(35) n/d
<i>Escherichia coli</i> (–)	–	(0) – (0)
<i>Pseudomonas aeruginosa</i> (–)	–	(0) – (0)
<i>Staphylococcus aureus</i> (+)	+++	(751) + (39)
<i>Staphylococcus epidermidis</i> (+)	+++	(513) + (vs)
MRSA 16a (+)	+++	(318) n/d
MRSA 252 (+)	+++	(148) n/d
<b>Fungi</b>		
<i>Candida glabrata</i>	–	(0) n/d
<i>Candida neoformis</i>	–	(0) n/d
<i>Candida</i> sp.	–	(0) – (0)
<i>Saccharomyces cerevisiae</i>	–	(0) n/d

Palmitoleic acid potently inhibited the growth of staphylococcal species, including MRSA, but had no effect on the marine and Gram-negative bacteria or the fungal strains tested. HTA inhibited the growth of Gram-positive and Gram-negative species but not fungi. Actual areas of growth inhibition are given in brackets in  $\text{mm}^2$  (vs: very slight growth inhibition around the disc)

– not susceptible, + weakly susceptible (area of growth inhibition  $\leq 60 \text{ mm}^2$ ), ++ highly susceptible (area of growth inhibition  $>60$  but  $\leq 120 \text{ mm}^2$ ), +++ very highly susceptible (area of growth inhibition  $>120 \text{ mm}^2$ )



antibacterial assay. Palmitoleic acid, HTA and EPA are probably released during extraction by lipases cleaving the polar lipid species that constitute cellular membranes (Parrish and Wangersky 1987; Budge and Parrish 1999; Jüttner 2001; Cutignano et al. 2006), as unsaturated fatty acids are not found in their free form to any great extent in healthy diatom cells (Jüttner 2001; Pohnert 2002).

To investigate the importance of the double bond(s) in the antibacterial activity of the C16 fatty acids, the saturated palmitic acid (C16:0) was tested against the four species that HTA inhibited (*L. anguillarum*, *P. citreus*, *S. aureus*, *S. epidermidis*). However, palmitic acid shows no activity against these bacteria indicating that the presence of carbon–carbon double bond(s) in C16 fatty acids is crucial for the antibacterial action (data not shown).

The antibacterial potency of free fatty acids usually increases with the unsaturation of the carbon chain for fatty acids of equal length (Kabara et al. 1972; Knapp and Melly 1986; Sun et al. 2003). In contrast to these previous reports, palmitoleic acid was approximately twice as potent as HTA against *S. aureus* but this could be due the positions of the double bonds, which are also known to affect antibacterial activity (Kabara et al. 1973, 1977). The exact mechanism by which free fatty acids exert their bactericidal action remains unresolved but suggestions have been put forward that these molecules initiate peroxidative processes (Knapp and Melly 1986; Wang and Johnson 1992) and inhibit bacterial fatty acid synthesis (Zheng et al. 2005). However, most observations indicate cellular membranes as the main site of action (Galbraith and Miller 1973; Miller et al. 1977; Thormar et al. 1987; Shin et al. 2007). Free fatty acids may interact with these membranes causing leakage of molecules from the cells, reduction of nutrient uptake or inhibition of cellular respiration (Borst et al. 1962; Sheu and Freese 1972; Galbraith and Miller 1973).

Finally, considering their potency, rapidity of killing, low toxicity, lack of bacterial resistance to their action (Laser 1952; Lacey and Lord 1981; Sun et al. 2003) and the encouraging preliminary in vivo results of Lacey and Lord (1981) and Clarke et al. (2007), it is clear that free fatty acids, such as palmitoleic acid and HTA, warrant further examination as possible new therapies for topical or systemic MRSA infections.

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