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The identification and role of a novel eicosanoid in the reproductive behaviour of barnacles (*Balanus balanus*)

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Summary

Post-copulatory behaviour in barnacles involves a violent rocking movement of the opercular valves, which is thought to contribute to the expulsion of oocytes through the oviduct into the mantle cavity where they are fertilised. We demonstrate in this study that the seminal vesicles/testis of the subtidal barnacle *Balanus balanus* produce a biologically active factor, barnacle muscle stimulatory factor (BMSF), which causes a significant increase in cirral and body muscular activity. BMSF was identified using a combination of high performance liquid chromatography and mass spectrometry as a novel eicosanoid/oxylipin, 8,13-dihydroxyeicosapentaenoic acid. This is rapidly inactivated under mild acid conditions to form a complex range of triene and pentaene chromophore-containing products that have only been partially identified. Injection of purified BMSF into the mantle cavity of barnacles caused the rocking movements of the opercular valves as reported following fertilisation. In excised barnacles, it also caused muscular contractions of the whole body mass. The breakdown products of BMSF, however, were without such activities. The function of BMSF in facilitating fertilisation in barnacles is comparable to the role of other eicosanoids in human reproduction, reinforcing the view that these compounds have conserved activities in both invertebrates and vertebrates.

Key words: eicosanoid, barnacle, *Balanus balanus*, lipoxygenase, mass spectrometry.

Introduction

Eicosanoids are bioactive lipids derived from polyunsaturated fatty acids, such as the C20 arachidonic acid (AA; 20:4, n-6) that play a wide role in many physiological and immunological processes (Samuelsson et al., 1987; Smith, 1989). As well as AA, there are a number of alternate polyunsaturated fatty acid substrates, including eicosapentaenoic (EPA; 20:5, n-3) and docosahexaenoic (DHA; 22:6, n-3) acids, that can also yield a range of oxygenated products. Recent studies have shown that in mammals these alternative fatty acids can act as substrates for the biosynthesis of a range of novel compounds including the resolvins and neuroprotectins whose discovery goes some way to explain the beneficial effects of fish oils rich in these omega-3 fatty acids (Marcheselli et al., 2003; Mukherjee et al., 2004; Serhan et al., 2004). Aquatic animals, unlike their terrestrial counterparts, naturally incorporate high levels of EPA and DHA into their cellular phospholipids, and hence the diversity of the eicosanoids generated in such animals is unique within the animal kingdom. Furthermore, many marine invertebrates, such as corals, ascidians, bryozoans and barnacles, have been found to biosynthesize both ‘classical’ eicosanoids (i.e. those found in mammals) and novel forms including clavulones, mucosin, prostaglandin 1,15-lactones, and bromo- and iodo-vulones from these alternate fatty acid substrates (DiMarzo et al., 1991; Casapullo et al., 1997; Rowley et al., 2005). With a few notable exceptions (e.g. Valmsen et al., 2001), the mechanism of biosynthesis of these compounds is unknown and their biological significance is often unclear (Stanley, 2000).

Barnacles have been subject to extensive study on eicosanoid biosynthesis following the initial finding nearly 50 years ago that an unknown factor causes the stimulation of hatching in brooded larvae in the mantle cavity (Crisp, 1956) that was later found to consist of a complex mixture of several mono- and tri-hydroxy derivatives of EPA and AA (Vogan et al., 2003). In the present study, we demonstrate the biosynthesis of a novel, biologically active, EPA-derived eicosanoid in the subtidal barnacle, *Balanus balanus*, and confirm its role in the fertilisation process. The active principal has been named ‘barnacle muscle stimulatory factor’ (BMSF).

Materials and methods

Materials

HPLC grade solvents were obtained from Fisher Scientific (Loughborough, UK) while HPLC-MS solvents were obtained from BDH (Poole, UK). Eicosanoid standards were purchased from Cayman Chemicals (Ann Arbor, MI, USA). All other reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Animals

Adult *Balanus balanus* L. were provided by Millport Marine Biological Station (Scotland, UK). Upon return to the laboratory, animals were maintained in a circulating seawater aquarium at ca. 17°C until use.

BMSF and other eicosanoid biosynthesis

Disrupted barnacle tissues (body, testis/seminal vesicles, cirri) were prepared by dissection under a binocular microscope and mechanically disrupted in 5 ml cold 0.45 μm-filtered seawater (FSW). This disrupted material consisted of isolated cells and cell debris. It was subsequently challenged with 5 μmol l^{-1} calcium ionophore A23187 for 20 min at 12°C and cells and cell debris removed by centrifugation (1000 ***g***, 10 min, 4°C). Supernatants were removed and 200 ng PGB_1 internal standard added. In some cases, supernatants were briefly acidified (pH 3.5) using 10% acetic acid immediately prior to solid-phase extraction. Samples were added to prepared C_{18} Sep-Pak cartridges (Waters Chromatography, Watford, UK), pre-washed with 10 ml methanol followed by 10 ml ultra-high purity water. Samples were washed with 10 ml water, 2 ml hexane, and eluted with 5 ml methanol. Eluants were dried under a continuous stream of N_2, resuspended in 150 μl of the appropriate HPLC mobile phase and stored at –80°C until separation.

RP-HPLC

Samples containing BMSF were separated by reverse-phase high performance liquid chromatography (RP-HPLC) using an Ultrasphere C_{18} ODS column (5 μm packing, 25 cm×0.46 cm; Beckman Coulter, High Wycombe, UK) with a 40 min linear gradient changing from 100% water:methanol:acetonitrile: acetic acid (45:30:25:0.05) buffered to pH 7 or 5.7 to 100% methanol, with a flow rate of 0.6 ml min^{-1} (Pettitt et al., 1991). Peaks were identified using a Waters 996 diode-array detector and Waters Millennium software. Quantification was by reference to the extraction efficiency of the internal standard (PGB_1) using published molar extinction coefficients where possible.

Inhibitor studies

To determine the effect of cytochrome P450 and lipoxygenase inhibitors on the generation of BMSF and other products, barnacle tissues were disrupted and split into two portions. In each case, one portion was pre-incubated with either metyrapone (100 μmol l^{-1}), SKF-525A·HCl (Proadifen) (100 μmol l^{-1}), esculetin (5–50 μmol l^{-1}) or nordihydroguaiaretic acid (20 μmol l^{-1}) for 10 min at 12°C. The other portion was incubated under these conditions but with the same concentration of ethanol as employed in the stock solutions of the inhibitors. Both portions were incubated for 20 min at 12°C in the presence of 5 μmol l^{-1} calcium ionophore A23187. Results were expressed as % change compared to the control following extraction, separation and quantification as previously described (Pettitt et al., 1991).

Fatty acid precursors

The effects of the potential precursor fatty acids, eicosapentaenoic (EPA), arachidonic (AA) and docosahexaenoic acids (DHA) were tested on disrupted barnacle seminal vesicles/testis. Following disruption in 5 ml FSW, this material was split into 5×1 ml samples. Stock solutions of fatty acids dissolved in ethanol were added to give a final concentration of 0, 10, 20 or 50 μmol l^{-1} and incubated for 10 min at room temperature (RT). Calcium ionophore A23187 (5 μmol l^{-1}) was subsequently added and further incubated for 20 min at RT. Samples were then extracted and separated by RP-HPLC.

Stability of BMSF

RP-HPLC fractions containing the material under the peaks of interest (i.e. BMSF and its breakdown products) were dried under a stream of N_2 and resuspended in 750 μl methanol. These were placed in quartz cuvettes with a background reading of methanol already removed and the absorbance measured between 200–400 nm. Acetic acid (10%; 80 μl) was then added to each 750 μl sample to give an apparent pH of 3.5 and the absorbances measured at A_{240}, A_{269} and A_{330}, every 30 s for a period of 10 min.

Mass spectrometry

For liquid chromatography-mass spectrometry (LC-MS), samples were chromatographed on a Jasco modular HPLC system (Great Dunmow, Essex, UK) using a Hichrom rpb column (15×0.2 cm, Hichrom, Reading, UK) eluting at 200 μl min^{-1} with a linear 40 min gradient of acetonitrile:water (10:90–70:30). Initially the solvents contained 0.01% formic acid, but this was eliminated for the final stages of analysis to prevent BMSF inactivation. The flow was directed through a Jasco diode array UV spectrophotometer and split one third into a Micromass Quattro II triple quadrupole mass spectrometer (Waters Ltd., Elstree, Herts, UK) operated in the negative ion electrospray mode. The remaining two thirds sample was collected as 200 μl fractions for further analysis. Nanospray MS^n was conducted on a Thermo Finnigan LCQ Deca (Hemel Hempsted, Herts, UK) in both positive and negative ion modes. Samples were dissolved in 50% acetonitrile:water and sprayed into the ion source using EconoTip nanospray needles (New Objectives, Woburn, MA, USA). Gas chromatography-mass spectrometry (GC-MS) was carried out on a VG Trio 1000 instrument (originally from Micromass, Altrincham, Cheshire, UK). Fractions were converted to their methyl ester O-triethylsilyl ether derivatives

using freshly prepared ethereal diazomethane (20 min, RT) followed by either *bis* trimethylsilyl trifluoracetamide or $^2H_9$ *bis* trimethylsilylacetamide (CDN Isotopes, Pointe Claire, Quebec, Canada) overnight. Samples were chromatographed on a DB5 column (30 m, Jones Chromatography, UK) using a 10° min^{-1} gradient from 100 to 350°C with helium as the carrier gas. The flow was routed into the electron impact or chemical ionisation ion sources of the mass spectrometer.

Chemical reactions

Catalytic hydrogenation was carried out in methanol using a PtO_2 catalyst for up to 2 h. Peracetylation was undertaken by treatment with pyridine:acetic anhydride (10:1 v/v) for up to 2 h. Pyridine was purified by passage through neutral alumina. Methoximation was carried out in 1% methoxyamine HCl in either water or pyridine for up to 18 h at RT. Reduction was undertaken with sodium borohydride in methanol (1 mg ml^{-1}) for 1 h at RT. Isotope exchange studies were carried out by incubating either BMSF or 8,15-dihydroxyeicosatetraenoic acid (8,15-diHETE) standard in $^2H_2O/CH_3CN$ (3:1 v/v) for up to 5 h.

Functional studies

The *in vivo* effects of the eicosanoids generated by adult *B. balanus* (BMSF and its apparent breakdown products) were tested by drilling a small hole (ca. 1.5 mm diameter) through one of the wall plates of adult barnacles, which was then temporarily plugged with Blu-Tack™. These animals were left to acclimatise in a beaker of FSW for 10 min at RT, then 20 μl of the test or control fractions resuspended in FSW were injected through the hole and the resulting change in behaviour observed.

The potential effects of purified BMSF and its breakdown products (identified by mass spectrometry) on muscle activity was examined by removing adult *B. balanus* from their shells intact and placing individuals in solid watch glasses containing 1 ml FSW for 10 min at RT. Changes in cirral and body contractions were then observed under a binocular microscope and counted for 10 min to provide a basal rate. The FSW was removed and the RP-HPLC fractions containing products of interest added. Activity was counted in 10 min periods for a total of 40 min. Results were expressed as percentage change in activity compared to the base rate (100%).

B. balanus seminal vesicles were dissected out and chopped in 200 μl FSW to release spermatozoa. Samples (100 μl) were then added to RP-HPLC fractions containing products of interest or as a control the same time fractions from RP-HPLC separation of FSW only. Any change in structure or swimming activity of these spermatozoa was then observed under a phase contrast microscope for 15 min.

Results

Generation of barnacle muscle stimulatory factor (BMSF)

Ionophore challenge of *B. balanus* seminal vesicles/testis generated a number of UV-absorbing components that were well separated by RP-HPLC at pH 7 (Fig. 1). The predominant UV absorbing product (peak 1 in Fig. 1A) eluted at 9 min on RP-HPLC and had a λ_{max} of 240.3 nm. Fractions containing this product alone exhibited potent muscle stimulatory activity (see below) and the product was hence termed barnacle muscle stimulatory factor (BMSF). A number of conjugated triene-containing products were also observed (at 10–14 min) with varying λ_{max} of 269–274 nm; the main product (peak 2, Fig. 1B) in terms of peak area, eluted at 12 min and exhibited a λ_{max} of 269.8 nm. Smaller amounts of a later-eluting conjugated pentaene chromophore-containing compounds were also present (23–25 min retention time; λ_{max} of ca. 330 nm; e.g. peak 3 in Fig. 1C). In comparison, the general body tissues (muscle, gut, cirri) generated a similar profile, although substantially less material (<15%) in terms of equivalent wet mass was produced (not shown). Mechanical disruption of tissues alone was also found to be a trigger for the biosynthesis of some product generation in the absence of ionophore.

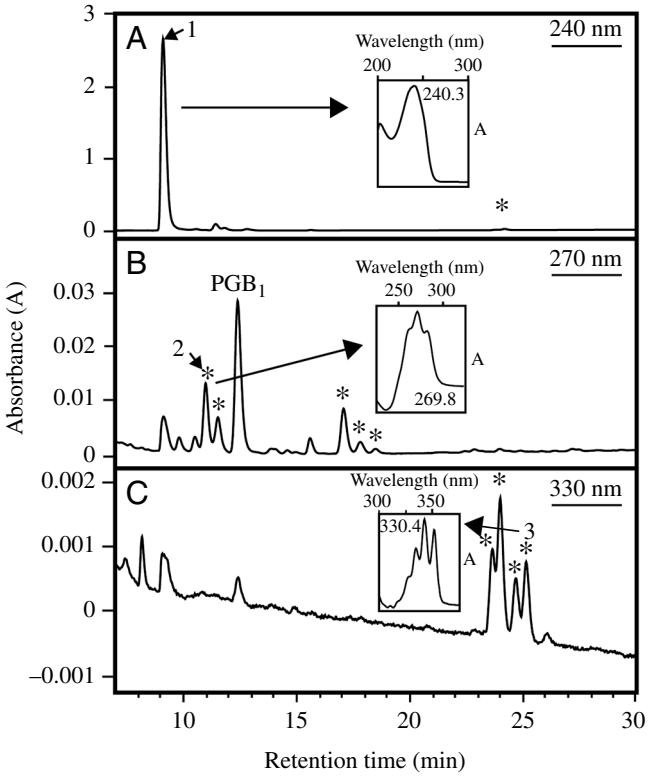

Fig. 1. RP-HPLC chromatograms of supernatants from A23187 ionophore-challenged barnacle seminal vesicles/testis separated at pH 7.0. (A) Biologically active BMSF (peak 1) elutes at 9 min. (B) Peak 2 (12 min); (C) Peak 3 (25 min). Insets show characteristic UV profiles of peaks indicated. Asterisks indicate peaks with a similar UV profile to that shown in insets.

Acidification of *B. balanus* supernatants to pH 3.5 prior to solid phase extraction resulted in a profound change in the product profile observed on RP-HPLC. In particular, the amount of BMSF (peak 1 in Fig. 1) was dramatically reduced and there was an increase in the number and amount of

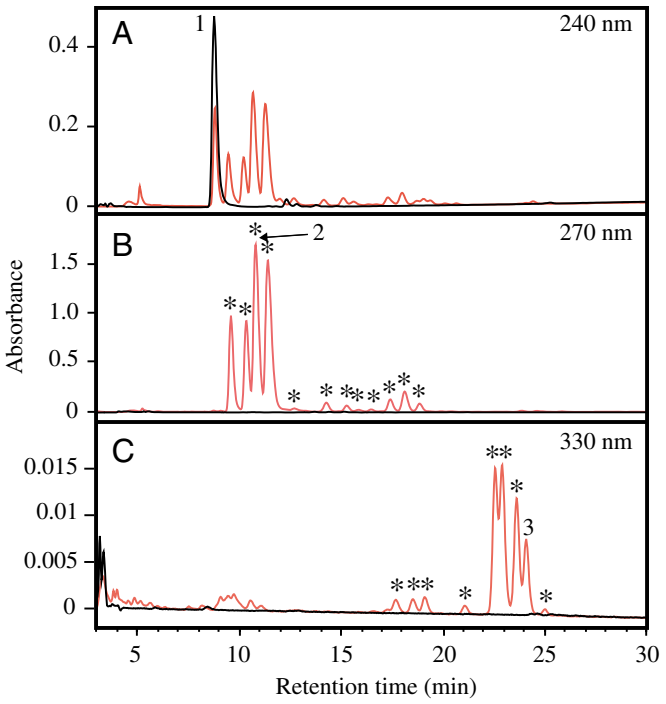

Fig. 2. Effect of pH prior to solid phase extraction, on the products produced by barnacle seminal vesicles/testis. Chromatograms shown are from RP-HPLC separation at pH 7.0 of material extracted at either pH 3.5 (red trace) or pH 7.0 (black trace). Note the significant reduction in peak 1 (BMSF; A) and the appearance of triene- and pentaene-containing compounds including peaks 2 (B) and 3 (C) following this brief period of acidification. Asterisks indicate peaks with similar UV profiles.

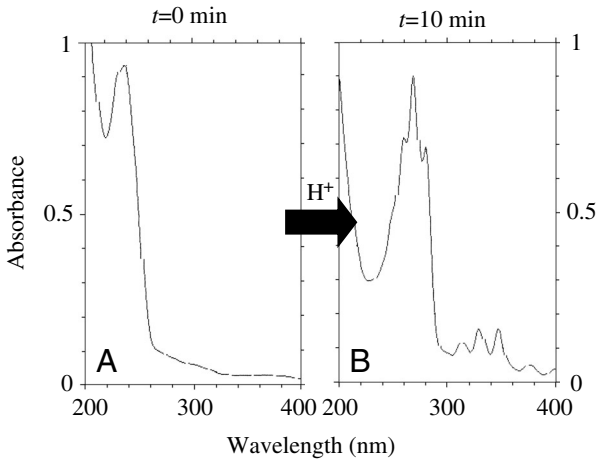

Fig. 3. Effect of acidification on BMSF. Product was collected from initial RP-HPLC separation at pH 7.0 (A), the pH was adjusted to 3.5 using acetic acid and the changes in UV absorbtion monitored spectrophotometrically at RT (B).

products containing conjugated triene and conjugated pentaene chromophores (Fig. 2). When HPLC-purified BMSF (i.e. collected from fractions post-separation at pH 7.0) was also acidified to pH 3.5, the UV profile altered from λ_{max} ~240 nm to 270 nm, with shoulders at ca. 259, 270 and 280 nm, characteristic of conjugated triene-containing products (Fig. 3). Smaller amounts of products with conjugated pentaenes (λ_{max} ca. 330 nm) were also apparent (Fig. 3). This acid-catalysed rearrangement occurred rapidly and was essentially complete within 10 min. To determine the nature of the potential breakdown products of BMSF (material under peak 1), fractions containing peaks 1 and 2 (see Fig. 1) from initial HPLC separation were acidified to pH 3.5, extracted and re-run on HPLC at pH 7.0. This showed that under such conditions BMSF (peak 1) broke down to yield a range of products including peak 2 (Fig. 4A–D). It also showed that peak 2 was relatively stable under acidic conditions (Fig. 4C).

As well as the novel compounds described, all *B. balanus* tissues biosynthesised both 8-hydroxyeicosapentaenoic acid (8-HEPE) and 8-HETE (identified by co-elution with authentic standards, as described in Maskrey et al., 2005; not shown).

Biosynthesis of BMSF

The addition of exogenous EPA (0–50 μmol l^{-1}) to the disrupted testis/seminal vesicle preparations caused a dose-dependent increase in the amount of A_{240}-absorbing BMSF generated (Fig. 5A). In contrast, the addition of AA or DHA (0–50 μmol l^{-1}) had no positive effect on the generation of either BMSF or its potential breakdown product 2 (data not shown). Production of BMSF was inhibited by the lipoxygenase inhibitors, esculetin (ca. 75% at 50 μmol l^{-1}), nordihydroguaiaretic acid (ca. 100% inhibition at 20 μmol l^{-1}) and the cytochrome P450 inhibitor, metyrapone (100 μmol l^{-1}; Fig. 5B). Another cytochrome P450 inhibitor, SKF525A at the same concentration, had no inhibitory effect (data not shown). In each case, similar levels of inhibition were observed for the acid-derived product 2. Together these data suggest that BMSF is a product of lipoxygenase-catalysed oxidation of EPA with some potential involvement of cytochrome P450 activities.

Functional significance of BMSF

The site-specific generation of BMSF by testis/seminal vesicles suggested a role for this compound in the reproductive process of barnacles. Addition of RP-HPLC purified BMSF to intact *B. balanus* removed from their shells resulted in a statistically significant increase in muscular activity of the cirri and body over time periods 10–40 min ($P<0.05$) when compared to the control (Fig. 6). Addition of fractions containing product 2 (produced by acid-catalysed breakdown of BMSF) resulted in no significant change in muscular contractions compared to the control (Fig. 6B). When BMSF at the same concentration as used in the *in vivo* studies was injected through the shell into whole barnacles it produced several changes in the animal's behaviour, which although not quantified, was manifested by the raising of the tergo-scutal plates (a process that occurs during feeding and fertilisation). In contrast, injection of the same volume of control material (same time fractions from FSW injection on RP-HPLC) had no effect.

BMSF had no obvious effect on the appearance of *B. balanus* spermatozoa observed by phase contrast microscopy. Most (ca. 95%) of these spermatozoa were immobile on initial observation, and incubation with fractions containing BMSF for periods of up to 30 min had no effect on the motility observed.

Structural characterisation of BMSF

The UV absorbance of BMSF is consistent with the presence of a conjugated diene, possibly constrained or partially conjugated to a heteroatom. BMSF eluted as a single peak at 27.3 min on microbore LC and generated an intense deprotonated molecular ion (M-H$^-$) at *m/z* 333, consistent with a dihydroxylated pentaene eicosanoid [C20:5(OH)$_2$]. This was confirmed by a series of simple chemical reactions. Following catalytic hydrogenation, BMSF was converted to the decahydro product (M-H$^-$ at *m/z* 343) together with smaller amounts of a hydroxyeicosanoic acid (*m/z* 327) and eicosanoic acid (*m/z* 311), formed as dehydration by-products. Peracetylation generated both the mono- and di-acetyl products (M-H$^-$ at *m/z* 375 and 417) and the monomethyl ester was formed by diazomethane treatment: *m/z* 371 (M+Na$^+$), *m/z* 349 (M+H$^+$), 331 (M+H$^+$ –H$_2$O). BMSF was not affected by either methoximation or borohydride reduction, which suggested that neither hydroperoxides nor simple ketones were present. The collisionally induced fragmentation (MS^2) of the M-H$^-$ ions of a number of eicosanoids were examined, including prostaglandins A$_2$, E$_2$, D$_2$, J$_2$, F$_{2\alpha}$, both AA- and EPA-derived mono- and dihydroxyeicosanoids, 12- and 15-HPETEs, cytochrome P450 products (EEPs and DHETres), carboxy leukotriene (LT) B$_4$, hepoxilin A$_3$, trioxilin A$_3$ and lipoxin B$_4$. They all fragmented with losses of H$_2$O and CO$_2$, and the majority also showed a characteristic cleavage at the allylic hydroxyl (–CH=CH-CH.OH-CH$_2$-R) with concomitant loss of RCHO. Collisionally induced fragmentation of the M-H$^-$ ion of BMSF (*m/z* 333) generated a spectrum with ions at m/z 315 (–H$_2$O), 297 (–2xH$_2$O), 289 (–CO$_2$), 271 (315 –CO$_2$), 219, 195, 193, 177 (195-H$_2$O), 171, 155 (OHC.C$_6$H$_{10}$.COO$^-$) and 149 (193-CO$_2$) (Fig. 7A). The presence of an 8-hydroxyl group was shown by the ion at *m/z* 155, which is present in the MS^2 spectra of all 8-hydroxylated eicosanoid standards tested. The interpretation of structure of ions at *m/z* 219, 195 and 193 was not immediately obvious.

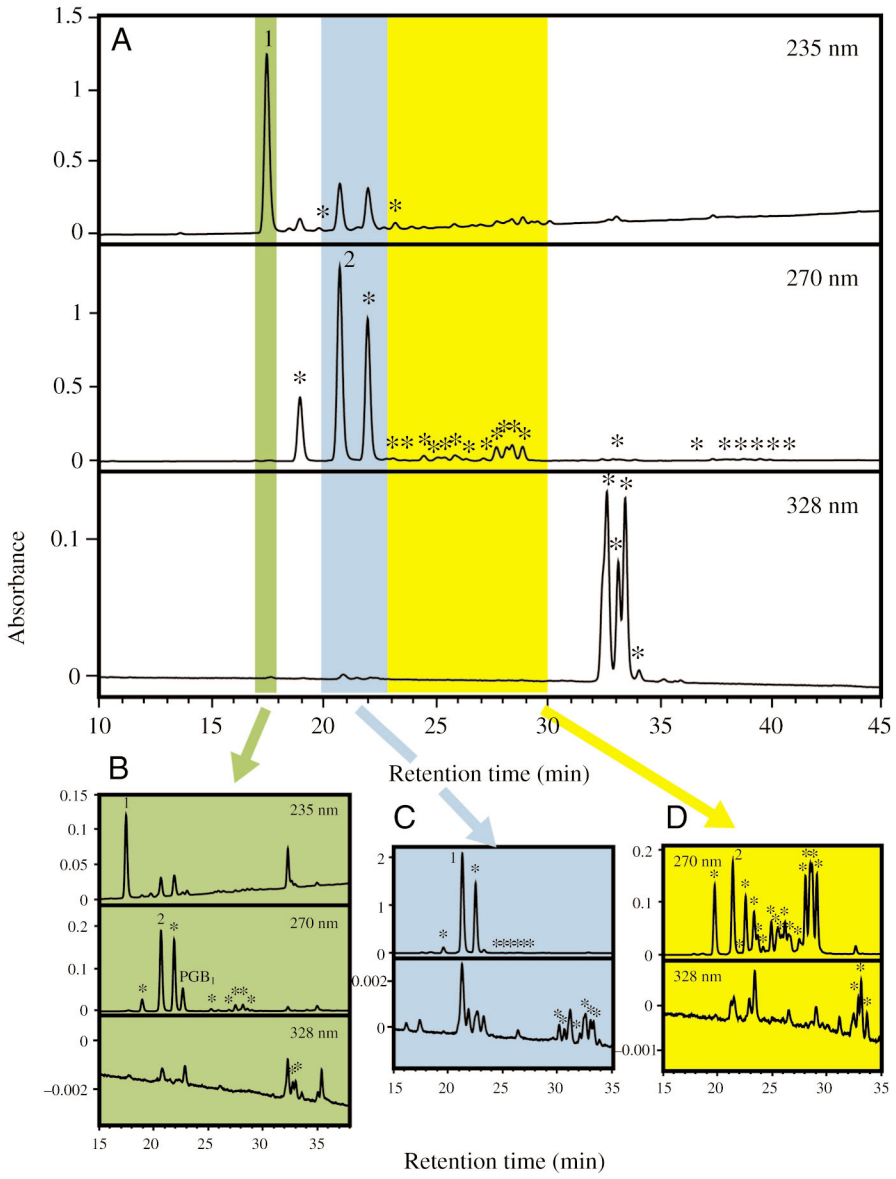

Fig. 4. Effect of acidification on post-HPLC separated products generated from ionophore-challenged barnacle seminal vesicles/testes. (A) The original RP-HPLC separation at pH 7.0 and the fractions collected (green, blue and yellow regions) that were subsequently acidified to pH 3.5, re-extracted and separated again by RP-HPLC at pH 7.0 (B–D).

They did not arise by simple cleavage at an allylic hydroxyl as was observed with the eicosanoid standards; however, they could all be further fragmented under MS^3 conditions with losses of CO$_2$ and H$_2$O, implying that they were oxygenated products containing a free carboxylic acid. Isotope exchange experiments in ^{2}H$_2$O showed that the characteristic ion *m/z* 193 was generated with hydrogen scrambling. This was in contrast to the 8-hydroxyl derived ion at *m/z* 155 and the ω-side chain loss (–C$_5$H$_{11}$CHO) from ^{2}H$_2$O-treated 8,15-diHETE used as control. Based on the known structural features of BMSF, together with MS^3 experiments, the characteristic ion at *m/z* 193 was judged to have arisen by cleavage between C11–C12 and cyclisation to form the pyran, [C$_4$H$_3$O].C$_6$H$_{10}$.COO$^-$]. The

ion at *m/z* 219 could then be CH_2=CH.[C_4H_2O].C_6H_{10}.COO^-, although this has not been confirmed. The ESP data are consistent with a structure for BMSF as 8,X-diHEPE. On acidification of BMSF with 5% formic acid, the M-H^- ion at *m/z* 333 was unchanged over 1 h eliminating the presence of an epoxide. Under the same conditions the hydroxy-epoxide, hepoxilin A_3 (M-H^-, *m/z* 335) was completely hydrolysed to trioxilin A_3 (M-H^-, *m/z* 353). The MS^2 spectrum of acid-treated BMSF showed that there had been complete conversion of BMSF to 8,15-dihydroxyeicosapentaenoic acid (8,15-diHEPE), with ions at *m/z* 333 (M-H^-), 315 (–H_2O), 297 (–2xH_2O), 289 (–CO_2), 271 (315 –CO_2), 235 (–C_5H_9CHO, allylic OH cleavage product), 155 (OHC.C_6H_{10}.COO^-).

BMSF and its acid-degraded products were then examined by capillary GC with both electron impact (EI) and chemical ionisation (CI) mass spectrometry. The methyl ester O-TMS ether of BMSF eluted as a broad peak on GC at 23.5–24.6 min, suggesting rearrangement on column. The CI spectrum showed an intense ion at *m/z* 493 ($M+H^+$) and 510 ($M+NH_4^+$), consistent with a monomethyl ester di-trimethylsilyl ether. It generated an EI mass spectrum with ions at *m/z* 492 (M^+.), 477 (–CH_3·), 463 (–C_2H_5·), 461 (–OCH_3·), 449 (–C_3H_7·), 429 (in some spectra), 402 (–TMSOH), 387 (477-TMSOH), 373 (463-TMSOH), 359 (449-TMSOH), 371 (461-TMSOH), 312 (402-TMSOH), 351 (–CH_2.CH=CH.$(CH_2)_3$.$COOCH_3$), 243 (TMSO=CH.CH_2.CH=CH.$(CH_2)_3$.$COOCH_3^+$), 261 (351-TMSOH), 221 ($C_6H_5TMS^{+}$·), 166 (C_6H_5.$OTMS^{+}$·), 151 (166-CH_3·), 129 and 73 (Fig. 7B). It was noted that the spectrum was similar to that of 8,15-diHEPE except that there was no evidence for ions at *m/z* 171/423 arising from 15-hydroxylation. The methyl ester O-[$^2H_9$] trimethylsilyl derivative gave analogous ions at 9u or 18u higher mass, confirming the 8,X-diHEPE structure. The acid-degraded product contained an 8,15-diHEPE, eluting at 24 min from GC with characteristic ions at *m/z* 492 (M^+.), 477, 461, 423 (–C_5H_9·), 402 (–TMSOH), 351 (– CH_2.CH=CH.$(CH_2)_3$.$COOCH_3$·), 333, 312, 282, 261 (351-TMSOH), 243 (TMSO=CH.CH_2.CH=CH.$(CH_2)_3$.$COOCH_3^+$), 217, 171 (TMSO=CH.$C_5H_9^+$) 129. The position of the second hydroxyl group in BMSF was determined from the hydrogenated

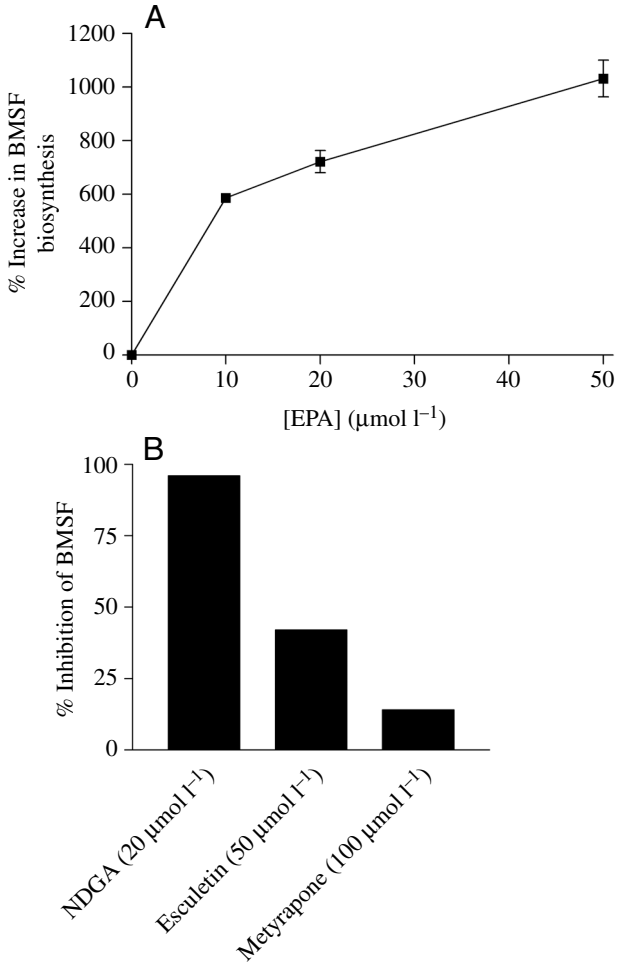

Fig. 5. (A) Exogenous EPA causes a dose-dependent increase in the generation of BMSF by disrupted *B. balanus* seminal vesicles/testis incubated at 12°C for 20 min. Products were separated by RP-HPLC at pH 7.0 and quantified as detailed in the Materials and methods. Values are means ± s.d. (B) BMSF production is inhibited by lipoxygenase inhibitors NDGA (20 µmol l^{-1}) and esculetin (50 µmol l^{-1}), and by the cytochrome P450 inhibitor, metyrapone (100 µmol l^{-1}). Representative data are shown from an experiment repeated several times.

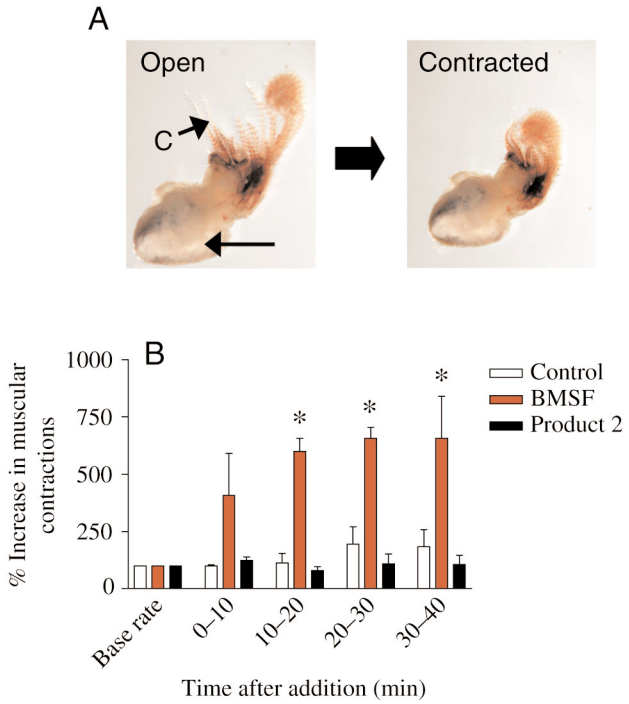

Fig. 6. (A) Spontaneous muscle contractions of an intact *B. balanus* following its removal from the shell. Note the contraction of the cirri (C) and generation reduction in size of the body mass following contraction. The whitish area highlighted (unlabelled arrow) is the region of the seminal vesicles/testis. (B) Effect of BMSF and breakdown product 2 on muscular contractions of *B. balanus* over time. Values are means ± s.e.m., *N*=3, **P*<0.05 compared with control (paired *t*-test).

product: the methyl-OTMS derivative of decahydro BMSF eluted at 32.2 min on GC and gave an electron impact spectrum with ions at *m/z* 487 (M^+.-CH_3·), 403 (–C_7H_{15}·), 359 [–.$(CH_2)_6$.$COOCH_3$], 313 (403-TMSOH), 269 (359-TMSOH), 245 [TMSO=$CH(CH_2)_6$.$COOCH_3^+$], 201 [TMSO=$CH(CH_2)_6$·CH_3^+] identifying it unequivocally as 8,13-dihydroxyeicosanoic acid (Fig. 7C). Some eicosanoic acid, 8-OH and 13-OH eicosanoic acids were also present, arising from facile dehydration during the catalytic hydrogenation. The fully hydrogenated acid-degraded product contained 8,15-dihydroxyeicosanoic acid, as expected.

Overall, these data are consistent with a structure for biologically active BMSF as 8,13-dihydroxyeicosapentaenoic acid (8,13-diHEPE; see Fig. 8) which, on acidification, degrades rapidly to a series of the biologically inactive products including at least one 8,15-diHEPE.

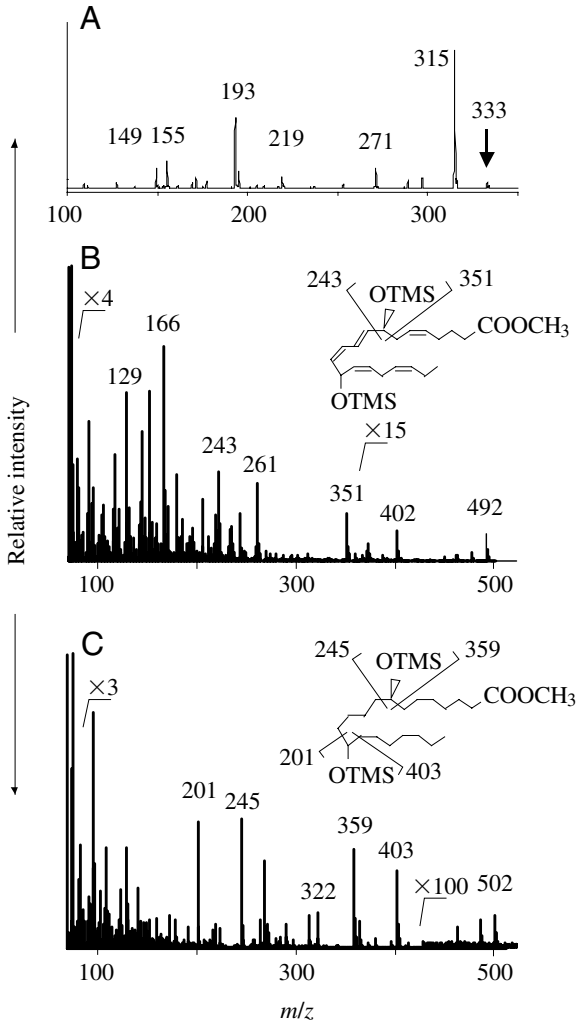

Fig. 7. (A) Collisionally induced dissociation of the deprotonated molecular ion BMSF. The ion at *m/z* 333 fragments to lose water and CO_2. Ions at *m/z* 155 (OHC.C_6H_{10}.COO^-) and 193 ([C_4H_3O].C_6H_{10}.COO^-), are characteristic; both lose CO_2 under further MS^3 dissociation. (B) Electron impact mass spectrum of the methyl ester O-trimethylsilyl ether of BMSF showing ions at *m/z* 492 (M^+.), 351 and 243 are characteristic of an 8,X-diHEPE. (C) Catalytic hydrogenation of the A_{240} material results in the formation of 8,13-dihydroxy eicosanoic acid, with characteristic ions at *m/z* 502 (M^+.), 201, 245, 322, 359 and 403.

Discussion

This current study has demonstrated that the intertidal barnacle *B. balanus* generates a novel EPA-derived eicosanoid/oxylipin, 8,13-dihydroxyeicosapentaenoic acid. Because this is capable of stimulating muscle activity we have termed this barnacle muscle stimulating factor (BMSF). Due to the tissue specificity for its generation in the testis/seminal vesicles, it was concluded that it might play a role in either spermatogenesis or the resulting fertilisation process. Barnacles are hermaphroditic and undergo internal fertilisation whereby a functioning 'male' inserts an extended penis into a receptive 'female', possibly following chemo-attraction from an odour cue produced by the cirral glands of the receptive female (Walley, 1967). Post-copulatory behaviour in such animals involves violent rocking movements of the opercular valves, which are controlled by the large internal opercular depressor muscles (Walley et al., 1971; Walker, 1980). This rocking behaviour was also observed in *B. balanus* in the current study after the injection of BMSF, suggesting that the natural active factor responsible for this activity is produced in the seminal vesicles/testis and delivered during copulation. Our current study has also shown a significant increase in muscular contraction in excised barnacles in the presence of BMSF, further strengthening the conclusion that this former compound aids fertilisation. It has been suggested that this muscular contraction following copulation causes the expulsion of oocytes and their passage through the oviduct into the mantle cavity (Barnes et al., 1977).

Addition of BMSF to barnacle spermatozoa was found to have no obvious effect on their morphology or motility. Indeed, in the current study only a small percentage (<5%) of sperm collected from the seminal vesicles were motile. Other studies on barnacle fertilisation have also reported a similar finding (Walley et al., 1971) and have implicated an

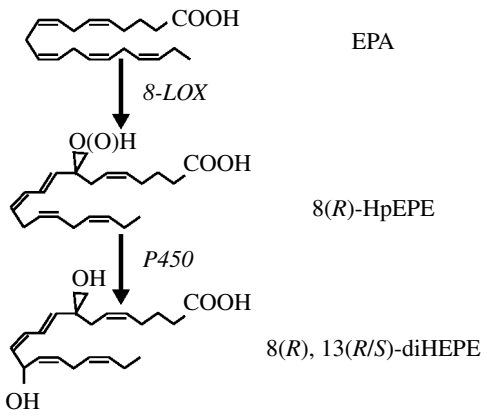

Fig. 8. Proposed pathway for the generation of BMSF (8,13-diHEPE). Note that the stereochemical assignments shown are tentative.

unidentified factor produced by the oviducal gland in the receptive 'female' that triggers sperm motility. Although BMSF is clearly not this factor, it is feasible that its presence in the 'semen' could act as a cue to stimulate the production of other eicosanoid 'oviducal factors'. Overall, there is an interesting parallel between the role of eicosanoids in barnacle and mammalian fertilisation. For instance, prostaglandins (PG), including PGE_1 and PGE_2 released into mammalian semen by the prostate gland, cause the contraction of smooth muscle in the uterus that may aid in fertilisation.

Under mild acidic conditions BMSF breaks down to form several compounds, which have no bioactivity in our assays. These include conjugated trienes, of which the major component is an 8,15-diHEPE, and pentaenes, which are probably hydroxyeicosahexaenoic acids formed by further elimination of water. Although these compounds were produced under non-physiological conditions in our study, it is possible that acid lability may act as method of limiting the duration of the biological activity of BMSF *in vivo*. However, it is difficult to envisage a situation *in vivo*, with the possible exception of the alimentary canal, where acidic conditions might exist. The pH of barnacle seminal fluid and ejaculate is also unknown.

Our studies only give a limited insight into the mechanism of biosynthesis of BMSF. EPA is the preferred substrate for this pathway, with neither AA nor DHA able to stimulate BSMF production suggesting that there is a specific requirement for this C20 n-3 polyunsaturated fatty acid (PUFA) in BMSF biosynthesis. Two oxygenation steps are required to generate a diHEPE. Our data showing the inhibitory effects of both esculetin and NDGA on BMSF synthesis are consistent with the involvement of a lipoxygenase as one of these steps. The presence of 8-lipoxygenase activity is common in aquatic invertebrates including corals (Bundy et al., 1986; Brash et al., 1996), starfish (Meijer et al., 1986), crabs (Hampson et al., 1992), sea squirts (Knight et al., 1999) and barnacles (Vogan et al., 2003). Such reports have shown that the hydroperoxides formed have an *R* rather than the *S* configuration, which is largely characteristic of vertebrate lipoxygenases (Brash et al., 1996; Schneider and Brash, 2002). There are no reports of a 13-lipoxygenase activity in any animals but a number of allylic monohydroxy fatty acid derivatives, including 13-HETE, can be synthesized *via* a cytochrome P450 dependent mechanism (Oliw et al., 1993; Brash et al., 1995; Bylund et al., 1998). Our preliminary data on metyrapone inhibition may indicate a potential involvement of P450s in BMSF synthesis. We therefore suggest that one possible mechanism of biosynthesis could involve initial lipoxygenation at C8 followed by direct hydroxylation at C13, perhaps by a P450 monoxygenase reaction, to form an 8,13-diHEPE (see Fig. 8). The possibility of other oxygenation mechanisms, and the involvement of intermediates such as epoxides, cannot be ruled out.

The stereochemistry of BMSF has not been defined; however, in common with many invertebrates (and other crustaceans; Hampson et al., 1992) an 8(*R*) hydroxyl group seems most likely. If the second hydroxylation occurs through a cytochrome P450 mechanism, then the 13-OH is probably a mixture of *R* and *S* forms (Oliw et al., 1993; Brash et al., 1995). It was possible to deduce the position of the double bonds in BMSF from the UV and MS data. The characteristic UV absorbance at 240 nm is immediately suggestive of a conjugated diene, bathochromically shifted by partial conjugation to a heteroatom. Fragment ions observed in the EI mass spectra analysis of BMSF (*m/z* 243 and 351) and its breakdown product, 8,15-diHEPE (*m/z* 171) would indicate that the $\Delta^{5,6}$ and $\Delta^{17,18}$ double bonds are present and are probably unchanged from the (all *Z*) EPA precursor. The mass spectrometric behaviour of the 13-hydroxyl group eliminated a simple *mono* allylic hydroxyl function. The data are consistent with *bis* allylic hydroxylation, and this is borne out by work from Brash et al. (1995) who showed that cytochrome P450-derived *bis* allylic (*Z*,*Z*) HETEs can rearrange under acidic conditions, with 13-HETE forming both 11- and 15-HETE. The rapid acid-mediated breakdown of BMSF to the isomeric 8,15-diHEPE (forming a $\Delta^{9,11,13}$ triene chromophore) is consistent with the presence of an (11*Z*, 14*Z*) allylic 13-hydroxyl moiety and would also imply the presence of a $\Delta^{14,15}$ non-conjugated (*Z*) double bond. The absence of any 8,11-diHEPE as a breakdown product would also indicate that the $\Delta^{11,12}$ double bond is not available for rearrangement and would be consistent with the presence of an 9,11 (*E,Z*) conjugated diene arising from 8-lipoxygenation. These data are consistent with a stereochemistry of BMSF as 8 (*R*), 13 (*R*/*S*) dihydroxy 5*Z*,9*E*,11*Z*,14*Z*,17*E* eicosapentaenoic acid.

In conclusion, we have shown for the first time that a novel eicosanoid, 8,13-diHEPE, is formed by barnacle testis/seminal vesicles. This compound probably acts by causing muscular contraction in the recipient 'female' during copulation that potentiates sperm–egg interaction. The full stereochemistry and confirmation of the proposed mechanism of biosynthesis remain to be resolved as does its mechanism of action on the muscular tissue of barnacles.

List of abbreviations

AA	arachidonic acid
BMSF	barnacle muscle stimulatory factor
CI	chemical ionisation
DHA	docosahexaenoic acid
EI	electron impact
EPA	eicosapentaenoic acid
FSW	filtered sea water
GC-MS	gas chromatography-mass spectrometry
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
LC-MS	liquid chromatography-mass spectrometry
LOX	lipoxygenase
LT	leukotriene
PG	prostaglandin
PUFA	polyunsaturated fatty acid

RP-HPLC	reverse phase-high performance liquid chromatography
RT	room temperature

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