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# 15-Hydroperoxy-PGE$_2$ – A Novel Intermediate in Mammalian and Algal Prostaglandin Biosynthesis

Hans Jagusch, Markus Werner, Oliver Werz*[b], and Georg Pohnert*[a]

**Abstract:** *Oxidized metabolites of $C_{20}$ fatty acids are potent lipid mediators contributing to the initiation and resolution of inflammation in mammals. One important class are the arachidonic acid-derived prostaglandins (PGs) with $PGE_2$ playing a central role in inflammation and numerous immunological reactions. The enzymes of $PGE_2$ biosynthesis are important pharmacological targets for anti-inflammatory drugs. Besides mammals, certain edible marine algae possess a comprehensive repertoire of bioactive arachidonic acid-derived oxylipins including PGs that may account for food poisoning. Here we describe the analysis of $PGE_2$ biosynthesis in the red macroalga* Gracilaria vermiculophylla *that led to the identification of 15-hydroperoxy-$PGE_2$, a novel precursor of $PGE_2$ and 15-keto-$PGE_2$. Interestingly, this novel precursor is also produced in human macrophages where it represents a key metabolite in an alternative biosynthetic $PGE_2$ pathway in addition to the well-established arachidonic acid-$PGG_2$-$PGH_2$-$PGE_2$ route. This alternative pathway of mammalian $PGE_2$ biosynthesis may open novel opportunities to intervene with inflammation-related diseases.*

Arachidonic acid (AA)-derived oxylipins are potent tissue hormones involved in numerous homeostatic biological functions but also in the pathogenesis of a variety of disorders.[1,2] In mammals, these lipid mediators are generated in response to extracellular stimuli by cyclooxygenases (COXs) and/or lipoxygenases (LOXs).[3] One class of these tissue hormones are the prostaglandins (PGs) that play a central role in regulating inflammation.[4] Increase of PG levels during acute inflammation is part of the immune responses to injury and infections. Chronically elevated PG levels are connected to the pathogenesis of arthritis, cancer, atherosclerosis, stroke, and neurodegeneration.[2,5,6] Non-steroidal anti-inflammatory drugs (NSAIDs) and coxibs (selective COX-2 inhibitors) block the biosynthesis of PGs.[7] $PGE_2$ **1** is involved in diverse biological processes that include ovulation, bone metabolism, blood vessel tone, and pain.[8] Its action is mediated *via* the G protein-coupled receptors EP1-4.[3,9] The well-known AA-$PGG_2$-$PGH_2$-$PGE_2$ biosynthetic pathway involving COX-1 and-2 as key enzymes (red arrows Figure 1) is a widely accepted route to **1** and no alternative branches for the generation of **1** in mammals were considered till today.

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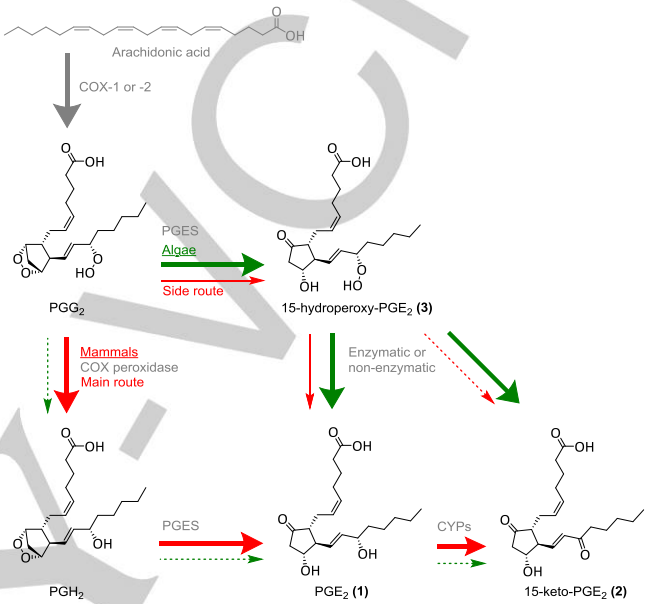


***Figure 1.*** Biosynthetic pathways for $PGE_2$ **1** and 15-keto-PGE2 **2** in mammals and algae. In the canonic pathway (thick red arrows), AA is converted by COX-1 or -2 to $PGG_2$ that is subsequently reduced by the peroxidase activity of the COX to $PGH_2$ and converted by $PGE_2$ synthase (PGES) to **1**. In the novel pathway introduced here (thin red and thick green arrows), $PGG_2$ is metabolized by a PGES-like activity to the novel intermediate 15-hydroperoxy-$PGE_2$ **3** that is reduced enzymatically or non-enzymatically to **1**. 15-keto-$PGE_2$ **2** occurs as a metabolic by-product. The contribution of both pathways to the formation of **1** differs between algae and mammals probably due to different COX peroxidase activity.

Besides mammals, also marine algae generate AA-derived oxylipins including PGs that function primarily as defense molecules.[10,11] PGs derived from the red macroalga *Gracilaria vermiculophylla* are involved in the chemical defense against grazers of the alga and are therefore associated with its invasive spreading after introduction from the northwest Pacific.[12,13] The oxylipins are rapidly biosynthesized in response to wounding of the algal tissue. They can also be induced in intact algae upon reception of signals from pathogens and serve to increase resistance.[10] Work on algal oxylipins has often stimulated novel concepts about oxylipin biosynthesis with implications for mammalian, plant, and algal pathways.[14,15] Edible red seaweeds of the genus *Gracilaria* contain high amounts of **1** related to numerous reported cases of food poisoning in East Asia upon ingestion of raw alga.[16] The algal formation of **1** and metabolic intermediates are not fully elucidated yet. To explore the algal biosynthesis of **1**, we systematically mined *G. vermiculophylla* for AA-derived oxylipins.

We collected fresh specimens of *G. vermiculophylla* from the Baltic Sea near Kieler Förde (Germany) for oxylipin extraction. To elucidate the complex oxylipin profile, we extracted *G. vermiculophylla* after mechanical wounding, a procedure that was previously shown to initiate the lipase- and

LOX-mediated formation of $C_{20}$ oxylipins.[11,13] Briefly, the alga was frozen in liquid nitrogen and ground. After thawing and incubation at room temperature, the oxylipins were taken up in methanol. Solid phase extraction (SPE) provided samples for ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS).[17] We identified the common algal $PGE_2$ **1** and 15-keto $PGE_2$ **2** as well as a novel putative oxylipin **3** (Figure 2).

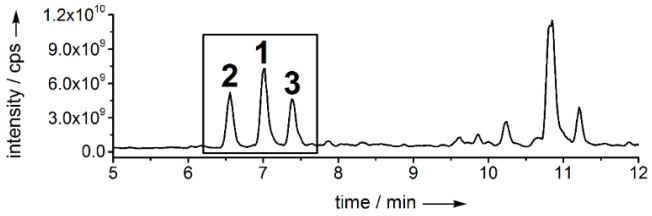


***Figure 2.*** UHPLC-MS profile of an extract from wounded *G. vermiculophylla*. The novel prostaglandin 15-hydroperoxy-$PGE_2$ **3** elutes at 7.38 min next to 15-keto-$PGE_2$ **2** at 6.56 min and $PGE_2$ **1** at 7.02 min. Dihydroxy-eicosatetraenoic acids (diHETEs) eluted at 9.50-11.50 min. The oxylipin profile was recorded in negative ionization mode and the total ion count in full MS is plotted.

The high-resolution mass of the pseudo molecular ion *m*/*z* 367.2116 $[M\text{-}H]^-$ suggested an elemental composition of $C_{20}H_{31}O_6$ (calculated mass 367.2120) consistent with a highly oxidized eicosanoid. For structure elucidation of **3**, we fractionated the algal extract by reversed-phase high-performance liquid chromatography (RP-HPLC) and obtained 21.4 µg compound $g^{-1}$ alga (fresh weight). The structure of **3** was assigned by 1D and 2D nuclear magnetic resonance (NMR) as well as by $MS^2$ (see Supporting Information SI-Figure 4 and SI-Table 1). A first comparison of NMR data suggested the molecule to be very similar to **1** with an intact 3-hydroxy-cyclopentanone core. The 5*Z*,13*E* double bond conformation was inferred by $^1H,^1H$-coupling. The UV spectrum with maximum absorption at 215 nm suggested a chromophore similar to that of **1** (see Supporting Information SI-Figure 5).[18] A hydroperoxy group at position $C_{15}$ was deduced from NMR and $MS^2$: The $^{13}C$-NMR signal of $C_{15}$-OOH (85.4 ppm) was downfield shifted compared the $C_{15}$-OH of **1** at 70.7 ppm.[19] The hydroperoxide **3** required lower energy for $MS^2$ fragmentation compared to hydroxy functionalized **1**. The $MS^2$ of **3** resembled that of **2**, which can be explained by an initial loss of water at $C_{15}$ from **3** and further similar fragmentation (see Supporting Information SI-Figure 4).

To determine the absolute configuration of **3**, we reduced the compound with $NaBH_4$ resulting in **1** and $PGF_{2\alpha/\beta}$. Co-injection with authentic standards confirmed the structures and further supported the hydroperoxy moiety in **3** (see Supporting Information SI-Figure 3). The circular dichroism (CD) spectra of **1** and **3** isolated from *G. vermiculophylla* were similar and matched with the literature data for mammalian **1**, thus confirming an identical configuration of **3** and mammalian 15*S*-configured **1** (see Supporting Information SI-Figure 6).[20] The novel oxylipin was thus elucidated as 15*S*-hydroperoxy-$PGE_2$ **3**. The structure and key NMR-correlations are shown in Figure 3.

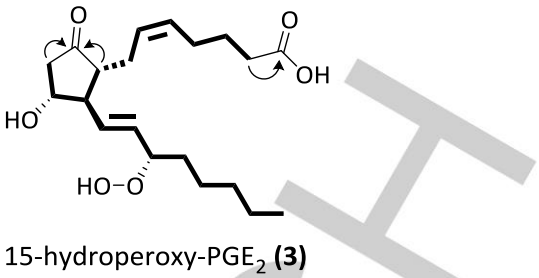


***Figure 3.*** Structure of 15-hydroperoxy-$PGE_2$ **3** with characteristic NMR correlations given as bold lines for $^1H,^1H$-COSY and as arrows for HMBC.

The hydroperoxide **3** was thermally unstable forming **1** and **2** (confirmed with commercially available standards Figure 4, SI-Figure 1 and SI-Figure 3). The ratio of both metabolites generated upon heating of **3** to 37 °C for 24 h was ca. 1:1, in accordance with a disproportion reaction. In wounded *G. vermiculophylla* this ratio was ca. 3:2 indicating an enzymatic contribution to their formation or metabolization.

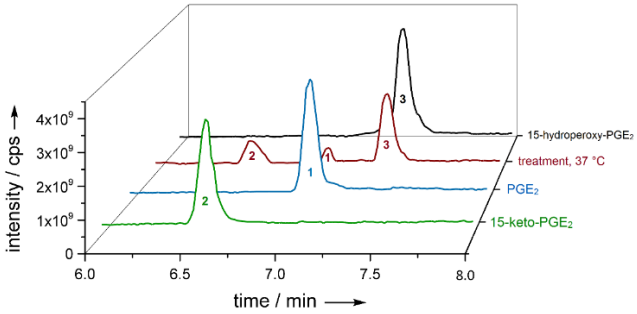


***Figure 4.*** UHPLC-MS profiles for 15-hydroperoxy-$PGE_2$ **3** (black, red at 7.36 min) and its degradation products 15-keto-$PGE_2$ **2** and $PGE_2$ **1** (red at 6.5 min and 7 min) formed at 37 °C (for 24 h) in comparison to PG standards: **1** (blue) at 7 min; **2** (green) at 6.5 min. All profiles were measured in negative ionization mode and the total ion count in full MS is plotted. Note that the detector response for the ionization of **1** and **2** is different and that, after normalization with external standards, observed peak areas in the treatment at 37°C correspond to equal molar amounts (see Supporting Information SI-Figure 8 and SI-Table 7).

In mammals, COX-1 or -2 catalyze the conversion of phospholipase-released AA to $PGG_2$. Subsequent transformation of the peroxide to $PGH_2$ is mediated by the peroxidase activity of the COX enzymes.[21] Subsequently, a PGES (three different isoenzymes are known) generates **1** from $PGH_2$.[22] Similar key enzymes were previously found in algae converting AA to **1**, however, isolation and elucidation of intermediates such as $PGH_2$ was not reported.[23,24] The novel labile PG **3** can be explained by the action of a COX converting AA, followed by a PGES-like transformation of the intermediate. The abiotic conversion of **3** to **1** and **2** suggests it to be an alternative intermediate in the biosynthesis of these PGs in the alga. The product ratio of the algal PGs indicates a mixed mechanism involving disproportionation of **3** and its enzymatic transformation. An algal pathway that does not follow the established AA-$PGG_2$-$PGH_2$-**1** route is supported by the fact that the COX of *G. vermiculophylla* shares just about 20 % of the amino acid sequence from the mammalian counterpart rendering the algal enzymes resistant to commonly available COX

inhibitors.[23] The peroxidase activity of the algal COX might be low, leading to the accumulation of $PGG_2$; the algal PGES might then open the endoperoxide of $PGG_2$ faster than the COX reduces the hydroperoxide yielding preferably **3**, thus privileging the novel route *via* **3** as compared to the well-known (mammalian) pathway *via* $PGH_2$.

Given the specificity of the abiotic transformation and the lability of **3** we reasoned that this molecule might also represent an overlooked intermediate in mammalian PG biosynthesis. We, therefore, surveyed lipid mediators in human monocyte-derived macrophages with an M1 phenotype that is known for substantial PG production during inflammation.[25,26] Stimulation of M1 with ionophore A23187 was used to induce PG biosynthesis.[27] Lipid mediator extracts were screened for **3** using the UHPLC-MS protocol described above. Indeed, **3** was detected (63 pg $2x10^6$ cells) besides **2** (109 pg $2x10^6$ $cells^{-1}$) and **1** (5370 pg $2x10^6$ $cells^{-1}$) in stimulated M1 macrophages. The amount of the hydroperoxide **3** increased upon A23187 stimulation, however it was still only detected in traces (ca. 1.2 %) compared to **1**. This would be in accordance with its role as an intermediate in $PGE_2$ biosynthesis (Figure 5).

Based on mechanistic considerations, **3** has been postulated in the early 1970s as an intermediate in the sequence AA-$PGG_2$-**3**-**1** but has, to our knowledge, never been confirmed.[28] Our finding now substantiates this pathway as an alternative route for the established sequence AA-$PGG_2$-$PGH_2$-**1**.[29] In comparison to M1 macrophages, we only found minor quantities of **3**, **2**, and **1** in M2 macrophages regardless of whether cells were stimulated or not. This is consistent with the fact that M2 express only low levels of COX-2 and PGES with moderate capacities to produce PGs (see also Supporting Information SI-Table 2).[30]

To confirm the hydroperoxide **3** as a direct precursor of **1**, we incubated either stimulated or non-stimulated M1 or M2 macrophages with **3** and analyzed the generated products by UHPLC-MS. The amounts of **1** in both phenotypes were significantly elevated in samples amended with **3** (Figure 6, see also Supporting Information SI-Tables 2 to 4). The amount of 15-keto-$PGE_2$ **2** determined in the samples was lower compared to **2** arising from the disproportionation of **3** in the medium control. This is possibly due to metabolization of **2** *via* reductases to 13,14-dihydro-15-keto-$PGE_2$ and eventually *via* β- and ω-oxidation to PGE-M (see Supporting Information SI-Table 3 and SI-Table 4, SI-Figure 9 and SI-Figure 10).[31] Although **3** accounts only for approx. 1.2 % in quantities compared to **1**, the contribution of this intermediate *via* the alternative biosynthetic branch to **1** is very likely higher because **3** is endogenously metabolized by human cells.

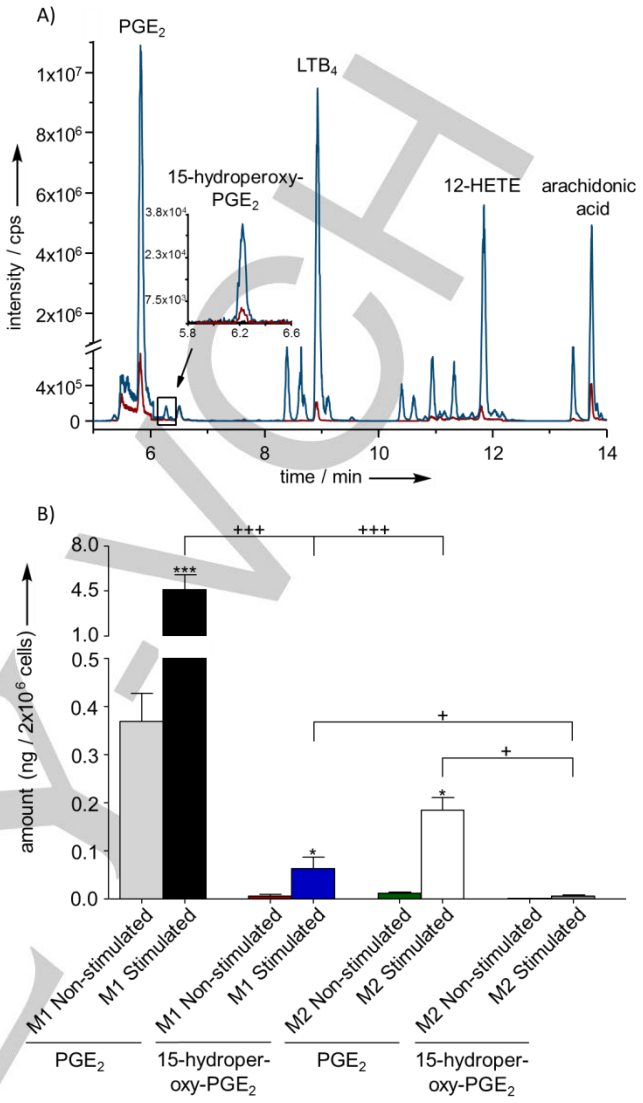


***Figure 5.*** A) UHPLC-MS profile of a lipid mediator extract from $2x10^6$ stimulated (blue) or non-stimulated (red) human M1 macrophages measured in negative ionization mode, plotted as total ion count in full MS. The enhanced region shows the extracted ion chromatogram for 15-hydroperoxy-$PGE_2$ **3**. The novel PG **3** elutes at 6.25 min. Further known lipid mediators were detected: $PGE_2$ **1** at 5.82 min, leukotriene $B_4$ ($LTB_4$) at 8.93 min, 12-HETE at 11.85 min, AA at 13.73 min. B) Amounts of **1** (grey, black, green, white) and **3** (red, blue, orange, dark grey) produced in $2x10^6$ stimulated (n=4) or non-stimulated (n=3) human M1 or M2 macrophages shown as means ± SEM. Cells were suspended in 1 mL PBS plus 1 mM $CaCl_2$ and incubated for 10 min at 37 °C with or without 2.5 µM A23187 or vehicle (0.5 % methanol). Statistical evaluation: one way ANOVA with Tukey Post-hoc test, $^{*/+}$ P≤0.05; $^{**/++}$ P≤0.01; $^{***/+++}$ P≤0.001, asterisks refer to comparison to non-stimulated conditions.

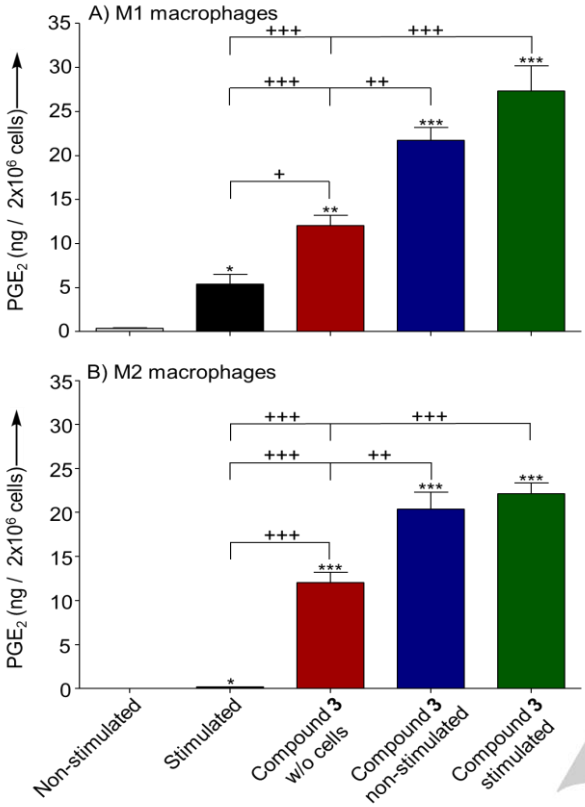


***Figure 6.*** Production of $PGE_2$ **1** in $2x10^6$ stimulated or non-stimulated A) M1 or B) M2 macrophages, either treated with 100 nM 15-hydroperoxy-$PGE_2$ **3** (stimulated, non-stimulated n=6, respectively) or vehicle (0.5 % methanol) (non-stimulated n=3; stimulated n=4). Cells were suspended in 1 mL PBS plus 1 mM $CaCl_2$ and pre-incubated with **3** or vehicle for 10 min at 37 °C. Cells were subsequently stimulated with 2.5 µM A23187 or vehicle (0.5 % methanol) and incubated for another 10 min at 37 °C. **3** was dissolved in 1 mL PBS plus 1 mM $CaCl_2$ and incubated in absence of cells for 20 min at 37 °C as control (n=6) to determine formation of **1** due to degradation. All values are shown as means ± SEM. Statistical evaluation: one way ANOVA with Tukey Post-hoc test, $^{*/+}$ P≤0.05; $^{**/++}$ P≤0.01; $^{***/+++}$ P≤0.001, asterisks refer to comparison to non-stimulated conditions.

In contrast to the transformation of **3** in the red macroalga *G. vermiculophylla*, where **1** and **2** were formed in a ratio of 3:2, M1 and M2 macrophages transformed **3** mainly to **1**, independently of A23187 stimulation. This indicates a COX-independent reducing activity in human cells. This process might be achieved enzymatically by reductases or mediated by reductants such as glutathione (GSH). It was previously shown that the catalytic activity of COX is affected by cellular conditions, such as oxidative stress, altered substrate, and GSH or hydroperoxide concentrations (*e.g.* 15-HPETE).[28,32,33] We thus assume that the second pathway to **1** *via* **3** may be an adaption toward inflammation that affects these cellular conditions. For therapeutic approaches, selective inhibitors that address the COX peroxidase domain without affecting the cyclooxygenase domain may be developed. This might aim towards inhibitors that reduce the carcinogenic effects caused by side products generated by COX peroxidase activity.[34] These alternative inhibitors might also alleviate gastrointestinal and cardiovascular side effects observed for non-steroidal anti-inflammatory drugs (NSAID) and coxibs.[35] Regarding ingestion of raw alga, increased levels of leukotriene $B_4$ and **1** are toxic for the gastrointestinal tract.[36,37] Both lipid mediators may be formed from labile precursors such as thermally unstable **3** or acid-labile 5*R*,8*R*-di hydroxyl eicosatetraenoic acid.[17]

In conclusion, we reveal here an alternative biosynthetic pathway to $PGE_2$ **1** in algae and in mammals. Upon cell activation, **1** is formed either *via* the canonical AA-$PGG_2$-$PGH_2$-**1** route catalyzed by COX, COX peroxidase and PGES or *via* the newly identified AA-$PGG_2$-**3**-**1** pathway mediated by COX, PGES, and hydroperoxide-reduction. The contribution of each pathway to the production of **1** differs between algae and mammals. Whereas the first route *via* $PGH_2$ dominates in mammals, algae preferentially rely on the second pathway *via* **3**, which represents a side route in human macrophages. These species-dependent preferences are probably due to differences in the COX proteins affecting the peroxidase activity. Interestingly, the hydroperoxide **3** is reductively converted mainly to **1** in human macrophages but in macroalgae conversion leads to a mixture of **1** and **2**.

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**Keywords:** alga · biosynthesis · inflammation · mammal · prostaglandins

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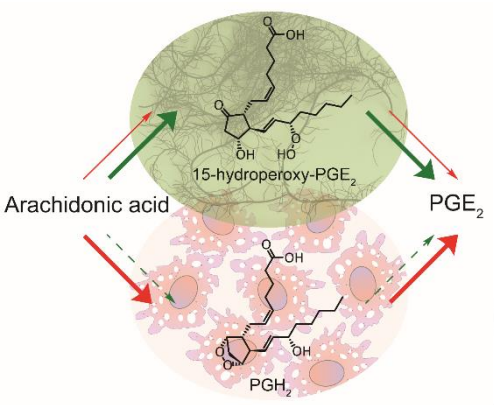
# COMMUNICATION

**A new pathway to a long-known lipid mediator** was discovered in the red alga *Gracilaria vermiculophylla* (top). Upon re-investigation of the mammalian biosynthesis 15-hydroxy-$PGE_2$ was also established as alternative pathway toward the inflammation mediating hormone.

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