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Discovery of a Highly Potent Anti-inflammatory Epoxyisoprostane-Derived Lactone

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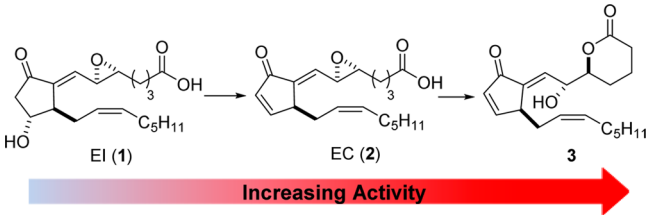
Supporting Information

ABSTRACT: Epoxyisoprostanes EI (**1**) and EC (**2**) are effective inhibitors of the secretion of proinflammatory cytokines IL-6 and IL-12. In detailed studies toward the investigation of the molecular mode of action of these structures, a highly potent lactone (**3**) derived from **1** was identified. The known isoprostanoids **1** and **2** are most likely precursors of **3**, the product of facile intramolecular reaction between the epoxide with the carboxylic acid in **2**.

Oxidized phospholipids (OxPLs) comprise a collection of biomolecules that have gained increasing attention as important, highly potent regulators of various physiological functions in higher organisms.[1] Among the different classes of OxPLs, the epoxyisoprostanes, such as EI (**1**) and EC (**2**) are particularly interesting, as their phosphatidylcholine-bound versions have been shown to play a pivotal role in the early development of atherosclerosis and other inflammatory conditions.[2] Recently, we disclosed a synthesis of epoxyisoprostanes **1** and **2** and, in accompanying biological studies, noted their unprecedented anti-inflammatory effects. This was manifest as the observed reduced secretion of proinflammatory cytokines IL-6 and IL-12 in bone-marrow-derived dendritic cells (BMDCs).[3] Additionally, we demonstrated that free acids **1** and **2** are more potent than their phospholipid-bound derivatives; significantly, **2** was shown to be more potent than **1**.

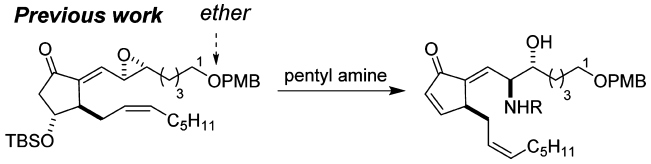
Over the course of studies aimed at understanding the chemistry and biology of **1** and **2**, we noted the ready formation of lactone **3** from **2** under physiologically relevant conditions (Scheme 1). Herein, we report the discovery of EC-derived lactone **3** as the most highly potent anti-inflammatory compound in the series as inhibitors of proinflammatory cytokines IL-6 and IL-12 secretion. We also document a collection of studies that underscore the role of the enone as the key structural feature responsible for biological activity as an anti-inflammatory agent.

Scheme 1. EI (1), EC (2), and EC-Derived Lactone 3 and Their Relative Activity as Inhibitors of Proinflammatory Cytokines IL-6 and IL-12 Secretion



The bioactivity of cyclopentenone (iso-)prostanes is versatile and resides both in interesting receptor-based mechanisms on their properties as potent electrophiles.[4−6] For the latter, OxPLs have been shown to undergo conjugate addition reactions with common nucleophiles, such as thiols found in intracellular domains.[7] This plays an important role in the oxidative stress response pathway, in which electrophiles such as eicosanoid enones bind to the cytoplasmic Keap1 complex. Upon binding, transcription factor Nrf2 is released from the complex, which then translocates into the nucleus, turning on the expression of antioxidant proteins.[8] In this context, the epoxyisoprostanes are especially intriguing because, in addition to electrophilic enones, they include an embedded allylic epoxide. The latter is expected to be highly susceptible to nucleophilic ring-opening reactions. In this respect, in previous work, treatment of an EI-derived ether with pentyl amine was observed to lead to an amino alcohol resulting from nucleophilic ring opening of the allylic epoxide (Scheme 2). This led to the proposal that similar reactions of

Scheme 2. Previous Model with Electrophilic Epoxide as Key to Biological Activity



lysines would account for the molecular mode of action for **1** and **2**.[9] However, it is important to note that these previous studies were conducted with an ether function at C1 rather than the native carboxylic acid.

The synthesis to prepare **1** and **2** provides convenient access to a wide range of derivatives of isoprostanoids. We surmised that these could serve as probes with which to examine the mode of action of this class of oxidized lipids. Because our initial investigations specifically underscored the high potency of dienone **2**, we sought to identify the origin of activity with enhanced resolution.

In our initial investigations, for the purposes of calibration, we compared the dose-dependent potency of **2** with known "anti-

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inflammatory prostaglandin" 15d-PGJ$_2$ (**4**, Figure 1a)[4a] and noted the superior potency of **2** in reduced secretion of

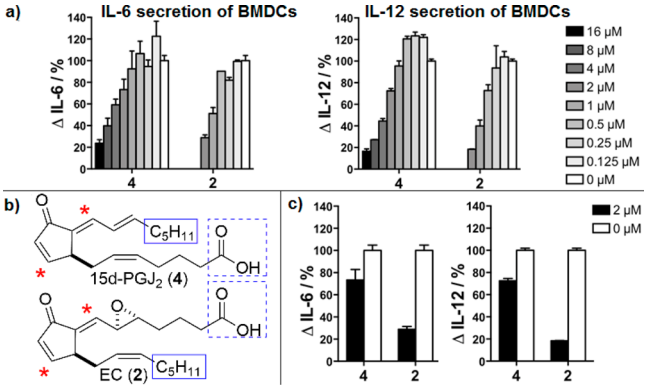


Figure 1. Comparison of the activity of prostaglandin 15d-PGJ$_2$ (**4**) and EC (**2**) to inhibit secretion of proinflammatory cytokines IL-6 and IL-12 in BMDCs. (a) Effects at varying doses of **4** and **2**. (b) Structural comparison of **4** and **2**. (c) Effects of **4** and **2** at a concentration of 2 μM compared to the solvent control.

proinflammatory cytokines IL-6 and IL-12 in BMDCs as it becomes even more obvious when compared at single concentration points (e.g., 2.0 μM, Figure 1c). This finding compelled us to compare and contrast the structures of the two lipids (Figure 1b), especially with respect to the influence of the Cα side chain. In this respect, **2** and **4** incorporate electrophilic endo- and exocyclic enones (red stars), but differ by the absence of the epoxide in the latter and the disposition of the carboxylic acid (Cα vs Cβ side chains, blue boxes). In our initial synthesis studies, we noted that, under mild acidic conditions, such as on silica gel, **2** undergoes isomerization into lactone **3** through a 6-exo-tet cyclization process (Scheme 1). This observation sparked our interest to investigate the contribution of the structure of the Cα side chain to anti-inflammatory effects and, specifically, to assess whether lactone **3** was active. If the latter were shown to be active, then the biological activity of this series of compounds would not be due to the electrophilic epoxide.

To assess the activity of lactone **3**, a set of EC analogues **5**−**7** with **3** was synthesized to systematically probe the influence of the Cα side chain (Figure 2). Compound **5** results from

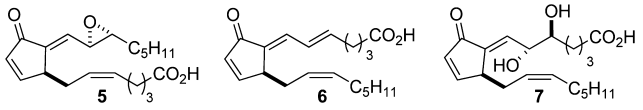
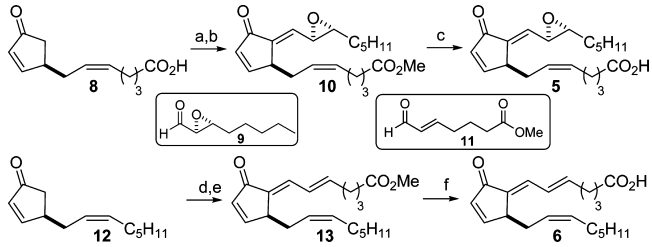


Figure 2. Set of EC analogues prepared and investigated.

switching the position of the carboxylic acid from the Cα to the Cβ side chain, so that the epoxide and the carboxylic acid are no longer proximal and thereby unlikely to participate in macrolactonization. Trienone **6** displays a 1,6-dienone similar to that found in **4**. Comparison of the potency of structures **5** and **6** with parent structures **2** and **4** could give insight into the effects of a 1,6-dienone versus a 1,4-(5,6-epoxy)enone on the electrophilicity of the oxidized lipid. Diol acid **7** was investigated to determine whether lactone **3** is a precursor to a more active diol seco-acid derivative.

Synthesis of **5** begins with cyclopentenone **8** (Scheme 3). Compound **8** is an intermediate in the synthesis of **4** and was prepared in analogy to **12**.[3] Stepwise aldol condensation of

Scheme 3. Synthesis of EC Analogues 5 and 6[a]

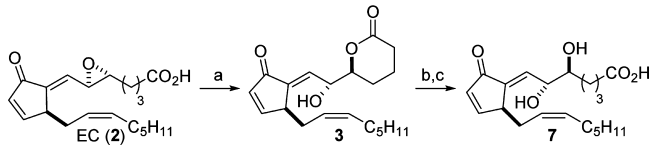


[a]Reagents and conditions: (a) *i*-Pr$_2$NLi, **9**, THF, −78 °C; then TMSCHN$_2$, C$_6$H$_6$/MeOH, rt; (b) MsCl, Et$_3$N, CH$_2$Cl$_2$, −78 °C; then Al$_2$O$_3$, CH$_2$Cl$_2$, rt; (c) Novozyme, buffer pH 7/THF (4:1), 46% over 3 steps; (d) LiHMDS, **11**, THF, −78 °C; (e) MsCl, Et$_3$N, CH$_2$Cl$_2$, −78 °C; then Al$_2$O$_3$, CH$_2$Cl$_2$, rt, 55% over 2 steps; (f) Novozyme, buffer pH 7/THF (4:1), 78%.

enone **8** and epoxyaldehyde **9** that is prepared by organocatalytic enantioselective epoxidation of (*E*)-oct-2-enal[10] afforded methyl ester **10**, which was transformed into acid **5** by enzymatic saponification. Synthesis of **6** was achieved in a similar fashion: aldol condensation of cyclopentenone **12** with (*E*)-enal **11** furnished methyl ester **13** that in turn was converted into trienone **6**.

Preparation of **3** and **7** commenced with **2** (Scheme 4). Lactone **3** had been observed to form as a side product during

Scheme 4. Synthesis of EC Analogues 3 and 7[a]



[a]Reagents and conditions: (a) SiO$_2$, CHCl$_3$, rt, 65%; (b) K$_2$CO$_3$, MeOH, rt; (c) Novozyme, buffer pH 7/THF (4:1), 30% over 2 steps.

column chromatographic purification of **2** on silica gel.[11] Accordingly, **2** was dissolved in a slurry of silica gel in CHCl$_3$ to afford **3** in 65% yield. Direct hydrolysis of lactone **3** to **7** under alkaline conditions proved difficult as a consequence of the instability of the product. Instead, a two-step procedure was enacted in which the lactone was first opened in alkaline methanol to furnish the corresponding methyl ester, prior to enzymatic hydrolysis to yield diol **7**.

Parent compounds **2** and **4** along with the various analogues synthesized were examined in assays to probe reduction in the secretion of IL-6 and IL-12 in a dose-dependent manner (Figure 3). All compounds were active as inhibitors, albeit with a range of efficiencies. Importantly, none of **5**−**7** were more active than **2**.

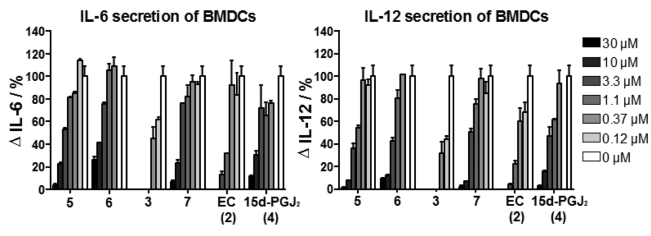


Figure 3. Comparison of the activity of prostaglandin 15d-PGJ$_2$ (**4**), EC (**2**), and analogues **3** and **5**−**7** in inhibiting secretion of proinflammatory cytokines IL-6 and IL-12 in BMDCs.

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Analogue **5** was more active than **4** and trienone **6**. This result gives insight into the inductive effect of the epoxide compared to the corresponding γ,δ-enone, where it becomes evident that the presence of the epoxide enhances the potency of the compounds. However, since **5** was also less active than parent **2**, the epoxide cannot be solely responsible for the superior potency of **2**.

Examination of lactone **3** and diol **7** led to a surprising observation. Although seco-acid **7** is less active than **2**, lactone **3** elicits the strongest effect of all compounds examined to date in inhibiting the secretion of proinflammatory IL-6 and IL-12.

The large concentration intervals used in the assays illustrated in Figure 3 resulted in only three assessment data points for lactone **3** before the cytotoxic concentration was reached. To examine the activity of **2** and **3** at greater resolution, a second titration series with smaller concentration intervals was conducted (Figure 4). The observed results show that **3** is

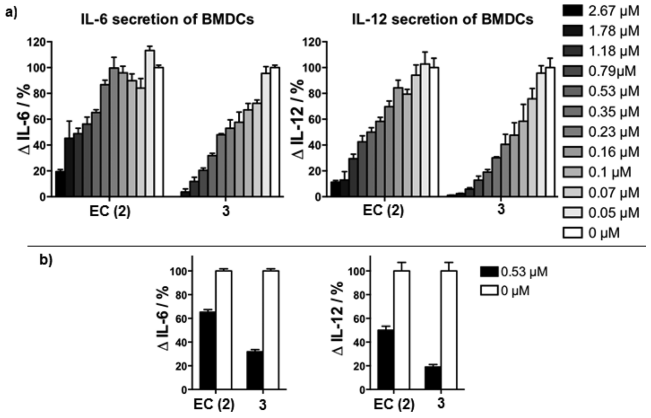


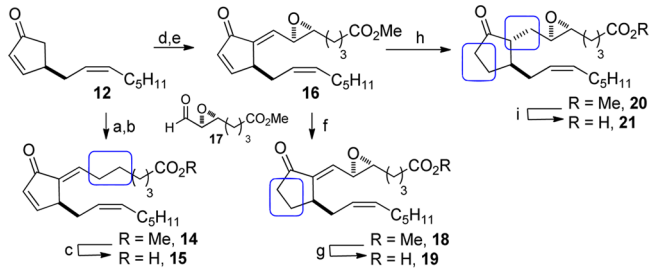
Figure 4. Titration assays of the highly potent compounds EC (**2**) and lactone **3** to establish their activity in inhibiting secretion of proinflammatory cytokines IL-6 and IL-12 in BMDCs. (a) Effects at varying doses of **2** and **3**. (b) Effects of **2** and **3** at a common concentration of 0.53 μM compared to the solvent control.

more potent than **2** even at very lowest concentrations, which is surprising in light of the prior proposal suggesting the epoxide as the reactive site.

Having established the superiority and unexpected activity of lactone **3**, we subsequently set out to identify the electrophilic site in **2** necessary for bioactivity in the assays. Analogue structures **15**, **19**, **21**, and **26** were prepared from cyclopentenone **12**, as shown in Schemes 5 and 6. Dienone **15**, lacking an epoxide, was accessed by aldol addition with methyl 7-oxoheptanoate, followed by elimination to generate enone **14**. Hydrolysis of methyl ester **14** to free acid **15** was carried out enzymatically using Novozyme. Dienone **16**, which incorporates an epoxide, served as precursor for **19** and **21**. In our previous synthetic studies to **3**, we observed that the endocyclic enone in **2** reacted selectively in the presence of 1 equiv of nucleophile, especially when the nucleophile is sterically demanding. Consequently, Stryker's reagent $[(PPh_3)CuH]_6$ was used to conduct enone reduction.[12] Selective conjugate reduction of the endocyclic enone was accomplished when 0.175 equiv of the hexameric reducing complex was employed to obtain methyl ester **18**. With 0.35 equiv, both enones were reduced to furnish methyl ester **20**. Enzymatic hydrolysis of **18** and **20** afforded free acids **19** and **21**.

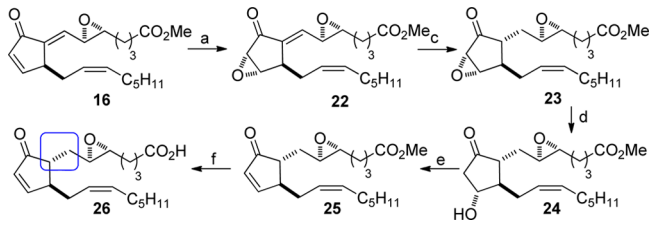
Analogue **26**, in which the exocyclic enone was reduced selectively, proved to be more challenging to prepare (Scheme

Scheme 5. Synthesis of the EC Analogues 15, 19, and 21[a]



[a]Reagents and conditions: (a) LiHMDS, THF, −78 °C; then methyl 7-oxoheptanoate; (b) MsCl, Et_3N, CH_2Cl_2, −78 °C; then Al_2O_3, CH_2Cl_2, rt, 57% (2 steps); (c) Novozyme, buffer pH 7/THF (4:1), rt, 77%; (d) LiHMDS, THF, −78 °C; then **17**; (e) MsCl, Et_3N, CH_2Cl_2, −78 °C; then Al_2O_3, CH_2Cl_2, rt, 64% (2 steps); (f) $[(PPh_3)CuH]_6$ (0.175 equiv), water, C_6H_6, rt, 47%; (g) Novozyme, buffer pH 7/THF, rt, 56%; (h) $[(PPh_3)CuH]_6$ (0.35 equiv), water, C_6H_6, rt, 65%; (i) Novozyme, buffer pH 7/THF, rt, 51%. Blue boxes indicate alterations with respect to EC (**2**).

Scheme 6. Synthesis of EC-Analog 26[a]



[a]Reagents and conditions: (a) *t*-BuOOH, DBU, THF, 0 °C, 74%; (b) Novozyme, buffer pH 7/THF (4:1), rt, 74%; (c) $[(PPh_3)CuH]_6$ (0.28 equiv), water, C_6H_6, rt, 74%, dr = 10:1; (d) SmI_2, THF−MeOH (7:3), −90 °C, 86%; (e) MsCl, Et_3N, CH_2Cl_2, 90%; (f) Novozyme, buffer pH 7/THF (4:1), rt, 65%. Blue boxes indicate alterations with respect to EC (**2**).

6). The higher reactivity of the endocyclic enone rendered the selective reduction of the exocyclic enone difficult. A method published by Evans and Fu for the selective reduction of (*E*)-enones, leaving (*Z*)- or endocyclic enones intact, was not successful.[13] Thus, we decided to exploit the increased reactivity of the endocyclic enone for the preparation of **26**. The endocyclic enone in dienone **16** was epoxidized selectively under nucleophilic conditions to provide epoxyketone **22**. Exposure of **22** to Stryker's reagent gave reduced epoxide **23** that, upon reductive epoxide opening with SmI_2, delivered β-hydroxy ketone **24**.[14] Mesylation of the β-hydroxy resulted in concomitant elimination and regeneration of the endocyclic enone to yield methyl ester **25**, which was further elaborated into **26**.

The new analogues obtained were tested together with parent **2** for their ability to reduce IL-6 and IL-12 secretion (Figure 5). Cyclopentanone **21** showed no biological activity, which indicates that the presence of an enone in the molecule is crucial for the investigated bioactivity. Compound **19**, which no longer contained an endocyclic enone, exhibited drastically diminished potency compared to **2**. In contrast, **26**, which is lacking the exocyclic enone, still displays considerable potency, although it is less active than the parent compound **2**. If no epoxide is present in the molecule, as in **15**, the potency of the molecule is reduced and comparable to that of **26**.

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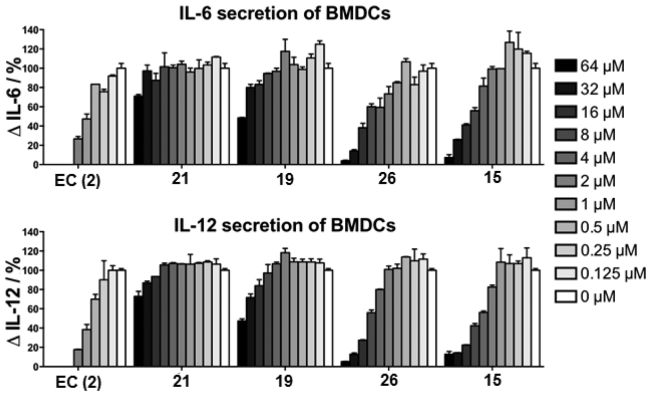

Figure 5. Effects of the analogues for inhibiting secretion of proinflammatory cytokines IL-6 and IL-12 in BMDCs.

The observation that **2** undergoes ready conversion to lactone **3** has provided key insights into the chemistry of this class of isoprostanes. Importantly, the greater activity of the lactone, which lacks an epoxide, underscores that this plays a minor role as an electrophilic site for decreasing IL-6 and IL-12 secretion. Additional investigations of structures in which the endocyclic enone is absent destroy the molecule's capability for cytokine inhibition completely, revealing that the endocyclic enone is crucial for biological activity. Prior investigations by us revealed that **1** readily undergoes elimination in aqueous media,[3] and indeed, it is well worth noting that analysis of the NMR spectra first reported for **1** reveals the presence of signals consistent with **2**.[2a] When combined with the results from this study, it leads to a hypothesis that lactone **3**, as the most active agent, elicits the observed anti-inflammatory effects. It can be speculated that highly potent lactone **3** is formed to some degree under physiological conditions from **2**. Lactone **3** is not only the chemically most stable compound from the series **1**−**3** but, in turn, might also constitute a longer lived version of free acid **2** being stabilized against β-oxidation and therefore exhibiting higher activity in vivo.[15] This explanation could account for the significant difference in biological activity between **4** and **2**, as only the latter is able to form the lactone. The results set the stage for additional challenging experiments involving detection of the various structures intracelullarly in real time. Additionally, it provides new leads for the development of anti-inflammatory therapeutics.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and characterization data for all reactions and products, including ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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