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Selective identification of specialized pro-resolving lipid mediators from their biosynthetic double di-oxygenation isomers

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Abstract

The n-3 polyunsaturated fatty acids are substrates for lipoxygenases and cyclooxygenases. During inflammatory processes, these enzymes form several distinct families of oxygenated polyunsaturated fatty acids coined specialized pro-resolving lipid mediators. Structural elucidation of these natural products using LC-MS/MS based metabololipidomics with the pico- to nanogram amounts of biosynthetic material available have been performed. The specialized pro-resolving lipid mediators display stereospecific and potent anti-inflammatory and pro-resolving actions. Most often the different families among these mediators are chemically characterized by two or three chiral, secondary alcohols, separated by either an *E,E,Z*-triene or an *E,Z,E,E*-tetraenemoiety. The lipoxygenases also form other oxygenated polyunsaturated natural products, coined double di-oxygenation products, that are constitutional isomers of the protectin and maresin families of specialized pro-resolving lipid mediators. Very often these products exhibit similar chromatographic properties and mass spectrometrical fragment ions as the pro-resolving mediators. In addition, the double di-oxygenation products are sometimes formed in larger amounts than the specialized pro-resolving lipid mediators. Thus, it is not always possible to distinguish between the specialized pro-resolving mediators and their double di-oxygenation isomers in biological systems, using LC/MS-based techniques. Herein, a convenient and easy-to-use protocol to meet this challenge is presented.

Graphical abstract

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Introduction

Polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (**1**, DHA), play a major role in the physiology of living organisms.¹ Recent efforts have established that DHA (**1**) is a substrate in the biosynthesis of several potent anti-inflammatory pro-resolving mediators such as protectin D1 (PD1, **2**),² maresin 1 (MaR1, **3**),³ resolvin D1 (RvD1, **4**),^{2a,4} and resolvin D2 (RvD2, **5**)^{2a,4} (see Figure 1 for chemical structures). Other examples of pro-resolving mediators are lipoxin A₄ (LXA₄, **6**)^{5,6} and lipoxin B₄ (LXB₄, **7**).^{5,6}

All of these are biosynthesized during the resolution phase of the acute-inflammatory response and have enabled new research areas related to many disease states associated with inflammation.^{7,8} For example, were cently reported additional evidence for the biosynthesis⁹ of protectin D1 (**2**) that confirmed earlier results.¹⁰ A human 15-lipoxygenase (15-LOX) type I mediated pathway in combination with enzymatic hydrolysis produces protectin D1 (**2**) *via* the two intermediates 17*S*-hydroperoxide intermediate (**10**) and 16*S*,17*S*-protectin epoxide (**11**) (Scheme 1).^{9,10} Alternatively, molecular oxygen addition at C10 of **10** yields the double di-oxygenation product 10*S*,17*S*-diHDHA (**8**),^{2a,10,11} also denoted protectin DX.¹¹

The biosynthesis of maresin 1 (**3**) and its double di-oxygenation product 7*S*,14*S*-diHDHA (**9**) is carried out by the action of 12-lipoxygenase (12-LOX),¹² and shares several steps with the biosynthesis outlined in Scheme 1. Both pathways result in the formation of two chiral, secondary alcohols separated by the six carbon atoms of an *E,E,Z*-triene moiety, see Scheme 2.

These docosanoid-based families of the different SPMs display high structural complexity due to the presence of several stereogenic centers, both in the form of chiral, secondary alcohols and conjugated *E*- and *Z*-double bonds,⁷ reflecting their biochemical origins, functions and stereo specific bioactions towards different G-coupled receptors (GPCRs).¹³ Moreover, the individual SPMs often exhibit identical chromatographic properties, resulting in incomplete separation of peaks.^{2,14} These properties are also found with other lipid mediators, such as the pro-inflammatory mediator leukotriene (LTB₄, **12**), that displays similar chromatographic behavior to its double lipoxygenation product 5*S*,12*S*-diHETE.¹⁵ Of the two, it is only LTB₄ (**12**), see Figure 2, that carries potent biological actions.¹⁵

Therefore, the unambiguous identification of closely related isomers of the distinct SPM families is of fundamental importance to the elucidation of their chemical structures, but also to the understanding of their biosynthetic origins and function in numerous biological processes. Of particular importance to the structural elucidation of SPMs products

biosynthesized during inflammatory processes, are their isomeric double di-oxygenation products.^{2a,7,11} Two examples are 10*S*,17*S*-diHDHA (**8**)² and 7*S*,14*S*-diHDHA (**9**),³ see Figure 1 for chemical structures. The double di-oxygenation products **8** and **9** also contain two chiral, secondary alcohols, but the double bond geometry of these two compounds constitute an *E,Z,E*-triene moiety amid the two alcohols. Of note, these oxygenated lipids display distinct activities and potencies from the SPMs.^{11,16} Isomerization of the chemically sensitive *E,E,Z*-triene, found in both protectin D1 (**2**) and maresin 1 (**3**), to their corresponding *E,E,E*-triene diastereomers, is also observed when analyzing SPMs produced *in vivo* by inflammatory exudates.⁷ Also, the n-6 PUFA arachidonic acid is biosynthetically converted into anti-inflammatory oxygenated compounds,¹⁷ such as the lipoxygenase interaction products termed lipoxin A₄ (**6**) and lipoxin B₄ (**7**), see Figure 1.^{5,6} Moreover, novel n-3 docosapentaenoic acid derived SPMs have also been reported,¹⁴ such as PD1_{n-3} DPA (**13**) (Figure 2), which is biosynthesized, via a pathway analogous to that described above for protectin D1 (**2**).¹⁸ PD1_{n-3} DPA also carries potent pro-resolution and anti-inflammatory actions.¹⁸ Hence, the isolation and structural elucidation of novel SPMs with such biologically fascinating actions is of considerable interest in the biomedical research community.¹⁹ Towards these ends, advances in LC-MS/MS metabololipidomics techniques have emerged.^{20,21} In parallel, an approach that may facilitate the exact structural elucidation of structurally challenging SPMs is chemical derivatization, and some derivatization protocols have been reported for the identification and structural elucidation of PUFAs.²² However, harsh derivatization conditions not suitable for the SPMs are employed, and the intensity of the ions obtained from the mass spectra is often low.

Given the small amounts of material and the chemically sensitive structures of the SPMs, which owes to the presence of two secondary allylic alcohols in conjugation with labile *E,E,Z*-trienes or *E,E,Z,E*-tetraenes, caution has to be exercised when deciding on an appropriate derivatization agent. As outlined above, the *in vivo* biosynthetic pathways of DHA (**1**) to protectin D1 (**2**) and maresin (**3**), yield both product mixtures with *E,E,Z*- or *E,Z,E*-triene geometries of the conjugated double bonds with different bioactions and biological potencies. These geometrical differences caught our interest, since a diene with an *E,E*-configuration is far more reactive in the Diels-Alder cycloaddition reaction than an *E,Z*-diene.²³ We were also aware of the guiding principles of click-chemistry, outlined by Sharpless and co-workers for cycloaddition reactions,²⁴ that have been applied in the development of synthetic protocols.^{25,26} For useful applications of the Diels-Alder cycloaddition reaction towards the structural elucidation of SPMs, a highly reactive dienophile must be employed. The 4-substituted-1,2,4-triazole-3,5-diones are examples of such dienophiles.^{27,28} In particular, the safe, easy-to-handle, commercially available solid, 4-phenyl-1,2,4-triazole-3,5-dione (PTAD, **14**), is a premier example of such a highly reactive dieneophile.²⁷

Only dienes that can adopt the *s-cis* conformation undergo the Diels-Alder reaction (Scheme 3). Any substitution pattern favoring the *s-trans* conformation, slows down the Diels-Alder cycloaddition, as is the case for *cis*-1,2-disubstituted dienes.²³ Hence, we envisioned that the double di-oxygenation products **8** and **9**, due to their *E,Z,E*-trienes, would be far less reactive in a Diels-Alder reaction with **14**. Given the importance of specific double bond geometries

to biological actions and potencies,^{3,10} our investigations towards development of a protocol for the selective identification of SPMs from their biosynthetic pathway markers are reported.

Results and Discussion

First, *E. coli* inoculation in mice was performed as reported earlier,²⁹ evoking a self-limited host response in which polymorphonuclear leukocyte (PMN) infiltration reached a maximum at approximately 12 h. After this period, the mice were euthanized and peritoneal exudates collected as reported.²⁹ In Figure 3, the chromatograms of the two DHA-derived SPMs protectin D1 (**2**) and maresin 1 (**3**), together with their respective double di-oxygenation isomers **10** and **11**, are depicted. To be able to develop a protocol for the selective identification of SPMs we employed validated synthetic protectin D1 (**2**)³⁰ and its congener PD1_{n-3}DPA (**13**)¹⁸ that were each separately reacted with 4-phenyl-1,2,4-triazole-3,5-dione (**14**) and separately profiled using LC-MS/MS-based lipid mediator metabololipidomics (see Experimental section for details).²¹ The results are depicted in Figure 4. For the two C22-derived SPMs, protectin D1 (**2**) and PD1_{n-3}DPA (**13**), the chromatographic behavior and the respective MS/MS spectra indicated the presence of Diels-Alder products formed between **2** and **14** (Figure 4A), but also between **13** and **14** (Figure 4B). Distinct m/z values for ions assigned to the PTAD-derivatized products of both **2** and **13** were observed at $m/z = 534$ and 536 , respectively. The MS/MS spectra for the Diels-Alder product of **2** (PTAD-PD1) displayed characteristic fragmentation patterns with the following fragments assigned: $m/z = 516$ [M-H⁺-H₂O]⁺, $m/z = 498$ [M-H⁺-2H₂O]⁺ and $m/z = 490$ [M-H⁺-CO₂]⁺, see Figure 4A. The PTAD-derivatized products exhibited prominent molecular ion peaks and diagnostic fragment ions due to the double bond position in the SPM **2**, as well as the PTAD-moiety. In particular, $m/z = 397$, $m/z = 371$ [M-H⁺-CO₂-PhCNO]⁺ and $m/z = 352$ were diagnostic, see Figure 4A for interpretations. A similar pattern was observed for the PTAD-PD1_{n-3}DPA product at m/z values 518, 500, 492, 399, 373 and 352, respectively (Figure 4B). The fragment at $m/z = 352$ is the same for the PTAD-derivatized products of both **2** and **13**, as the C15–C22 fragment is identical for both SPMs. Synthetic material of the isomer of **2**, namely maresin 1 (**3**), reacted just as smoothly with **14**, giving rise to $m/z = 534$ [M-H⁺]⁺ and $m/z = 514$ [M-H⁺-H₂O]⁺, see Figure 5. The loss of the C1–C7 fragment was prominent in the MS-spectra of the MaR1-PTAD product, $m/z = 392$. The method was conveniently extended to the two DHA-derived SPMs resolvin D1 (RvD1, **4**) and resolvin D2 (RvD2, **5**), Figure 6. The MS/MS-spectra of the PTAD-derivatives of **4** and **5** also showed diagnostic m/z values. For RvD1 (**4**), at $m/z = 550$ [M-H⁺]⁺ and $m/z = 506$ [M-H⁺-CO₂]⁺, and for RvD2 (**5**) at $m/z = 550$ [M-H⁺]⁺, $m/z = 532$ [M-H⁺-H₂O]⁺ and $m/z = 506$ [M-H⁺-CO₂]⁺. In both cases, the retro-Diels-Alder products were observed as the base peaks at $m/z = 176$. Moreover, the trihydroxy PTAD-derivatives of LXA₄ (**6**) and LXB₄ (**7**) showed a similar fragmentation patterns for both regioisomeric SPMs. The relevant m/z values were observed at 526 [M-H⁺]⁺, 508 [M-H⁺-H₂O]⁺, 490 [M-H⁺-2H₂O]⁺, 482 [M-H⁺-CO₂]⁺ and 464 [M-H⁺-CO₂-H₂O]⁺ (Figure 7). To our delight, no Diels-Alder reaction took place when reacting **14**, in individual experiments, with the two double di-oxygenation products 10*S*,17*S*-diHDHA (**8**) and 7*S*,14*S*-diHDHA (**9**), see Figure 8. These experiments were repeated in the presence of equimolar amounts of protectin D1 (**2**)

with the only observed Diels-Alder products observed being those derived from **2**. No PTAD-derivatives of **8** and **9** were detected. Hence, as elective identification of specialized pro-resolving lipid mediators as their PTAD-derivatives, from mixtures with their biosynthetic double di-oxygenation isomers **8** and **9**, was achieved. This protocol was further extended to *in vivo* biosynthetic material. To the mouse exudate extract obtained as described above, an excess of 4-phenyl-1,2,4-triazole-3,5-dione (PTAD, **14**) was added. Then, as proof of principle, PTAD-derivatives of both protectin D1 (**2**) and its congener PD1_{n-3} DPA (**13**), were investigated using multiple reaction monitoring (MRM)-mode. The results are depicted in Figure 9. These results and fragmentation patterns are in accord with the discussion outlined above for Figure 4A and Figure 4B, respectively. Moreover, we were also able to detect the Diels-Alder product LTB₄-PTAD after reaction of **14** with LTB₄ (**12**), see Figure 9A.³¹ Of importance, no formation of a Diels-Alder product from the reaction of **14** with the double lipoxygenation product 5*S*,12*S*-diHETE, was observed. Overall, the results depicted in Figure 9d demonstrate the utility of this easy-to-use protocol on materials present in picogram levels in biological samples.

Conclusions

To summarize, herein we present a straightforward protocol for the selective identification of pro-resolving and anti-inflammatory oxygenated polyunsaturated fatty acids, named specialized pro-resolving lipid mediators. This protocol is based on the guiding principles of click-chemistry, amenable for biological conditions,³² that we have now extended to the Diels-Alder reaction for the selective identification of SPMs. Distinct MS/MS fragmentation patterns were observed for all cycloaddition products obtained upon reaction of the dienophile 4-phenyl-1,2,4-triazole-3,5-dione (**14**) with different SPMs. In contrast, the non-resolving double di-oxygenation compounds were unreactive towards this reagent. For challenging and difficult-to-separate, co-eluting compounds, biosynthesized simultaneously with the SPMs, this fast, reproducible and easy-to-use protocol facilitates the identification of novel SPMs using LC-MS/MS based metabololipidomic techniques. Of note, the MRM chromatograms depicted in Figures 3 – 7 and 9, contain the entire structures of each individual SPM-derivative, attesting to the chemical stability of these Diels-Alder products. The protocol can easily be extended beyond the analysis of novel SPMs to other natural occurring olefinic compounds and their further metabolites that can be present at very low levels in biological samples. Such efforts have indeed been reported after we initiated our investigations on the SPMs.³³

Experimental section

Synthetic materials of resolvin D1 (RvD1, **4**), resolvin D2 (RvD2, **5**), lipoxin A₄ (LXA₄, **6**), lipoxin B₄ (LXB₄, **7**), 5*S*,12*S*-diHETE, 10*S*,17*S*-diHDHA (**8**), 7*S*,14*S*-diHDHA (**9**) and leukotriene B₄ (**12**) were obtained from Cayman Chemicals, while protectin D1 (**2**), maresin 1 (MaR1, **3**), and PD1_{n-3}DPA (**13**) were obtained by total synthesis as in ref.^{18,30,34} 4-Phenyl-1,2,4-triazole-3,5-dione (**14**), dry MeOH and CH₂Cl₂ were all purchased from Sigma-Aldrich.

Derivatization of SPM with 4-phenyl-1,2,4-triazole-3,5-dione (**14**)

A 5% molar excess of a stock solution of **14** (83 mg/mL in CH₂Cl₂) was added to an EtOH solution containing the SPM, the double di-oxygenation product or the exudate of interest at room temperature. The vial was then sealed and kept on ice for 30 minutes in the dark. The solvent mixture was evaporated under a gentle stream of nitrogen, the reaction mixture re-dissolved in 100 µL of CH₂Cl₂, and then filtered through a 0.1 cm plug of SiO₂-gel in a Pasteur pipette by eluting with 500 µL of hexane:EtOAc (1:1). Solvents were evaporated again and the residue re-dissolved in 100 µL of MeOH. This sample was analyzed by LC-MS/MS using lipid mediator metabololipidomics.²¹ The same procedure was employed when an inflammatory exudate was analyzed. The derivatization with **14** can be monitored either visually, since the deep red color of the PTAD-reagent **14** disappears when the reaction is complete. Alternatively, the reaction with an individual SPM can be monitored by UV-spectroscopy and the change in the intensity of the λ_{max} -value of the SPM, before and after addition of 4-phenyl-1,2,4-triazole-3,5-dione (**14**). Observation of the complete disappearance of the λ_{max} -peak assures a full conversion into the Diels-Alder product.

Microbial-initiated inflammation *in vivo* by *E. coli* inoculation

Male FVB mice (6 to 8 weeks old) purchased from Charles River Laboratories were fed ad libitum Laboratory Rodent Diet 20–5058 (Lab Diet, Purina Mills). Animal experimental procedures were approved by the Standing Committee on Animals of Harvard Medical School (protocol no. 02570) and complied with institutional and US National Institutes of Health (NIH) guidelines. Mice were given vehicle together with live *E. coli* (serotype O6:K2:H1; 10⁷ CFU) or saline injections (mock infection) i.p. After 12 h, mice were euthanized and the peritoneal exudate was collected, see reference 29 for details. The local eicosanoid levels and identification PTAD-products were assessed by targeted lipid mediator metabololipidomics as described below.

Lipid Mediator Metabololipidomics.²¹

The LC-MS/MS system included a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector (Shimadzu) paired with a QTrap 5500 (ABSciex) coupled to a Poroshell 120 EC column (100 mm × 4.6 mm × 2.7 µm; Agilent) maintained at 50 °C using a column oven (ThermaSphere TS-130; Phenomenex) employed for identification and quantification. For product profiling the following mobile phase was employed: MeOH/H₂O/acetic acid of 55:45:0.1 (vol:vol:vol) with a gradient ramped to 80:20:0.1 (vol:vol:vol) for 8 min, that was held for 3 min and then ramped to 98:2:0.1 (vol:vol:vol) for the next 0.1 min and maintained at 98:2:0.1 (vol:vol:vol) for 4 min, with a flow rate maintained at 0.6 mL/min. The QTrap 5500 was operated in negative ionization mode using scheduled multiple reaction monitoring coupled with information-dependent acquisition (IDA) and an enhanced product ion scan (EPI).

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Figure 1. Chemical structures of some SPMs and the two the double di-oxygenation products 10*S*, 17*S*-diHDHA (**8**) and 7*S*,14*S*-diHDHA (**9**).

Figure 2.
Chemical structures of leukotriene B₄ (LTB₄, **12**) and PD1_{n-3} DPA (**13**).

Figure 3.

Chromatographic separation of protectin D1 and maresin 1 from their double di-oxygenation isomers. Mice were administered *E. coli* (10^7 CFU), exudates obtained 12h later and the products extracted and subject to LC-MS/MS metabololipidomics. MRM chromatograms for exudates: (a) Protectin D1 (PD1, **2**) and 10S,17S-diHDHA (**8**) (m/z 359->153). (b) Maresin 1 (MaR1, **3**) and 7S,14S-diHDHA (**9**) (m/z 359->250). Results are representative of $n=12$ mouse exudates.

Figure 4.

Characterization of the di-hydroxy PTAD-derivatives of docosahexaenoic acid- and n-3 docosapentaenoic acid-derived mediators protectin D1 (PD1, **2**) and PD1_{n-3DPA} (**13**). Products were analyzed using LC-MS/MS metabololipidomics. MRM chromatograms obtained from using (a) m/z 534 $\rightarrow$ 181 and (b) m/z 536 $\rightarrow$ 183. Results are representative of 3 independent preparations.

Figure 5.

Characterization of the di-hydroxy PTAD-derivative of maresin 1 (MaR1, **3**). Products were analyzed using LC-MS/MS metabololipidomics. MRM chromatograms obtained using m/z 534- $\rightarrow$ 370. Results are representative of 3 independent preparations.

Figure 6.

Characterization of the tri-hydroxy PTAD-derivatives of docosahexaenoic acid-derived mediators resolvins D1 (RvD1, **4**) and resolvins D2 (RvD2, **5**). Products were analyzed using LC-MS/MS metabololipidomics. MRM chromatograms obtained using m/z 550->141. Results are representative of 3 independent preparations.

Figure 7.

Characterization of the tri-hydroxy PTAD-derivatives of arachidonic acid derived-mediators lipoxin A₄ (LXA₄, **6**) and lipoxin B₄ (LXB₄, **7**). Products were analyzed using LC-MS/MS metabololipidomics. MRM chromatograms obtained using m/z 526->115. Results are representative of 3 independent preparations.

Figure 8.

4-Phenyl-1,2,4-triazole-3,5-dione (PTAD, **14**) reacts selectively with mediators carrying *E,E*-conjugated double bonds and not their double di-oxygenation isomers. Products were analyzed using LC-MS/MS metabololipidomics. Results are representative of 3 independent preparations.

Figure 9.

Chromatographic separation of PTAD-products, obtained when reacting **14** with SPMs and their double di-oxygenation isomers. Mice were administered *E.coli* (10^7 CFU), exudates obtained 12h later and products analyzed using LC-MS/MS metabololipidomics. MRM chromatograms for exudates: (a) Product of leukotriene B₄ (LTB₄, **12**) and PTAD (**14**) (m/z 510->176). (b) Protectin D1 (PD1, **2**) and PTAD (**14**) (m/z 534->181), (c) PD1_{n-3}DPA (**13**) and PTAD (**14**) (m/z 536->183). Results are representative of $n = 12$ mouse exudates.

Scheme 1.

Outline of the biosynthetic pathways of protectin D1 (**2**) and the isomeric double di-oxygenation product 10*S*,17*S*-diHDHA (**8**).

Scheme 2.

Outline of the biosynthetic pathways of maresin (3) and the isomeric double di-oxygenation product 7*S*,14*S*-diHDHA (9).

Scheme 3.

General outline of the Diels-Alder reaction between a 1,4-disubstituted *E,E*-olefin, present in SPMs, and **14**.