From Vietnamese plants to a biflavonoid that relieves inflammation by triggering the lipid mediator class switch to resolution

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Abbreviations: 5-H(p)ETE, 5-hydro(pero)xy-eicosatetraenoic acid; 12-HHT, 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid; COX, cyclooxygenase; DAD, diode array detector; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ECD, electronic circular dichroism; ESI, electrospray ionization; FCS, fetal calf serum; HPLC, high performance liquid chromatography; HR, high resolution; IFN, interferon; IL, interleukin; LOX, lipooxygenase; LT, leukotriene; LTC4S, leukotriene C4 synthase; MaR, maresin; mPGES-1, microsomal prostaglandin E2 synthase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBMC, peripheral blood mononuclear cells; PD, protectin; PG, prostaglandin; PMNL, polymorphonuclear neutrophils; RP, reversed phase; Rv, resolvin; sEH, soluble epoxide hydrolase; SPE, solid phase extraction; SPM, specialized pro-resolving mediators; TX, thromboxane; UPLC–MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry.

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1. Introduction

Inflammation is a self-defensive process of the host microcirculation against external stimuli like pathogen infection and injury\(^1\). Both initiation and termination of inflammation are regulated through a network of oxygenated lipid mediators, which are produced from polyunsaturated fatty acids by a limited set of biosynthetic enzymes that are differentially expressed in innate immune cells and are spatially and temporally controlled\(^2\). Inflammation is initiated and maintained by the production of pro-inflammatory mediators, among which leukotrienes (LTs) and prostaglandins (PGs) are dominating\(^3\). The physiological inflammatory response is actively terminated in the resolution phase, a homeostatic process that is governed by the biosynthesis of specialized pro-resolving mediators (SPM)\(^4,5\). Impaired resolution together with the excessive production of pro-inflammatory mediators and the failure to dampen their signaling allow acute inflammation to turn into a chronic state, which causes permanent tissue damage and is closely related to chronic inflammatory pathologies, such as osteoarthritis, atherosclerosis, asthma, and cancer\(^6\). Classical strategies to treat inflammation mainly focus on suppressing pro-inflammatory signaling, while novel approaches aim at promoting resolution programs.

LTs and PGs are produced by lipoxygenases (LOXs) and cyclooxynases (COXs) in concert with further redox- and non-redox-active enzymes\(^7\), whose inhibition relieves inflammation-related diseases like asthma, allergy, and osteoarthritis\(^8,9\). However, current anti-inflammatory drugs targeting COX and 5-LOX are associated with gastrointestinal, renal, and cardiovascular side effects\(^10\text{--}12\) and were proposed to impair resolution\(^13\). Additional targets within eicosanoid biosynthesis, \(i.e.,\) microsomal prostaglandin E\(_2\) synthase 1 (mPGES-1), leukotriene C\(_4\) synthase (LTC\(_4\)S), and soluble epoxide hydrolase (sEH), were reported\(^14,15\), and polypharmacological strategies were explored\(^16,17\), including dual inhibition of LT biosynthesis and mPGES-1\(^18\). While some of these approaches favorably modulate pro-inflammatory lipid mediator signaling, strategies that alter lipid mediator networks from a pro-inflammatory to a pro-resolving state have not yet been realized, despite their proclaimed high therapeutic potential\(^19,20\).

SPM biosynthesis essentially depends on the activity of 12-LOX and 15-LOX, which are abundantly expressed in anti-inflammatory (M2-like) macrophage subsets and other immune cells\(^21,22\). 5-LOX also participates in SPM biosynthesis but its role is diffuse. Increasing evidence shows that SPM inhibit neutrophil trafficking, scavenge pro-inflammatory cytokines, stimulate efferocytosis and bacterial clearance, promote tissue regeneration, and protect from oxidative stress\(^4,5\).

To identify small molecules that induce a lipid mediator class switch from inflammation to resolution, we started from an in-house library of Vietnamese medical plants to access structurally diverse pharmacophores\(^23,24\). Detailed investigations disclose the isomers of 8-methylsorcolin-4′-ol (2) that limit inflammation by suppressing pro-inflammatory lipid mediator formation and inducing SPM biosynthesis, with the 2\(\delta\),7-isomer being most active. Compound 2 inhibits LT formation across human innate immune cells while specifically triggering SPM production in macrophages of the M2 phenotype. The lipid mediator class switch by compound 2 was confirmed \(i.v.\) using an experimental mouse model of zymosan-induced peritonitis.

2. Materials and methods

2.1. Materials

Solvents used for extract fractionation and compound isolation were purchased from VWR International (Darmstadt, Germany). Solvents for high performance liquid chromatography (HPLC) were obtained from Merck (Darmstadt, Germany). Ultrapure water was produced by a Sartorius Arium 611 UV water purification system (Göttingen, Germany). Deuterated and non-deuterated lipid mediator standards for ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) were purchased from Cayman Chemicals (Ann Arbor, MI, USA).
Zymosan, indomethacin, staurosporine, and all other reagents were purchased from Merck (Darmstadt, Germany) unless indicated otherwise.

2.2. General experimental procedures for extract fractionation and compound isolation

Optical rotations were determined with a Perkin−Elmer 341 polarimeter (Wellesley, MA, USA) at 20 °C. Electronic circular dichroism (ECD) and UV spectra were measured on a JASCO J-1500 CD spectrometer (Tokyo, Japan). 1D- and 2D-NMR experiments were recorded on a Bruker DRX 300 (Bruker Biospin, Rheinstetten, Germany) operating at 300.13 MHz (1H) and 75.47 MHz (13C) or Bruker Advance II 600 operating at 600.19 MHz (1H) and 150.91 MHz (13C) at 300 K; NMR solvents: methanol-d4/CDCl3/DMSO-d6 with 0.03% TMS (Eurisotop, GIF-Sur-Yvette, France), which was used as internal standard.

HPLC-DAD analysis was carried out using a Prominance UFLC-XR system (Shimadzu, Kyoto, Japan) equipped with degasser, binary pump, autosampler, column thermostat, and diode array detector (DAD). Separations were performed on a HyperClone ODS (C18) 120 Å (150 mm × 4.6 mm i.d., 3 μm, Phenomenex, Torrance, CA, USA) and a Merck (VWR, Darmstadt, Germany) LiChroCART 4-4 guard column with LiChrospher 100 RP18 (5 μm) packing. A mobile phase consisting of 0.02% TFA in H2O (v/v) (solvent A) and methanol (solvent B) was employed with gradient elution (0 min, 30% B; 10 min, 50% B; 25 min, 65% B; 40 min, 70% B; 50 min, 98% B; 60 min, 98% B). The thermostat was set to 35 °C, the flow rate was 0.5 mL/min, the injection volume 10 μL, and the detection wavelength 280 nm. Chiral Analytical separations were carried out at an Agilent 1100 HPLC system (Agilent Austria, Vienna, Austria) equipped with DAD detector, auto-sampler, and column thermostat. LC parameters: stationary phase: Phenomenex Lux 3 μm Cellulose-1, 250 mm × 4.6 mm or Phenomenex Lux 3 μm i-amylose-3, 250 mm × 4.6 mm, flow: 0.5 mL/min, 20 °C, isocratic elution, detection at 280 nm. Solvent composition, sample concentration, and injection volume for each sample were individually optimized as indicated in Supporting Information Section S1.

For low resolution LC−electrospray ionization (ESI)-MS experiments, the HPLC system was coupled to a Bruker (Bruker Daltonics, Bremen, Germany) Esquire 3000plus iontrap, replacing solvent A with a solution of 0.5% formic acid in H2O (v/v). LC−MS−LC-parameters: HP 1100 system (Agilent, Waldbronn, Germany) equipped with auto-sampler, DAD, and column thermostat. MS-parameters: Esquire 3000plus (Bruker Daltonics, Bremen, Germany); ESI, temperature: 340 °C; dry gas: 10.00 L/min; nebulizer gas 30 psi; full scan mode: m/z 100−1500. High resolution-(HR)-ESI-MS analysis was performed on a Bruker microOTOF-QII mass spectrometer after LC separation (identical to LC−MS), using the following parameters: ESI-MS parameters: 1.5 split from HPLC, dry temp.: 220 °C; dry gas: 5.00 L/min; nebulizer 1.6 bar; full scan mode: m/z 100−1500; ion polarity: positive; capillary voltage: 4.5 kV; end plate offset: −0.5 kV.

Semi-preparative HPLC was performed on a Dionex UltiMate 3000 preparative HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with degasser, pump, autosampler, column compartment, variable wavelength detector, fraction collector, and Chromelone software. Centrifugal partition chromatography was performed using a FCPC C apparatus (Kromaton, Angers, France) with a rotor volume of 55 mL equipped with an LC-10AD pump system (Shimadzu, Kyoto, Japan) and a 5 mL sample loop. Column chromatography was performed with Sephadex LH-20 (Pharmacia Biotech AB, Stockholm, Sweden) or silica gel 60 (0.040−0.063 mm; Merck, VWR, Darmstadt, Germany) as stationary phases. Thin layer chromatography (TLC) was carried out on silica gel 60 F254 plates (VWR, Darmstadt, Germany).

2.3. Plant material

The red wood of *D. cambodia*na*na* was collected in Tinh Bien (An Giang, Vietnam) in March 2014, identified by Prof. Dr. Tran Hung (Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy of HoChiMinh city, Vietnam) and air-dried. A voucher specimen (AG3032014) is stored at the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy of HoChiMinh City, Vietnam.

2.4. Extraction, isolation and chiral separation

Milled and air-dried red wood of *D. cambodia*na*na* (5 kg) was extracted with 20 L of 96% ethanol under reflux. The plant material was recovered by filtration and the extraction process was repeated twice with 20 and 10 L of 96% ethanol, respectively. The filtrates were combined and the obtained solution was evaporated to dryness under reduced pressure at a temperature of 40 °C yielding 628 g crude extract. The initial separation was performed by means of liquid−liquid extraction; 300 g of crude extract were suspended in 1.0 L water and extracted with dichloromethane (500 mL × 4), ethyl acetate (500 mL × 6), followed by n-butanol (500 mL × 4). Individually combined organic solutions as well as aqueous solution were evaporated to dryness, affording dichloromethane (44.2 g), ethyl acetate (104.3 g), n-butanol (38.5 g) and water subfractions (128.2 g). A part of the ethyl acetate subfraction (52.4 g) was subjected to silica gel column chromatography (CC) using a stepwise gradient of dichloromethane and methanol (100:0 to 1:100, v/v) as mobile phase to obtain subfractions 1 to 20. Subfraction 5 (1.04 g) was subjected to silica gel CC (petroleum ether/acetone, 95.5:0.5 to 85:15, v/v) to afford compound 9 (179.8 mg). Subfraction 7 (3.7 g) was separated by silica gel CC (petroleum ether/acetone, 95:5 to 70:30, v/v) to afford compound 10 (30.4 mg) and a subfraction (7-9), which was purified by Sephadex-LH-20 CC using methanol as a mobile phase to yield compound 8 (27.4 mg). A further subfraction (7-15) was separated by semi-preparative HPLC (Phenomenex Aqua 5 μm C18 125 Å; 250 mm × 10.0 mm, gradient acetonitrile/water, 70:30 to 86:14 in 20 min; flow rate 1.2 mL/min, 35 times) to obtain compound 3 (11.7 mg). Subfraction 8 (1.4 g) was combined with subfraction 9 (1.0 g) and subjected to silica gel CC (petroleum ether/acetone, 90:10 to 70:30, v/v) to yield compound 14 (317.8 mg). Subfraction 10 (840 mg) was separated by Sephadex LH-20 CC with methanol as eluent to afford 9 fractions (10-1 to 10-9). Compound 15 (17 mg) precipitated from the methanolic solution of subfraction 10−3. Subfraction 10-4 was separated by Sephadex LH-20 CC (dichloromethane/acetone, 85:15, v/v) to yield compounds 5 (24.7 mg) and 6 (23.9 mg); a further subfraction of this step, 10-4-6, was further purified by silica gel CC (petroleum ether/ethanol 6:4) to obtain compounds 11 (10 mg) and 16 (6.5 mg). Subfraction 11 (786.0 mg) was subjected to silica gel CC (petroleum ether/acetone, 7:3) to yield 6 fractions (11-1 to 11-6). Subfraction 11-6 (549.8 mg) was purified by Sephadex LH-20 CC (methanol as mobile phase) and then separated with semi-preparative HPLC (Phenomenex Aqua
The molecular docking simulation was conducted with GOLD 5.2 (CCDC, Cambridge, UK). Scoring was calculated using the CHEMPLP (https://www.ccdc.cam.ac.uk/support-and-resources/support(caseid=5d1a2fc0-c93a-49c3-a8e2-f95c472dcef0) scoring function.

PubChem entry ncf10, stable 5-LOX, co-crystalized with 3-O-acetyl-11-keto-β-boswellic acid (AKBA) (www.inteligand.com/ligandscout). A re-docking of AKBA was conducted to optimize the docking settings. The best ranked pose with the final docking settings had an RMSD of 1.061 compared to the co-crystallized ligand.

2.7. Isolation of primary cells from human blood

Human blood (3.13% sodium citrate) and human leukocyte concentrates from freshly withdrawn venous blood (16 I.E. heparin/mL blood) were provided by the Institute for Transfusion Medicine of the University Hospital Jena (Germany) with informed consent of the volunteers. The selected fasted (12 h) healthy male and female blood donors (18–65 years) were registered and regularly donated blood every 8–12 weeks. They were physically inspected by a clinician and free of obvious infections and inflammatory or allergic disorders and neither had taken antibiotics nor anti-inflammatory drugs within 10 days before blood collection.

Human polymorphonuclear leukocytes (PMNL), peripheral blood mononuclear cells (PBMC) and platelets were freshly isolated from leukocyte concentrates as described. Briefly, PMNL, PBMC and platelets were separated by dextran sedimentation, followed by density gradient centrifugation using lymphocyte separation medium (LSM 1077, GE Healthcare, Freiburg, Germany). Platelets and PBMC were collected from the supernatant and the intermediate layer, respectively, whereas PMNL were obtained from the pellet cells after removal of erythrocytes by hypotonic lysis. To enrich monocytes, PBMC were seeded in RPMI 1640 medium plus 5% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (GE Healthcare) in culture flasks (Greiner Bio-one, Frickenhausen, Germany) and incubated for 1.5 h at 37 °C.

2.8. Monocyte differentiation and macrophage polarization

For differentiation of monocytes to macrophages and further polarization towards M1 and M2 phenotypes, human primary monocytes were first stimulated with 20 ng/mL GM-CSF or M-CSF (Peprotech, Hamburg, Germany) for 6 days in macrophage medium (RPMI 1640 supplemented with 10% FCS, 2 mM/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin) to obtain monocyte-derived macrophages. These macrophages were further polarized for 48 h in macrophage medium with 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL interferon (IFN)-γ (Peprotech, Hamburg, Germany) to obtain M1 or with 20 ng/mL interleukin (IL)-4 (Peprotech) to obtain M2 phenotypes.

5 μm C18 125 Å; 250 mm × 10.0 mm, gradient methanol/water, 70:30 to 85:15 in 30 min; flow rate 1.2 mL/min, 80 times) to afford compounds 1 (21.7 mg) and 2 (25.3 mg). Preparative chiral separation of compound 1 and 2 was carried out at a Prominence UFLC-XR HPLC system (Shimadzu) equipped with auto-sampler, DAD, and column thermostat using the following conditions: stationary phase: Phenomenex Lux 3 μm Cellulose-1, 250 mm × 4.6 mm, flow: 0.5 mL/min, 30 °C, isotropic elution with ethanol abs. and n-hexane (3:7, v/v), detection at 280 nm injection volume: 2 μL; sample concentration: 9.0 mg/mL mobile phase for compound 1 and 5.6 mg/mL mobile phase for compound 2. Collected fractions of 80 injections were evaporated to dryness and passed through Sephadex LH-20 (diameter 0.5 cm, bed height 3 cm, swollen in mobile phase; flow rate 0.5 mL/min, methanol as solvent) to remove stationary material yielding enantiomeric pure compound 1a (1.18 mg), 1b (1.00 mg), 2a (0.80 mg), and 2b (1.26 mg). Compound 18 (11.2 mg) was isolated from subfraction 12 (830.2 mg) by Sephadex LH-20 CC (methanol as mobile phase) and then purified by silica gel CC (petroleum ether/ethyl acetate, 6:4). Subfraction 13 (1.12 g) was separated by silica gel CC (petroleum ether/acetone, 10:0 to 6:4) to obtain 13 subfractions (13-1 to 13-13). Sub-fractions 13-4 and 13-8 were purified by Sephadex LH-20 CC to yield compounds 13 (119.2 mg), 17 (6.6 mg), and 4 (20.4 mg). Subfraction 16 was separated by FCPC (dichloromethane/methanol/water, 4:3:3, v/v; lower phase: mobile phase) and then purified by Sephadex LH-20 CC (methanol as mobile phase) to afford compounds 7 (60.5 mg) and 12 (40.0 mg). Structure elucidation of known compounds isolated was carried out by means of mass spectrometry (LC−(HR)-ESI-MS) and NMR spectroscopy as well as by comparison of physical and spectral data with those of reference compounds reported in the literature. Structure elucidations of new compounds are described in Supporting Information Sections S2−4.

2.5. Computational methods for ECD calculation

3D structures of 1 and 2 were drawn and subjected to a conformational search by using OPLS-3 force field in MacroModel v.9 (Schrödinger Ltd., New York, NY, USA) in water. The number of steps was set high enough to include all low energy conformers. Conformers occurring in the energy window of 10 kcal/mol from the global minima were subjected to geometrical minimization and optimization in the DFT/B3LYP/6-31+G(d,p) basis set and level of theory and using the conductor-like polarizable continuum model (CPCM) in ethanol, using Gaussian 16 v. A.03 software (Gaussian, Inc., Wallingford, CT, USA) (Supporting Information Fig. S1). Frequency calculations were performed at the same level and no imaginary frequencies were observed. Calculation of the excitation energy (nm), rotatory strength dipole velocity (Rvel) and dipole length (Rlen) were performed by using time-dependent density-functional theory (TD-DFT) at PBE0PBE/def2-SV/6-31+G(d,p)/CPCM level in ethanol. ECD curves were simulated on the basis of rotatory strengths using SpecDis v.1.7 with half-band of 0.2 eV and UV shift of 10–30 nm.

2.6. Docking simulation

The molecular docking simulation was conducted with GOLD 5.2 (CCDC, Cambridge, UK). Scoring was calculated using the CHEMPLP (https://www.ccdc.cam.ac.uk/support-and-resources/support?caseid=5d1a2fc0-c93a-49c3-a8e2-f95c472dcef0) scoring function.
2.9. Determination of cell-free 5-LOX activity

Human recombinant 5-LOX enzyme was purified by affinity chromatography on an ATP-agarose column. In brief, Escherichia coli BI21 (DE3) was transformed with pT3-5LO plasmid and lysed in lysis buffer containing 50 mM/L trithanolamine, 5 mM/L EDTA, 60 μg/mL soybean trypsin inhibitor, 1 mM/L phenylmethylsulphonyl fluoride, 1 mM/L dithiothreitol, and 1 mg/mL lysozyme. The supernatant was pre-warmed for 30 s at 37 °C before arachidonic acid (20 μM) and CaCl2 (2 mM) were added. The reaction was stopped by addition of an equal volume of ice-cold methanol. Lysates were sonicated (3 times, 15 s, each) and centrifuged twice (first at 10,000 × g, 15 min, 4 °C; then the supernatant at 40,000 × g, 70 min, 4 °C). The supernatant was applied to an ATP-agarose column, which was extensively washed with PBS pH 7.4 plus 1 mM/L EDTA, phosphate buffer (50 mM/L containing 0.5 mol/L NaCl and 1 mM/L EDTA) and phosphate buffer (50 mM/L plus 1 mM/L EDTA). Semi-purified 5-LOX enzyme was eluted with phosphate buffer (50 mM/L containing 1 mM/L EDTA and 20 mM/L ATP.

For determination of 5-LOX activity, semi-purified enzyme (0.5 μg) was pre-treated with vehicle (DMSO) or test compounds in PBS pH 7.4 containing 1 mM/L EDTA and 1 mM/L ATP for 10 min at 4 °C. The mixture was pre-warmed for 30 s at 37 °C before arachidonic acid (20 μM/L, if not indicated otherwise) and CaCl2 (2 mM/L) were added. After 10 min at 37 °C, the reaction was stopped by addition of an equal volume of ice-cold methanol. Formed lipid mediators were extracted by solid phase extraction (SPE) using Sep-Pak C18 35 cc Vac Cartridges (Waters, Milford, MA, USA). 5-LOX products (all-trans isomers of LTB4 and 5-hydro(pero)xy-eicosatetraenoic acid (5-H(p)ETE)) were separated by reversed phase (RP)-HPLC on a Nova-Pak C18 Radial-Pak Column (4 μm, 100 mm × 5 mm, Waters) under isocratic conditions (73% methanol/27% water/0.007% trifluoroacetic acid) at a flow rate of 1.2 mL/min and detected at 235 and 280 nm. The selective 5-LOX inhibitors zileuton (3 μM/L; Tocris Bioscience, Bristol, UK) and BWA4C (0.5 μM/L, Merck) used as reference suppressed 5-LOX product formation by 70.7 ± 2.7% and 59.1 ± 6.9%, respectively.

To investigate whether compounds 2 and 5 reversibly inhibit 5-LOX activity, the purified enzyme was pre-incubated with the test compounds at 30 or 3 μM/L for 15 min and then 10-fold diluted to a final compound concentration of 3 or 0.3 μM/L, respectively. Arachidonic acid (20 μM/L) and CaCl2 (2 mM/L) were added and after 10 min at 37 °C 5-LOX products were extracted and analyzed as described above.

2.10. Determination of cellular 5-LOX, 15-LOX, and 12-LOX activity

Freshly isolated human PMNL (1 × 10⁷ cells/mL) were suspended in PBS pH 7.4 plus 1 mg/mL glucose and 1 mM/L CaCl2. PMNL were pre-treated with vehicle (DMSO) or test compounds for 10 min before addition of arachidonic acid (20 μM/L) and/or calcium ionophore A23187 (2.5 μM/L) and incubation for another 10 min at 37 °C. The reaction was terminated by addition of an equal volume of ice-cold methanol. Lipid mediators were extracted by solid phase extraction using Sep-Pak C18 35 cc Vac Cartridges, and 5-LOX products (LTB4, its all-trans isomers and 5-H(p)ETE), 15-HETE (15-LOX product), and 12-HETE (12-LOX product) were analyzed by RP-UPLC-HPLC as described for the determination of 5-LOX metabolites from cell-free assays. The reference inhibitors zileuton (3 μM/L) and BWA4C (0.5 μM/L) inhibited the biosynthesis of 5-LOX metabolites by 64.1 ± 6.3% and 78.6 ± 9.7%, respectively.

2.11. Sample preparation and metabololipidomics analysis of lipid mediators

Lipid mediator biosynthesis in human PMNL, M1/M2 macrophages or citrated blood were treated with A23187 (30 μM/L, PMNL or blood, 15 min), pathogenic E. coli (serotype O6:K2:H1, ratio 1:50, 90 min for macrophages; 1 × 10⁷/mL blood, 180 min for blood) or Staphylococcus aureus (6850)-conditioned medium (SACM, 1%, 180 min). SACM was prepared by growing the bacteria for 17 h at 37 °C in brain heart infusion medium (Carl Roth, Karlsruhe, Germany), followed by centrifugation at 3500 × g for 10 min and sterile-filtration through a Millex-GP filter unit (0.22 μm; Millipore) for the indicated time.

Supernatants of treated cells (PMNL or M1/M2 macrophages in 1 mL PBS pH 7.4 plus 1 mM/L CaCl2), blood (1 mL) or exudates from murine peritonitis (1 mL) were mixed with ice-cold methanol (2 mL) supplemented with deuterium-labeled internal standards (10 μL; 200 mM/L d₅-LTB₄, d₅-LXA₄, d₅-RvD₂, d₅-PGE₂, and 10 μM/L d₃-arachidonic acid). Lipid mediators were extracted by SPE on Sep-Pak C18 35 cc Vac Cartridges (Waters) in brief, samples were placed at 4 °C for at least 45 min to allow protein precipitation. After centrifugation (1200 × g, 4 °C, 10 min), 8 mL acidified H₂O (pH = 3.5) was added to the supernatants, and the samples were loaded onto equilibrated RP-columns. After extensive washing with H₂O and n-hexane, lipid mediators were eluted with methyl formate. Eluates were brought to dryness (TurboVap LV, Biotage, Uppsala, Sweden) and dissolved in methanol/water (50/50) for UPLC–MS/MS analysis. Lipid mediators were analyzed by UPLC–MS/MS using an Acquity UPLC system (Waters) coupled to a QTRAP 5500 mass spectrometer (Sciex, Darmstadt, Germany), which was equipped with a Turbo V™ electrospray ionization source. Analytes were separated on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm; Waters) at 50 °C with a flow rate of 0.3 mL/min. The mobile phase consisting of methanol, water, and acetic acid (42:58:0.01) was ramped to 86:14:0.01 over 12.5 min and then switched to 98:2:0.01 for isotropic elution (3 min). Diagnostic ions were detected in the negative ionization mode by scheduled multiple reaction monitoring using optimized MS parameters for each lipid mediator. Retention times were confirmed using external standards (Cayman Chemicals). Automatic peak integration was performed with Analyst 1.6 software (Sciex) using InteLLiQuan default settings. Lipid mediator amounts were calculated from signal intensities normalized to internal standards using external calibration curves.

2.12. Determination of cell viability

Mitochondrial dehydrogenase activity was measured by MTT assay to assess cell viability briefly. Monocytes were pre-incubated with vehicle (DMSO), test compounds or staurosporine (1 μM/L, cytotoxic reference) for 15 min and incubated for 24 h at 37 °C and 5% CO₂, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and cells were incubated for 2–4 h. After cells were lysed and the formazan crystals solubilized with SDS (10% in 20 mM/L HCl), the absorption was read at 570 nm using a Multiskan Spectrum Microplate Reader (Thermo Fisher Scientific).
2.13. Determination of radical scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH, Merck; 50 mmol/L) was incubated with vehicle (DMSO), test compounds or ascorbic acid (50 μmol/L, reference compound for radical scavenging) in ethanol for 30 min.27 Absorbance was measured at 520 nm using a Multiskan Spectrum Microplate Reader.

2.14. Determination of COX-1 and COX-2 activity

For the determination of cell-free COX activity, isolated ovine COX-1 (50 units, Cayman Chemicals) or human recombinant COX-2 (20 units, Cayman Chemicals) were pre-treated with vehicle (DMSO) or test compounds in 100 mmol/L Tris pH 8 (supplemented with 5 mmol/L glutathione, 5 μmol/L hemoglobin and 100 μmol/L EDTA) for 5 min at 4 °C. The mixture was pre-warmed for 1 min at 37 °C, and arachidonic acid was added (5 μmol/L for COX-1 and 2 μmol/L for COX-2). The reaction was stopped by addition of an equal volume of ice-cold methanol, and formed 12-HHT was extracted by SPE using Sep-Pak C18 35 cc Vac Cartridges (Waters) and analyzed by RP-UV–HPLC on a Nova-Pak C18 Radial-Pak Column as described for 5-LOX products. The COX-1/2 inhibitor indomethacin (10 μmol/L) was used as control.

For the determination of cellular COX-1 activity, freshly isolated human platelets (1 × 10⁸) were pre-treated with vehicle (DMSO), test compounds or indomethacin (10 μmol/L) for 5 min at room temperature, and arachidonic acid (5 μmol/L) was added. After 5 min at 37 °C, the reaction was stopped by addition of an equal volume of ice-cold methanol, and formed 12-HHT was extracted and analyzed as described above.

2.15. Determination of LTC₄S activity

Human embryonic kidney (HEK)293 cells (ATCC, Manassas, VA, USA) were cultivated in DMEM/high glucose (4.5 g/L; GE Healthcare) supplemented with heat-inactivated FCS (10%) and penicillin/streptomycin at 37 °C and 5% CO₂. Confluent cells were reseeded at 1 × 10⁶/cm² after being detached with trypsin/EDTA (GE Healthcare). Microsomal membranes (2.5 μg total protein) from HEK293 cells that stably express LTC₄S were pre-treated with vehicle (DMSO) or test compounds in potassium phosphate buffer (0.1 mol/L, pH 7.4, containing 5 mmol/L glutathione) for 10 min at 4 °C. The reaction was started by addition of LTA₄ methyl ester (1 μmol/L, Cayman Chemicals) and stopped after 10 min at 4 °C by addition of an equal volume of ice-cold methanol. LTC₄S-d₂ methyl ester (5 ng, Cayman Chemicals) was used as internal standard. Formed LTC₄S was extracted by SPE and analyzed by UPLC–MS/MS.28 MK886 (10 μmol/L), Cayman Chemicals) was used as reference for LTC₄S inhibition.

2.16. Determination of mPGES-1 activity

Human lung adenocarcinoma epithelial A549 cells (ATCC) were cultivated in DMEM/high glucose (4.5 g/L) supplemented with heat-inactivated FCS (10%) and penicillin/streptomycin at 37 °C and 5% CO₂. Confluent cells were reseeded at 1 × 10⁶/cm² after being detached with trypsin/EDTA. mPGES-1 activity was determined in microsomal membranes of A549 lung carcinoma cells that were first incubated with IL-1β (2 ng/mL) for 48 h.28 Then, cells were harvested, frozen in liquid nitrogen, and incubated with ice-cold homogenization buffer (0.1 mol/L potassium phosphate buffer pH 7.4 plus 1 mmol/L phenylmethanesulfonyl fluoride, 60 μg/mL soybean trypsin inhibitor, 1 μg/mL leupeptin, 2.5 mmol/L glutathione, and 250 mmol/L sucrose) for 15 min at 4 °C. After sonication (3 times, 20 s, each, at 4 °C), the microsomal membrane fraction was obtained by differential centrifugation (first 10,000 × g, 10 min, 4 °C; then 174,000 × g, 1 h, 4 °C). Microsomal membranes (containing 2.5–5 μg total protein) were suspended in potassium phosphate buffer (0.1 mol/L, pH 7.4, plus 2.5 mmol/L glutathione) and pre-treated with vehicle (DMSO), test compounds or the reference inhibitor MK886 (10 μmol/L) for 15 min at 4 °C. PGH₂ (20 μmol/L, final concentration) was added and the reaction terminated after 1 min on ice by addition of stop solution (40 mmol/L FeCl₂, 80 mmol/L citric acid) supplemented with 1 mmol 11β-PGE₂ as internal standard (Cayman Chemicals). Formed PGE₂ was extracted by SPE using Sep-Pak C18 35 cc Vac Cartridges, separated by RP-UV–HPLC on a Nova-Pak C18 Radial-Pak column (4 μmol/L, 5 mm × 100 mm, Waters) under isocratic conditions (30% acetonitrile, 70% water, 0.007% trifluoroacetic acid) at a flow rate of 1 mL/min and detected at 195 nm.

2.17. Determination of sEH activity

Human recombinant sEH enzyme was purified from baculovirus-infected Sf9 insect cells by benzylthio-sepharose affinity chromatography.28 Briefly, cells were lysed in 50 mmol/L NaHPO₄ pH 8 containing 300 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 60 μg/mL soybean trypsin inhibitor. After sonication (3 times, 10 s, each, at 4 °C) and centrifugation (100,000 × g, 60 min, 4 °C), supernatants were collected and subjected to benzylthio-saheraphine affinity chromatography. The sEH enzyme was eluted with 4-fluorochalcone oxide in PBS pH 7.4 containing 1 mmol/L dithiothreitol and 1 mmol/L EDTA. Purified sEH (60 ng) was dialysed, concentrated and pre-treated with vehicle (DMSO), test compounds or the reference inhibitor AUDA (100 mmol/L, Cayman Chemicals) for 10 min in 25 mmol/L Tris buffer, pH 7 plus 0.1 mg/mL BSA. PHOME (50 μmol/L, Cayman Chemicals) was added and converted by sEH to fluorescent 6-methoxy-napthaldehyde within 1 min at room temperature in the dark. The reaction was stopped by 200 mmol/L ZnSO₄, and the fluorescence was determined using a NOVosstar fluorescence microplate reader (BMG Labtech, excitation: 330 nm, emission: 465 nm).

2.18. Flow cytometry

Macrophages were stained in PBS pH 7.4 containing 0.5% BSA, 2 mmol/L EDTA and 0.1% sodium azide by Zombie Aqua™ Fixable Viability Kit (Biolegend, San Diego, CA, USA) to determine cell viability (5 min, 4 °C) and by fluorochrome-labelled antibodies (20 min, 4 °C): FITC anti-human CD14 (clone MSE2, #555397, BD Bioscience, San Jose, CA), APC-H7 anti-human CD80 (clone L307.4, #561134, BD Bioscience), PE-Cy7 anti-human CD54 (clone HA58, #5353115, Biolegend), PE anti-human CD163 (clone GHI/61, #556018, BD Bioscience), APC anti-human CD206 (clone 19.2, #550889, BD Bioscience) to determine M1 and M2 surface marker expression using a LSRFortessa™ cell analyzer (BD Bioscience), and data were analyzed using FlowJo X Software (BD Bioscience).31
2.19. SDS-PAGE and Western blot

M2 macrophages were treated with vehicle (DMSO) or compound 2 for 24 h at 37 °C and 5% CO2 and then lysed in ice-cold lysis buffer containing Tris-HCl (20 mM/L, pH 7.4), NaCl (150 mM/L), EDTA (2 mM/L), NaF (5 mM/L), phenylmethanesulphonyl fluoride (1 mM/L), leupeptin (1 mg/mL), soybean trypsin inhibitor (60 µg/mL), sodium vanadate (1 mM/L), and sodium pyrophosphate (1 mM/L). After lysates have been sonicated (3 x 3 s, on ice) and centrifuged (21,100 × g, 5 min, 4 °C), cell supernatants were collected and their protein concentration determined by DC-protein assay kit (Bio-Rad Laboratories GmbH, Munich, Germany). Aliquots were mixed (1:1, v/v) with 1 × SDS-PAGE loading buffer containing Tris-HCl (125 mM/L, pH 6.5), 25% sucrose, 5% SDS, 0.25% bromophenol blue, and 5% β-mercaptoethanol, and heated at 95 °C for 5 min. Equal aliquots (corresponding to 12.5 µg protein) were loaded and separated on 10% SDS-PAGE gels and then transferred onto hybond ECL nitrocellulose membranes (GE Healthcare, Freiburg, Germany). The membranes were incubated with the following primary antibodies: mouse monoclonal anti-15-LOX-1, 1:5000 (ab197774, Abcam, Cambridge, UK); rabbit polyclonal anti-15-LOX-2, 1:1000 (ab23691, Abcam); mouse monoclonal anti-15-LOX, 1:1000 (610,695, BD Biosciences, NJ, USA), and mouse monoclonal anti-β-actin, 1:1000 (3700 S, Cell Signaling). Immuno reactive bands were stained with the following fluorescence-labeled antibodies: DyLight® 680 Cross-Adsorbed Goat anti-Mouse IgG (H + L) (10,797,775, Invitrogen, Thermo Fisher Scientifics, MA, USA), DyLight® 800 Cross-Adsorbed Goat anti-Mouse IgG (H + L) (13,477,327, Invitrogen), DyLight® 680 Cross-Adsorbed Goat anti-Rabbit IgG (H + L) (10,517,364, Invitrogen), DyLight® 800 Cross-Adsorbed Goat anti-Rabbit IgG (H + L) (13,477,187, Invitrogen), and visualized by a Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France). Data from densitometric analysis were background-corrected.

2.20. Zymosan-induced peritonitis in mice

Male CD-1 mice (33–39 g, 6–8 weeks, Charles River Laboratories, Calco, Italy) were housed at 21 ± 2 °C, provided with standard rodent chow and water and subjected to a 12 h light/12 h dark cycle. Mice were randomly assigned for the experiments and allowed to acclimate for four days. The experimental procedures comply with the ARRIVE guidelines and were approved by the Italian Ministry (744/2019) and conducted according to European and Italian regulations for animal protection (directives 2010/63/EU and DL 26/2014).

Mice (n = 6 per experimental group) received vehicle (0.5 mL, 2% DMSO in saline), compound 2 (10 mg/kg) or MK886 (3 mg/kg) by intraperitoneal injection (i.p.) 30 min prior to peritonitis induction by zymosan (0.5 mL, 2 mg/mL suspension in 0.9% saline, i.p.)[14]. Mice were euthanized 120 min post zymosan injection with CO2, followed by a peritoneal lavage with 3 mL of cold PBS pH 7.4. Exudates were collected from the peritoneal cavity, and cells were immediately counted by vital trypan blue staining. After centrifugation (18,000 × g, 5 min, 4 °C), supernatants were collected, and lipid mediators were extracted by SPE and analyzed by UPLC/MS/MS. LTB4 levels in the exudates were analyzed by enzyme immunoassays (Enzo Life Sciences, Lörrach, Germany) according to manufacturer’s instructions. To measure myeloperoxidase activity, cell pellets were sonicated in PBS buffer (50 mM/L, pH 6) with 0.5% hexadecyltrimethylammonium bromide, freeze-thawed three times, and centrifuged (32,000 × g, 20 min, 4 °C). Cell supernatants were collected, and the reaction was initiated by addition of α-dianisidine (0.167 mg/mL) and 0.0005% hydrogen peroxide. Changes in absorbance were time-dependently monitored using an iMark microplate Reader (Bio-Rad, Segrate, Milan, Italy). Myeloperoxidase activity was determined from the calibration curve using human neutrophil myeloperoxidase as reference standard and is given in U/mL[15].

2.21. Statistical analysis

Data are presented as mean ± SEM of n observations, where n represents the number of independent experiments (including independent immune cell preparations) or the number of animals per group, as indicated. GraphPad Prism 8 software (San Diego, CA, USA) was used for statistical calculations. Normal distribution of the data was addressed by Kolmogorov–Smirnov tests. Samples were blinded for screening of plant extracts, fractions and natural products and for counting infiltrated cells in the mouse peritoneum. Data were log-transformed for statistical analysis where indicated. Outliers were determined using a Grubb’s test. For multiple comparison, ordinary or repeated-measures one-way ANOVA with Tukey or Dunnett’s post hoc tests or ordinary two-way ANOVA with Tukey post hoc tests were applied. For comparison of two groups, paired t-tests were used. The criterion for statistical significance was *P < 0.05. IC50 values were calculated by non-linear regression using GraphPad Prism 8.

3. Results

3.1. Targeting LOX isoenzymes by 8-methylsocotrin-4′-ol from Vietnamese plant extracts

To identify novel natural product-derived pharmacological agents that induce a lipid mediator class switch from inflammation to resolution, we evaluated an in-house library of 94 extracts from 31 Vietnamese medicinal plants in activated PMNL (Fig. 1A). Our aim was to identify extracts, which potently suppress 5-LOX-dependent LT biosynthesis (IC50 ≤ 10 µg/mL) while enhancing 15-LOX (residual activity ≥ 100%) and optionally 12-LOX product formation (Supporting Information Table S1[23]). The methanolic red wood extract of Dracaena cambodiana Pierre ex Gagnep. (Asparagaceae) showed the most favorable pharmacological profile. Biological testing confirmed the potent bioactivity of the ethanolic extract on 5-LOX product formation in PMNL. Evaluation of the subfractions revealed strong 5-LOX-inhibitory activity of the dichloromethane and the ethyl acetate subfraction, while the n-butanol fraction turned out to be inactive (Fig. 1E). Therefore, the ethyl acetate subfraction, which showed a strong 5-LOX-inhibitory potential, was selected for further bioactivity-guided fractionation. Separation by silica gel column chromatography yielded 20 subfractions, which were analyzed for their 5-LOX-
inhibitory activity in PMNL. At 3 μg/mL, subfractions 5–18 showed a statistically significant 5-LOX inhibition, with subfractions 11 and 12 being most active (Fig. 1F and G). By using different chromatographic techniques, 18 (poly)phenolic compounds were isolated from the bioactive subfractions (Table 1, Fig. 1F), among them three new natural products (3–5) and another three (9, 10, 16) that have not yet been reported in Dracaena species (Supporting Information Section S5). Details on the elucidation of the chemical structures and the obtained physical and spectral data of the new compounds 3–5 are given in Sections S2–4. Due to the observed small optical rotation values of all chiral compounds (1–11), with the exception of compound 10, we assumed the presence of racemic/seedlike mixtures of the respective compounds. This assumption could be verified by chiral HPLC analysis, which revealed for all chiral compounds, except for 10, at least two peaks (Section S1).

Potent 5-LOX inhibition in PMNL was observed for two series of dimeric compounds, either comprising a 2-(4-hydroxyphenyl) chromanol (1–4) or 4,4'-propane-1,3-diyldiphenol moiety (5–7) (Table 1). Compounds within these series differed only slightly in their substitution pattern with methoxy- or hydroxy-groups. The mixtures of the 8-methylsocotrin-4'-ol isomers 1 and 2 were most active and inhibited the biosynthesis of 5-LOX products in PMNL with an IC50 of 0.3–1.1 μmol/L. Interestingly, the formation of 15-LOX products was markedly enhanced by the dimeric compounds 1–7 (Fig. 2A), whereas 12-LOX product formation was not affected or slightly reduced (Fig. 2B). The monomeric compounds 8–16 were considerably less potent 5-LOX inhibitors (Table 1).

All seven dimeric compounds 1–7 directly inhibited human recombinant 5-LOX in a cell-free assay (IC50 = 0.3–1.1 μmol/L) (Table 1). Neither cytotoxic (Fig. 2C) nor radical scavenging activities (Fig. 2D) were observed at relevant concentrations. The two compound series, represented by compound 2 and 5, inhibited 5-LOX independently of the substrate concentration (Fig. 2E) in a reversible manner (Fig. 2F). Together, we identified a group of potent 5-LOX inhibitors from a library of medicinal plant extracts, which shift the balance from 5-LOX to 15-LOX product formation in PMNL.

3.2. Stereoisomeric requirements for 5-LOX inhibition

As major constituents of the most active subfractions 11 and 12 from activity-guided fractionation (Supporting Information Fig. S2), we separated the 8-methylsocotrin-4'-ol isomers (1...
and 2) by chiral chromatography and determined their absolute configuration by comparing simulated and experimental ECD spectra (Fig. 3A). The isomers 1a, 1b, 2a, and 2b have 2R, γS, 2S, γR, 2R, γR, and 2S, γS configuration, respectively, and were investigated individually for their potency to inhibit 5-LOX.

The two stereoisomers 1a and 1b of the scalemic mixture 1 (Section S1) inhibited human recombinant 5-LOX (IC50 = 0.7 μmol/L) (Fig. 3B) and suppressed 5-LOX product formation in PMNL with comparable potency [IC50 (A23187) = 1.7 μmol/L, IC50 (A23187 plus arachidonic acid) = 2.1 μmol/L] (Fig. 3B). In contrast, the scalemic mixture 2 consists of the potent stereoisomer 2b [5-LOX enzyme: IC50 = 0.5 μmol/L; PMNL: IC50 (A23187) = 0.4 μmol/L, IC50 (A23187 plus arachidonic acid) = 0.8 μmol/L] and the considerably less active 2a [5-LOX enzyme: IC50 = 1.5 μmol/L; PMNL: IC50 (A23187) = 11.3 μmol/L, IC50 (A23187 plus arachidonic acid) = 13.4 μmol/L]. The ratio of 2b to 2a in mixture 2 is 62.8/37.2 (Section S1). While the isomers 1a, 1b and 2b comparably inhibit 5-LOX in the cell-free assay, only compound 2b maintains its high inhibitory potency in PMNL (Fig. 3B).

Molecular docking studies on the allosteric binding site of 5-LOX suggest that the most active stereoisomer 2b forms hydrogen bonds with Lys140, Glu136, and Arg101 (Fig. 3C, Supporting Information Table S2). While the first two bonds are also formed by 1a and 1b, 2a is turned around in the binding site, due to steric hindrance, and forms other interactions, which could explain the loss of activity (Fig. 3D).

Due to the poor accessibility of the purified stereoisomers, we selected the racemic/scalemic mixtures 2 and 5 (as representatives

### Table 1  Inhibitory activity of natural products isolated from *D. cambodiana* against 5-LOX.

| Compd. | Activated PMNL | Human recombinant 5-LOX | Compd. | Activated PMNL |
|--------|----------------|-------------------------|--------|----------------|
| 1      | 0.34 ± 0.03a   | 0.50 ± 0.17a            | 10     | 65.7 ± 5.7a    |
| 2      | 0.28 ± 0.06a   | 0.50 ± 0.23a            | 11     | 89.2 ± 6.7b    |
| 3      | 0.80 ± 0.14a   | 0.30 ± 0.07a            | 12     | 5.0 ± 1.4a     |
| 4      | 0.46 ± 0.12a   | 0.90 ± 0.26a            | 13     | 60.1 ± 8.2b    |
| 5      | 0.51 ± 0.12a   | 1.10 ± 0.28a            | 14     | 8.9 ± 1.6a     |
| 6      | 0.85 ± 0.15a   | 0.30 ± 0.09a            | 15     | 6.5 ± 0.4a     |
| 7      | 0.59 ± 0.16a   | 0.60 ± 0.16a            | 16     | 4.4 ± 0.7a     |
| 8      | 67.4 ± 6.2b    | n.d.                    | 17     | 7.2 ± 0.9a     |
| 9      | 5.7 ± 0.9a     | n.d.                    | 18     | n.i.           |

*aIC50 values (μmol/L).

bResidual activities at 10 μmol/L (% control) are given as means ± SEM, n = 3. n.i., no inhibition; n.d., not determined.
Figure 2  Dimeric phenolic compounds induce a shift from 5- to 15-LOX metabolites. (A, B) Human PMNL were treated with compounds 1–7 isolated from the D. cambodiana ethyl acetate extract subfraction. LOX product formation was initiated by arachidonic acid plus A23187, and metabolites were analyzed by RP-UV–HPLC. 15-LOX (A) and 12-LOX product formation (B). Results are given as means ± SEM, percentage of vehicle control, ng/5 × 10⁶ cells, n = 3. Data were log-transformed for statistical analysis. ***P < 0.001, *P < 0.05 compared to vehicle control; repeated measures one-way ANOVA plus Dunnett’s post hoc test. (C) Cell viability of monocytes upon 24 h of treatment with compounds 1–7; STS, staurosporine (1 μmol/L). (D) Scavenging of DPPH radicals by compounds 1–7; ascorbic acid (50 μmol/L). (C, D) Results are given as means ± SEM, percentage of vehicle control, n = 3, **P < 0.01, *P < 0.05, compared to vehicle control, compound 1–7; repeated measures one-way ANOVA plus Tukey post hoc tests; control inhibitors (STS or ascorbic acid): paired t-test. (E) Dependency of 5-LOX inhibition on the substrate (arachidonic acid, AA) concentration. (F) Reversibility of 5-LOX inhibition. Human recombinant 5-LOX was pre-incubated with vehicle or compound and then 10-fold diluted before AA was added. Numbers in brackets indicate the diluted compound concentration after pre-incubation. (E, F) Products of human recombinant 5-LOX were quantified by RP-UV–HPLC. Results are given as means ± SEM, percentage of vehicle control, n = 3; ***P < 0.01, *P < 0.05; paired t-test.

3.3. Subordinate modulation of lipid mediator biosynthesis

Multiple enzymes besides LOXs cooperate in accomplishing the complex lipid mediator networks, thereby regulating initiation, maintenance, and resolution of inflammation.⁵⁻⁶ Compounds 2 and 5 substantially inhibited mPGES-1 in a cell-free assay (IC₅₀ ~ 1 μmol/L) (Fig. 4A) and to some extent weakly COX-1, both as purified enzyme (IC₅₀ > 10 μmol/L) (Fig. 4B) and in activated human platelets (IC₅₀ > 1 μmol/L) (Fig. 4C). sEH activity was slightly suppressed at 10 μmol/L, exclusively by compound 5 (Fig. 4D), and both 2 and 5 showed a minor inhibitory effect on LTC₄S by trend, again only at 10 μmol/L (Fig. 4E). Neither of the two compounds affected COX-2 activity (Fig. 4B). Together, in addition to prominent 5-LOX inhibition and the efficient increase of 15-LOX product formation in PMNL, we identified mPGES-1 and COX-1 as subordinated targets of 2 and 5.

3.4. Efficient inhibition of 5-LOX in blood and in activated PMNL

To investigate the effect of compounds 2 and 5 on more complex lipid mediator profiles, we performed metabololipidomics studies on human blood, which contains PMNL and monocytes as major cells contributing to large lipid mediator networks.⁷ When lipid mediator production was initiated by A23187, compound 2 efficiently suppressed the biosynthesis of LTs (LTB₄, LTB₄ isomers, 20-OH-LTB₄) and other 5-LOX products (5-HETE, 5,6-diHETE, 5-HEPE) (Fig. 5A and Supporting Information Table S3). The formation of the 15-LOX product 15-HETE and to a minor extend the 12-LOX-derived 12-HETE, 12-HEPE, and 14-HDHA was moderately elevated, with strong inter-individual differences, whereas the generation of COX-derived prostanoids (PGE₂, PGD₂, thromboxane (TXB₂)), and the release of fatty acid substrates (arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid) was not influenced. Comparable effects on 5-LOX production were observed when pathogenic E. coli was used as physiological stimulus (Fig. 5B and Supporting Information Table S4). Compound 5, on the other hand, failed to suppress the formation of 5-LOX products in human blood, even at the highest concentration tested (30 μmol/L), potentially due to unfavorable plasma protein binding.

PMNL, which are the major 5-LOX-expressing cells in blood, predominantly produce LTs and other pro-inflammatory lipid mediators.⁶ Compound 2 potently suppressed the biosynthesis of 5-LOX products in activated PMNL and moderately elevated the formation of the 15-LOX products 15-HETE and 15-HEPE at concentrations in the range of 0.3 and 3 μmol/L (Fig. 6). SPM biosynthesis was instead not upregulated. Compound 2 equally inhibited the formation of resolvins, lipoxins, and 5-LOX products. Comparable changes in the lipid mediator profile were evident for the 5-LOX reference inhibitor zileuton, which underlines a role for 5-LOX in SPM biosynthesis. It should be noted that PMNL dominate during the initial phase of inflammation while SPM are mainly produced by cells related to the resolution phase, i.e., macrophages.⁷

3.5. Induction of SPM biosynthesis in M2 macrophages

Macrophages with M2-like phenotype strongly express 15-LOX-1 and are the major innate immune cells responsible for SPM production at sites of inflammation during resolution.⁸⁻¹⁰ The M1 subtype, on the other hand, excels in generating pro-inflammatory lipid mediators, including PGs and LTs, and is associated with...
acute and unresolved inflammation. To obtain M1 and M2 subsets, we differentiated human primary monocytes into macrophages and subsequently induced their polarization. By monitoring the expression of typical M1 (CD54 and CD80) and M2 surface markers (CD163 and CD206), we could exclude that compound 2 affects macrophage polarization (Supporting Information Fig. S3).

We then investigated the two subsets for the effect of compound 2 on the lipid mediator network and confirmed that the formation of LTs and other 5-LOX products is strongly attenuated, regardless of whether cells were stimulated with E. coli or S. aureus (Supporting Information Table S6). Most remarkably, M2-like macrophages produced substantial amounts of SPM, including resolvins (RvE3, RvD5), protectins (PD1, AT-PD1, PDX), and maresins (MaR1), as well as mono-hydroxylated precursors derived from 12- and 15-LOX, when treated with compound 2. In M1 macrophages, compound 2 moderately increased the biosynthesis of 15-LOX products (i.e., 15-HETE, 15-HEPE, 17-HDHA) without substantially influencing the minute SPM generation (Fig. 7 and Table S5). Neither protein expression of 15-LOX-1, 15-LOX-2 nor 5-LOX were affected by compound 2 in M2 macrophages within 24 h (Supporting Information Fig. S4). Together, compound 2 efficiently suppresses pro-inflammatory LT biosynthesis throughout innate immune cells and selectively stimulates M2 macrophages to generate SPM.

3.6. Lipid mediator class switch to relieve inflammation in murine peritonitis

Whether the lipid mediator class switch from LTs to SPM, which we observed in vitro, also occurs at inflammatory sites in vivo, was...
investigated for zymosan-induced mouse peritonitis—a self-resolving model of acute inflammation that is initiated by LTs (Fig. 8A)46. Mice were sacrificed at 2 h after zymosan administration to allow the simultaneous analysis of LTs and SPM. Intraperitoneal administration of compound 2 prior to zymosan effectively reduced 5-LOX product levels, including LTs, in peritoneal exudates but neither substantially altered the release of PGs nor of polyunsaturated fatty acid substrates (Fig. 8B). Resolvin (RvE3 and RvD5) concentrations decreased, though this 2- to 3-fold drop was overcompensated by the strong increase of 12-LOX- and 15-LOX-derived SPM precursors and the 2.5- to 15-fold upregulation of protectins (PD1 and PDX) and MaR1 (Fig. 8B). MK886, an inhibitor of 5-LOX-activating protein (FLAP), had a similar but less prominent effect. The vehicle control (0.5 mL, 2% DMSO in saline) did not substantially affect the inflammatory reaction in the peritoneal cavity within 30 min to 4 h after zymosan injection, as suggested by the barely altered cell infiltration, myeloperoxidase activity (as marker for infiltrated neutrophils), and peritoneal LTC4 levels (Supporting Information Fig. S5). Together, compound 2 shifts the lipid mediator profile in experimental peritonitis from a pro-inflammatory to a pro-resolving signature, thereby attenuating inflammation, as indicated by the drop of immune cell infiltration into the peritoneal cavity (Fig. 8C).

4. Discussion

Chronic inflammation and inflammation-related diseases are accomplished by pro-inflammatory signaling, which essentially orchestrates the acute phase of inflammation but becomes pathological when being persistent6. Current pharmacological strategies to combat inflammatory disorders, i.e., NSAIDs, focus on the pro-inflammatory branch of inflammation, but are confronted with major challenges, including i) lack of selectivity and efficacy, ii) undesired gastrointestinal, renal, and cardiovascular safety issues, and iii) impaired resolution16,11,13,14. By identifying a unique natural product that induces the lipid mediator class switch from pro-inflammatory LTs to SPM, our study gives access to a novel anti-inflammatory strategy that, in addition to suppressing LT-driven inflammation, may actively promote resolution and the return to homeostasis.

We took advantage of a comprehensive library of 94 Vietnamese medicinal plant extracts (31 different plants), from which we identified two structural different series of dimeric phenolic compounds that i) are present in D. cambodiana, ii) potently inhibit 5-LOX, and iii) trigger 15-LOX product formation. D. cambodiana is an important source for the preparation of ‘Dragon’s Blood’, a dark red plant resin, which is used in many cultures as antimicrobial, antiviral, antitumor, and anti-inflammatory remedy37,38. The promising anti-inflammatory and analgesic activities of ‘Dragon’s Blood’ were confirmed in pre-clinical and clinical studies39–45. Furthermore, we showed that ‘Dragon’s Blood’ provides protective effects against radiation-induced oxidative stress, brain injury and myelosuppression in rodents41–44, and as a cream, improved wound healing in a randomized, double-blind and placebo-controlled trial on 60 patients with removed skin tags45. We speculate that the bioflavonoids identified in this study might contribute to these activities by increasing the biosynthesis of SPM that actively terminate neutrophil transmigration, suppress oxidative stress, stimulate phagocytosis and promote tissue regeneration44, and as a cream, improved wound healing in a randomized, double-blind and placebo-controlled trial on 60 patients with removed skin tags45. We speculate that the bioflavonoids identified in this study might contribute to these activities by increasing the biosynthesis of SPM that actively terminate neutrophil transmigration, suppress oxidative stress, stimulate phagocytosis and promote tissue regeneration44, and as a cream, improved wound healing in a randomized, double-blind and placebo-controlled trial on 60 patients with removed skin tags45.

![Figure 4](image)

Figure 4 Subordinate effects of compounds 2 and 5 on lipid mediator biosynthesis. Effects of compound 2 and 5 on (A) mPGES-1 in microsomal preparations of IL-1β-treated A549 cells; (B) isolated ovine COX-1 and human recombinant COX-2; (C) COX-1-dependent 12-HHT formation in platelets; (D) human recombinant sEH; (E) human recombinant LTC4S. MK886 (10 μmol/L), indomethacin (Indo, 10 μmol/L) or AUDA (1 μmol/L) were used as control. Results are given as means ± SEM, percentage of vehicle control, n = 3, except n = 2 (E, LTC4S, 10 μmol/L). Data were log-transformed for statistical analysis. ***P < 0.001, **P < 0.01, *P < 0.05, compound 2 or 5 compared to vehicle control, repeated measures one-way ANOVA plus Tukey post hoc tests; control inhibitors (MK886, Indo or AUDA) compared to vehicle control, paired t-test.
For the natural products 1, 2, and 6, which are representatives of the two identified series of dimeric phenols, anti-Helicobacter pylori, anti-thrombin, and anti-diabetic activities have been reported in vitro. However, their anti-inflammatory potential remained elusive. Our findings suggest that especially the biflavonoid isomer 2b contributes to the anti-inflammatory activity of D. cambodiana by suppressing the biosynthesis of pro-inflammatory 5-LOX-derived LTs and causing a lipid mediator class switch towards SPM that promote resolution. Flavonoids are excellent scaffolds for 5-LOX inhibition, but neither were adducts of them reported (as realized in the two biflavonoid series 1–7 of this study) nor effects on SPM biosynthesis addressed. Strong differences between the two biflavonoid series seem to exist for plasma protein binding. While the IC_{50} value of compound 2 for 5-LOX inhibition was only slightly shifted to higher values in human blood as compared to PMNL, compound 5, which is comparably effective to 2 in PMNL, failed to inhibit 5-LOX. Our finding is in agreement with a previous study, showing that compound 6, which differs from 5 only in its substitution pattern, is prone to human serum albumin binding.

5-LOX inhibitors can target the active site iron (redox-active compounds and iron chelators) or compete with arachidonic acid or fatty acid peroxides for binding to 5-LOX (non-redox type inhibitors). Higher efficacy under clinical settings is expected for allosteric inhibitors that interact with the ATP or membrane binding site. Recently, we identified a series of allosteric 5-LOX inhibitors and proposed that other reported inhibitors actually bind to this site. We confirmed this theory by

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**Figure 5** Effect of compound 2 on the lipid mediator profile of activated blood. Lipid mediator profiles of human blood elicited by A23187 (A) or pathogenic *E. coli* (B) as determined by UPLC–MS/MS. Heatmaps show mean percentage changes upon treatment with compound 2 or 5 relative to the vehicle control; zileuton (3 μmol/L). 20-OH-LTB₄, 20-hydroxy-LTB₄; HEPE, hydroxy-eicosapentaenoic acid; (di)HETE, (di)hydroxy-eicosatetraenoic acid; HDHA, hydroxy-docosahexaenoic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Line charts present data in pg/mL blood for selected lipid mediators as means ± SEM, n = 3 (A), n = 4 (B). Data were log-transformed for statistical analysis. ***P < 0.001 compared to vehicle control; repeated measures one-way ANOVA plus Tukey post hoc tests.
co-crystallizing 5-LOX with the pentacyclic triterpene acid AKBA. Since compound 2 inhibits 5-LOX like AKBA in a substrate concentration-independent manner and exhibits poor radical scavenging activity, we explored the structure-activity relationship at this allosteric binding site. The scoring function did not directly correlate with the 5-LOX-inhibitory activity of compounds 1–18, but the analysis of the docking simulation suggests three key interaction partners on the protein side, Lys140, Glu134, and Glu136, which form hydrogen bonds to those compounds that display IC50 values in the low or even submicromolar range (Table S2).

While strong 5-LOX expression in PMNL and the pro-inflammatory macrophage subtype M1 enables efficient LT biosynthesis, macrophages of the M2 phenotype instead express substantial 15-LOX-1, generate high amounts of SPM, and promote the resolution phase of inflammation as well as tissue repair. Compound 2 inhibits LT biosynthesis throughout the cellular systems investigated in this study, i.e., PMNL, the macrophage subtypes M1 and M2, and blood, and slightly enhanced the formation of 15-LOX products (e.g., 15-HETE, 15-HEPE) in most settings. However, a strong increase of both 12- and 15-LOX products was mainly evident for M2 macrophages. Accordingly, the formation of distinct SPM and their precursors was exclusively elevated in this macrophage subtype.

Substrate redirection of polyunsaturated fatty acids might partially explain the moderately increased biosynthesis of 15-LOX products throughout innate immune cells and blood. In support of this hypothesis, compound 2 also increased the production of PGE2 and other prostanoids in vitro and in vivo. However, this effect is weak, and we hypothesize that the shunting of arachidonic acid towards prostanoid biosynthesis is buffered by the inhibition of the low-affinity targets COX-1 and mPGES-1. Compound 2 (COX-1: IC50 = 6.8 µmol/L; mPGES-1: IC50 = 1.5 µmol/L) inhibits COX-1 less potent than indomethacin (IC50 = 0.02 µmol/L) but is comparably efficient in inhibiting mPGES-1 as the control inhibitor MK886 (IC50 = 1.6 µmol/L).

Accordingly, PGE2 formation slightly increased at low concentrations (≤3 µmol/L) of compound 2 in PMNL and again decreased at effective concentrations to inhibit COX-1 and mPGES-1 (10 µmol/L). Depending on the spatial and temporal distribution, PGE2 and other prostanoids display pro-inflammatory or anti-inflammatory activities during the course of inflammation. Helminth larval products have been proposed to exploit this bias by lowering LT and enhancing PG production. However, the balance between pro- and anti-inflammatory PG activities is sensitive, and we therefore consider the switch from pro-inflammatory LT to pro-resolving SPM as more robust and safer for pharmacotherapy.

Pharmacological strategies that trigger a lipid mediator class switch from inflammation to resolution are still rare. Endogenous metabolites such as PGE2 and oxidized phosphatidylcholine inhibit LT formation while enhancing the biosynthesis of lipoxins,
a class of 5-LOX-derived SPM, though they do qualify for therapeutic application. The allosteric 5-LOX inhibitor AKBA (IC$_{50} = 3$ μmol/L) increases the formation of 12-LOX (270%, at 10 μmol/L) and less 15-LOX metabolites (210%, at 10 μmol/L) in human neutrophils with unknown consequences on SPM production. Endogenous long-chain vitamin E metabolites excel in 5-LOX inhibition (IC$_{50} = 0.035–0.075$ μmol/L) but the increase in SPM is mainly restricted to systemic RvE3 levels during the resolution of murine peritonitis. Other small molecules that trigger SPM formation in human neutrophils or macrophages include benzene sulfonamide derivatives (110%–140% at 10 μmol/L), ginkgolic acid (110%–240% at 10 μmol/L), benzoaxanthene lignans (130%–170% at 10 μmol/L), and oxymetazoline (130% at 400 μmol/L). While oxymetazoline failed to inhibit 5-LOX product formation (at 1000 μmol/L), the other compounds inhibited 5-LOX with IC$_{50}$ values of 1.2–2.3 μmol/L, being considerably less potent than compound 2 (IC$_{50} = 0.3$ μmol/L), except for ginkgolic acid (IC$_{50} = 0.2$ μmol/L). Evidence for a lipid mediator class switch in vivo was limited to the benzoaxanthene lignans 1 and 2, which were less effective in raising SPM (precursor) levels in...
inflamed peritoneal exudates than compound 2. Additional mechanisms besides substrate redirection are needed to explain the extraordinarily strong increase in 12-LOX and 15-LOX metabolites as well as SPM in activated M2 subsets both in vitro and in the inflamed peritoneal cavity in vivo. On the one hand, binding of AKBA to the allosteric site that is also addressed by compound 2 changes the regioselectivity of 5-LOX from C5- to C12-hydroperoxidation of arachidonic acid. It is therefore tempting to speculate that compound 2 exploits a similar mechanism to reprogram the 5-LOX enzyme, thereby triggering the generation of 15-LOX products in innate immune cells. On the other hand, compound 2 might indirectly act on signaling pathways that are specific for M2 macrophages. Along these lines, we have recently shown that 5-LOX inhibition by zileuton neither enhanced the formation of SPM (precursors) in M2 macrophages nor in mouse peritonitis. How macrophages switch between LT and SPM biosynthesis was recently ascribed to HMGB1-C1q complexes that crosslink receptor for advanced glycation end product (RAGE) and leukocyte-associated immunoglobulin-like receptor (LAIR-1). Whether compound 2 targets this pathway besides direct inhibition of 5-LOX needs further investigation. However, such a mechanism would explain why SPM upregulation is limited to macrophages, while 5-LOX inhibition occurs also in other immune cells.

The compound 2-induced switch from LT to SPM biosynthesis is not equally efficient for all SPM. In zymosan-induced mouse peritonitis, which is driven by infiltrated PMNL and peritoneal macrophages, compound 2 lowered LT and elevated protectin (PD1, PDX) and MaR1 levels, whereas resolvins...
A biflavonoid triggers the lipid mediator class switch in inflammation

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Conflicts of interest

The authors declare no conflicts of interest. The above mentioned funding sources were neither involved in study design, data collection, analysis, and interpretation nor in writing and submission of the manuscript.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2021.04.011.

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