Published in final edited form as:

Biochem Pharmacol. 2017 August 15; 138: 107–118. doi:10.1016/j.bcp.2017.04.031.

***In vivo* α -hydroxylation of a 2-alkylindole antagonist of the OXE receptor for the eosinophil chemoattractant 5-oxo-6,8,11,14-eicosatetraenoic acid in monkeys¹**

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Abstract

We have developed a selective indole antagonist (**230**) targeting the OXE receptor for the potent eosinophil chemoattractant 5-oxo-ETE (5-oxo-6,8,11,14-eicosatetraenoic acid), that may be useful for the treatment of eosinophilic diseases such as asthma. In previous studies we identified ω 2-oxidation of the hexyl side chain of racemic **230** as a major metabolic route in monkeys, but also obtained evidence for another pathway that appeared to involve hydroxylation of the hexyl side chain close to the indole. The present study was designed to investigate the metabolism of the active S-enantiomer of **230** (**S230**) and to identify the novel hydroxy metabolite and its chirality. Following oral administration, **S230** rapidly appeared in the blood along with metabolites formed by a novel and highly stereospecific α -hydroxylation pathway, resulting in the formation of α S-hydroxy-**S230**. The chirality of α -hydroxy-**S230** was determined by the total synthesis of the

¹WSP and JR hold a patent on OXE receptor antagonists related to compounds **230** and **264** (Powell WS and Rokach J, 5-Oxo-ETE receptor antagonist compounds, US patent US-2014-0323535-A1 (2014)).

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COMPOUNDS

5S-HETE (PubChem CID: 5280733); 5-oxo-ETE (PubChem CID: 5283159); BINOL (PubChem CID: 11762); 5-chloro-1-methyl-1H-indole-2-carbaldehyde (PubChem CID: 23004695); pentyl magnesium bromide (PubChem CID: 121513990); methyl 5-chloro-3-methyl-5-oxopentanoate (PubChem CID: 10888500); Indo-1 AM (PubChem CID: 123918); TBDMSCl (PubChem CID: 28928); BDMAEE (PubChem CID: 18204); tBuOMe (PubChem CID: 15413)

Database link

Data for the crystal structure of compound **7** has been deposited with the Cambridge Crystallographic Data Centre (deposition number, CCDC 1527527).

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relevant diastereomers. Of the four possible diastereomers of α -hydroxy-**S230** only α S-hydroxy-**S230** has significant OXE receptor antagonist activity and only this diastereomer was found in significant amounts in blood following oral administration of **S230**. Other novel metabolites of **S230** identified in plasma by LC-MS/MS were α S, ω 2-dihydroxy-**S230** and glucuronides of **S230** and ω 2-hydroxy-**S230**. Thus the alkyl side chain of **S230**, which is essential for its antagonist activity, is also the major target of the metabolic enzymes that terminate its antagonist activity. Modification of this side chain might result in the development of related antagonists with improved metabolic stability and efficacy.

Graphical abstract

Keywords

Eicosanoids; 5-Lipoxygenase products; Inflammation; Granulocytes; Drug metabolism; Chiral analysis

1. Introduction

5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is a product of the 5-lipoxygenase pathway, along with leukotrienes B₄ and C₄. It is formed by the NADP⁺-dependent oxidation of 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5S-HETE) by 5-hydroxyeicosanoid dehydrogenase [1], which is highly expressed in a variety of both inflammatory and structural cells [2]. The formation of 5-oxo-ETE is regulated by the availability of the cofactor NADP⁺, which is normally present at very low intracellular concentrations, but is dramatically elevated under conditions of oxidative stress, activation of the respiratory burst, and cell death [3]. 5-Oxo-ETE is a potent activator of human and monkey [4] eosinophils and is the only 5-lipoxygenase product with potent chemoattractant effects for these cells [5]. It acts via the selective OXE receptor, which is highly expressed on eosinophils and to a lesser extent on neutrophils and macrophages [6]. Intradermal injection of 5-oxo-ETE in humans results in the infiltration of eosinophils into the skin and this response is enhanced in asthmatic subjects [7].

The potent chemoattractant effects of 5-oxo-ETE on eosinophils, suggest that it may be an important proinflammatory mediator in eosinophilic disorders such as asthma and allergic rhinitis. For this reason, we sought to develop selective antagonists against the human OXE receptor, using calcium mobilization in human neutrophils as a screening test. We tested a series of indoles containing two side chains, one mimicking carbons 1–5 of 5-oxo-ETE and the other, carbons 15–20 of 5-oxo-ETE. We found that an indole containing a hexyl group in

the 2-position and a 5-oxovalerate group in the 3-position selectively blocked 5-oxo-ETE-induced calcium mobilization. Modification of this compound led to the identification of the chiral compound **230** (Fig. 1) as a potent and selective OXE receptor antagonist [8], with the antagonist activity residing almost exclusively in the S-enantiomer (**S230**) [9].

To determine whether **S230** may be useful therapeutically it will have to be tested in an animal model. Orthologs of the OXE receptor exist in many species but unfortunately not in rodents. We initially investigated the possibility that cats, which have an OXE receptor ortholog and are quite susceptible to asthma, might be a suitable experimental model for this purpose. Although 5-oxo-ETE is an extremely potent activator of feline eosinophils, **230** is only a weak antagonist in this species, presumably because of significant differences between the human and feline receptors [10]. We therefore plan to test **230** in monkeys. To do this it is first important to understand its metabolism and to establish whether adequate blood levels can be achieved. In an initial study we investigated the pharmacokinetics and metabolism of racemic **230**, as at the time that the study was initiated we did not have a procedure for the synthesis of sufficient amounts of the pure S-enantiomer. We found that **230** is a potent OXE receptor antagonist *in vitro* in monkeys and that it rapidly appears in the blood, along with a number of metabolites, following oral administration [4]. Chiral analysis of **230** recovered from the blood revealed that the S-enantiomer was depleted more rapidly than the R-enantiomer. Although this study was not designed to follow the time courses of the metabolites, ω 1- and ω 2- oxidation products were identified by comparison with authentic standards in a blood sample taken 4 h after administration of **230**. A number of other unidentified metabolites were also detected, including a pair of isomers that exhibited distinct chromatographic behaviour and may have been formed due to hydroxylation of the hexyl side chain α or possibly β to the indole moiety (see Fig. 1 for designation of the different carbons of the hexyl group).

The objective of the current study was to investigate the pharmacokinetics and metabolism of the active S-enantiomer of **230**, which was made possible by a novel synthetic procedure that we recently developed [11]. We particularly wanted to conclusively identify the putative α - or β -hydroxy metabolite referred to above and to examine its antagonist activity, as hydroxylation of 5-oxo-ETE in the equivalent (ω 6) position does not abolish its agonist activity [2].

2. Materials and Methods

2.1. Animal experiments

Three female cynomolgus monkeys weighing between 4.9 and 5.3 kg and housed at INRS-Institut Armand-Frappier, Laval, Quebec, were used for these studies. All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the local institutional animal care committee.

S230, synthesized as described previously[11], was dissolved in EtOH at a concentration of 75 mg/mL and the appropriate volume added to 10 volumes of 20 mM NaHCO₃ and the mixture vortexed. The resulting suspension (4.4 mL/kg) was administered to three monkeys by oral gavage at a dose of 30 mg/kg. Blood samples were collected in heparinized tubes just

prior to gavage (1 mL) and 1, 4, 8, 12, and 24 h after gavage (4.5 to 5 ml for each time point, depending on the weight of the monkey). After centrifugation, plasma samples were frozen and stored at -80°C .

2.2. Analysis of S230 and its metabolites by RP-HPLC

Solvents used for chromatographic analysis were purchased from Fisher Scientific, Markham, ON, Canada. Plasma samples were thawed and diluted by the addition of MeOH (2 volumes), followed by addition of the internal standard (1 μg 5-(5-chloro-1-methyl-2-(5-methylhexyl)-1H-indol-3-yl)-3-methyl-5-oxopentanoate; identical to **S230** except that it contains an isoheptyl group instead of a hexyl group; synthesized as previously described [4]). After storing overnight at -80°C the samples were thawed and centrifuged. The concentration of MeOH was adjusted to 30% by the addition of water and each sample was loaded onto a Sep-Pak C18 cartridge (Waters Corporation, Milford Massachusetts) [12], which was washed with 30% MeOH prior to the elution of indole metabolites with 100% MeOH. After evaporation of the solvent using a rotary evaporator the residue was dissolved in 30% MeOH and analyzed by precolumn extraction/RP-HPLC [13]. The precolumn was a C18 SecurityGuard cartridge (3.2×8 mm ID) from Phenomenex, Torrance, CA, whereas the analytical column was a Kinetex C18 column (5 μm particle size; $4.6 \text{ mm} \times 250 \text{ mm}$; Phenomenex;) with a linear gradient between 35 and 66% MeCN containing 0.02% HOAc over 62 min at a flow rate of 1 mL/min and a column temperature of 35°C . Fractions corresponding to peaks of interest were collected and combined for subsequent analysis by LC-MS/MS.

The concentrations of **S230** and its major metabolites were determined by measuring the ratios of their peak areas with that of the internal standard. The identities of the metabolites that were quantitated were confirmed by LC/MS/MS in all cases, as well as by cochromatography with authentic standards when these were available (**S230**, ω 2-OH-**S230**, ω 2-oxo-**S230**, and α S-OH-**S230**). As we did not have standards for the glucuronide metabolites or α S, ω 2-diOH-**S230** we assumed that their extinction coefficients were identical to those of their precursors. The HPLC conditions were as described above except that a methanol gradient was used: 60 to 80% MeOH in H_2O with 0.02% HOAc over 60 min at a temperature of 35°C and a flow rate of 1 mL/min. The properties used for the quantitation of each compound were as follows: α , ω 2-di-OH-**S230** (t_R , 13.9 min; λ , 311 nm; ϵ , 12,830), ω 2-OH-**S230** glucuronide (t_R , 15.3 min; λ , 305 nm; ϵ , 12,710), ω 2-oxo-**S230** (t_R , 16.6 min; λ , 305 nm; ϵ , 12,710), ω 2-OH-**S230** (t_R , 19.3 min; λ , 305 nm; ϵ , 12,710), α -OH-**S230** (t_R , 35.1 min; λ , 311 nm; ϵ , 12,830), **S230** glucuronide (t_R , 38.6 min; λ , 305 nm; ϵ , 12,710), **S230** (t_R , 43.7 min; λ , 305 nm; ϵ , 12,710), internal standard (1 μg 5-(5-chloro-1-methyl-2-(5-methylhexyl)-1H-indol-3-yl)-3-methyl-5-oxopentanoate (t_R , 50.4 min; λ , 305 nm; ϵ , 12,710).

2.3. Chiral HPLC

Chiral HPLC was carried out using a Lux Cellulose-2 column (5 μm particle size; 250×4.6 mm; Phenomenex). The mobile phases used are described in the legends to the relevant figures. A flow rate of 1 mL/min and a column temperature of 45°C were used throughout.

2.4. Identification of S230 metabolites by LC-MS/MS

Fractions corresponding to the HPLC peaks shown in Fig. 5A were collected, with identical fractions from different time points being pooled. These fractions were then analyzed by LC/MS/MS using a model 1100 HPLC system (Agilent Technologies, Santa Clara, CA) connected to an LTQ Velos Orbitrap high resolution mass spectrometer via a heated electrospray ionization source (Thermo Scientific, San Jose, CA). Chromatographic separation was performed using a Phenomenex Kinetex C18 column (2.6 μ m particle size; 50 mm $\times$ 2.1 mm) at the flow rate of 0.3 mL/min and a column temperature of 25 °C. The mobile phase was a gradient between solvents A (0.02% HOAc in H₂O) and B (0.02% HOAc in MeCN) as follows: 0 min, 30% B; 1 min, 30% B; 20 min, 65% B. Samples were injected in a volume of 10 μ L and analyzed by negative electrospray ionization (ESI) mode using the following settings: capillary temperature, 350°C; source heater temperature, 300 °C; sheath gas flow, 20; auxiliary gas flow, 10; capillary voltage, -3.0 kV. The MS settings were: S lens RF level, 60%; automatic gain control (AGC) target, 1×10^6 ions; mass range, m/z 250 to m/z 700; resolution, 100,000. Multiple levels of MSⁿ analysis with data dependent acquisition (DDA) mode were used for the identification and elucidation of antagonist metabolites. In DDA mode, the selection of the precursor ion for MS² analysis was based on the chlorine isotope pattern and/or isolation of the top three most intense ions from the full MS scan for fragmentation with dynamic exclusion disabled; activation type, collision-induced dissociation (CID); signal threshold, 5000; normalized collision energy, 35; isolation width, 2 Da; activation time, 30 s. MS³ used parent and product mass lists to trigger MS³ for selected ions and was performed with CID as the activation type; minimal signal threshold, 5000; isolation width, 2 Da; activation time, 30 s; normalized collision energy, 45.

2.5. Evaluation of potencies of α -hydroxy-230 diastereomers in human neutrophils

The antagonist effects of α -hydroxy-230 diastereomers on 5-oxo-ETE-induced calcium mobilization in human neutrophils were examined as described previously [8]. Neutrophils prepared by dextran sedimentation and centrifugation over Ficoll-Paque (GE Healthcare, Mississauga, ON, Canada) were loaded with indo-1 AM (ThermoFisher Scientific, Waltham, MA) and placed in a thermostatted cuvette at 37 °C. After stabilization of baseline fluorescence, the α -hydroxy compound was added. 5-Oxo-ETE (10 nM; synthesized as previously described [14]) was added 2 min later followed 1 min later by digitonin (final concentration 0.1%). Fluorescence was measured using a Cary Eclipse spectrofluorometer (Agilent Technologies, Santa Clara, CA).

2.6. Chemistry

All reagents and solvents used for chemical synthesis were purchased from Sigma-Aldrich, Saint Louis, MO. All reactions were carried out under an argon atmosphere using dried, nitrogen-purged glassware and dry solvents. Reaction progress was monitored using Merck TLC Silica gel 60 F₂₅₄ plates. The plates were visualized using 254 nm UV light, iodine chamber and anisaldehyde dip where appropriate, followed by gentle warming. ¹H NMR and ¹³C NMR spectra were recorded on a BRUKER AMX 400 MHz spectrometer at rt in CDCl₃, using TMS as an internal standard. The following abbreviations are used for the

description of NMR spectra: s- singlet; d- doublet; t- triplet; q- quartet; dd- doublet of doublets and m- multiplet. High resolution mass spectrometry (HRMS) was performed using an AccuTOF mass spectrometer with positive ion ESI mode and DART as an ion source. The purity of all tested compounds was determined to be >95% by a combination of HPLC, NMR and HRMS.

2.7. X-ray Single Crystal Diffractometry

Single crystals suitable for X-ray diffraction were mounted on a Mitegen micromesh mount with the help of a trace of mineral oil. Data were collected on a Rigaku R-axis curved image plate diffractometer equipped with a MicroMax002+ high-intensity copper X-ray source with confocal optics and examined with Cu K α radiation ($\lambda = 1.54184 \text{ \AA}$). Data were collected using the d*TREK option of CrystalClear (Rigaku, The Woodlands, TX). All data sets were processed using HKL3000 [15] and were corrected for absorption and scaled using Scalepack [15]. The space group was assigned using XPREP from the Shexl suite of programs (SHELXTL (2003) Bruker Advanced X-ray Solutions, Bruker AXS Inc., Madison, Wisconsin: USA) and the structures were solved by direct methods with SHELXS [16] and refined by full matrix least squares against F^2 with all reflections using SHELXL-2014 [17] using the graphical user interface ShelXle [18]. H atoms attached to carbon and oxygen atoms were positioned geometrically and constrained to ride on their parent atoms, with carbon hydrogen bond distances of 0.95 $\text{\AA}$ for aromatic and alkene C-H, and 0.99 and 0.98 $\text{\AA}$ for aliphatic CH₂ and CH₃ moieties. $U_{\text{iso}}(\text{H})$ values were set to 1.5 times times $U_{\text{eq}}(\text{C/O})$ for methyl and hydroxyl hydrogen atoms, and to 1.2 times $U_{\text{eq}}(\text{C})$ for CH and CH₂ moieties. Complete structures in CIF format have been deposited with the Cambridge Crystallographic Database. CCDC 1527527 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

2.8. 1-(5-chloro-1-methyl-1H-indol-2-yl)hexan-1-ol (3)

To a stirred solution of 5-chloro-1-methyl-1H-indole-2-carbaldehyde (**1**) (1.675 g, 8.651 mmol) in THF (25 mL) was added pentyl magnesium bromide (**2**) (9 mL, 2.0 M solution in Et₂O) at -78°C dropwise over a period of 5 min. The reaction mixture was stirred at -78°C for 20 min and then at rt for 15 min. Aqueous NH₄Cl (20 mL) was added and the organic layer was extracted with EtOAc (3 $\times$ 25 mL). The combined organic extracts were washed with brine (50 mL), dried over Na₂SO₄, and the solvents were evaporated to obtain the crude mixture. Silica gel (from VWR International, Radnor, PA) column chromatography (15% EtOAc/hexane) afforded the product as a yellow solid (2.089 g, 91%). HRMS (ESI) m/z calcd for [C₁₅H₂₀ClNO + H]⁺, 266.1311; found 266.1616. ¹H NMR (400 MHz, CDCl₃): δ 7.53 (d, $J = 1.6 \text{ Hz}$, 1H), 7.21 (d, $J = 8.7 \text{ Hz}$, 1H), 7.15 (dd, $J = 8.7 \text{ Hz}$, 1.9 Hz, 1H), 6.37 (s, 1H), 4.84 – 4.80 (m, 1H), 3.80 (s, 3H), 2.00 – 1.94 (m, 2H), 1.62 – 1.34 (m, 6H), 0.91 (t, $J = 6.9 \text{ Hz}$, 3H). ¹³C NMR (CDCl₃): δ 143.4, 136.4, 128.1, 125.1, 122.0, 120.0, 110.1, 98.6, 67.3, 36.2, 31.7, 30.3, 25.8, 22.6, 14.0.

2.9. 2-(1-((tert-butyldimethylsilyl)oxy)hexyl)-5-chloro-1-methyl-1H-indole (4)

To a stirred solution of 1-(5-chloro-1-methyl-1H-indol-2-yl)hexan-1-ol (**3**) in CH₂Cl₂ (8 mL) was added TBDMSCl (200 mg, 1.327 mmol) followed by imidazole (150 mg, 2.203 mmol) and the reaction mixture was stirred at rt for 14 h. H₂O (8 mL) was added and the organic layer was extracted with CH₂Cl₂ (3 × 5 mL). The combined organic extracts were washed with aqueous NaHCO₃ (20 mL), dried over Na₂SO₄, concentrated to dryness, and purified using silica gel column chromatography (5% EtOAc in hexane) to obtain the product as a pale-yellow viscous oil (290 mg, 87%). HRMS (ESI) *m/z* calcd for [C₂₁H₃₄ClNOSi + H]⁺, 380.2176; found 380.2048. ¹H NMR (400 MHz, CDCl₃): δ 7.49 (d, *J* = 1.8 Hz, 1H), 7.19 (d, *J* = 8.7 Hz, 1H), 7.12 (dd, *J* = 8.7 Hz, 1.9 Hz, 1H), 6.22 (s, 1H), 4.83 (t, *J* = 7.0 Hz, 1H), 3.80 (s, 3H), 1.93 – 1.84 (m, 1H), 1.79 – 1.71 (m, 1H), 1.46 – 1.26 (m, 6H), 0.88 – 0.84 (m, 12H), 0.00 (s, 3H), –0.18 (s, 3H). ¹³C NMR (CDCl₃): δ 143.7, 136.6, 128.4, 125.0, 121.3, 119.7, 109.8, 99.5, 70.1, 38.1, 31.5, 31.0, 25.8 (3C), 25.7, 22.6, 18.1, 14.0, –4.8, –5.2.

2.10. Methyl 5-(2-(1-((tert-butyldimethylsilyl)oxy)hexyl)-5-chloro-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoate (6)

To a 100 mL round bottom flask containing 2-(1-((tert-butyldimethylsilyl)oxy)hexyl)-5-chloro-1-methyl-1H-indole (**4**) (127 mg, 0.334 mmol) in CH₂Cl₂ (16 mL) was added methyl 5-chloro-3-methyl-5-oxopentanoate (**5**) (80 mg, 0.448 mmol) in CH₂Cl₂ (1 mL). The solution was cooled to 0°C, and Me₂AlCl (0.43 mL, 1.0 M solution in hexanes) was added slowly over a period of 5 minutes. The reaction mixture was stirred at 0 °C for 1 h and then at rt for 2 h. Aqueous NaHCO₃ solution (10 mL) was then added to quench the reaction. The aqueous layer was extracted with EtOAc (3 × 8 mL), and the combined organic extracts were washed with brine (20 mL), dried over Na₂SO₄, the volatiles removed under reduced pressure, and the resulting crude product was purified using silica gel column chromatography (2% EtOAc/hexane) to afford compound **6** as a pair of diastereomers (67 mg, 38%). HRMS (ESI) *m/z* calcd for [C₂₈H₄₄ClNO₄Si + H]⁺, 522.2806; found 522.2985.

2.11. 5-(5-chloro-2-(1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid (α-OH-230)

To a stirred solution of methyl 5-(2-(1-((tert-butyldimethylsilyl)oxy)hexyl)-5-chloro-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoate (**6**) (10 mg, 0.0191 mmol) in CH₃CN (5 mL) was added HF.Pyridine (0.1 mL, 30% pyridine, 70% HF) at 0 °C. The reaction mixture was allowed to warm to rt and stirred for 30 min. Aqueous NaHCO₃ solution (5 mL) was added, the aqueous layer was extracted with EtOAc (3 × 5 mL), and the combined organic extracts were dried over Na₂SO₄. Evaporation of the solvents *in vacuo* gave the crude product as a pale yellow solid that was dissolved in THF (2.5 mL) and H₂O (0.6 mL). To this solution was added LiOH.H₂O (32 mg, 0.763 mmol) and MeOH (0.1 mL). The reaction mixture was stirred at rt for 14 h. H₂O (5 mL) was added and the aqueous layer was extracted with EtOAc (3 × 5 mL), and the organic extract dried over Na₂SO₄, concentrated to dryness, and the product purified using silica gel column chromatography (4% MeOH/CH₂Cl₂) to isolate the product as a pair of diastereomers (pale-yellow viscous oil, 6.1 mg, 80% over two steps). HRMS (ESI) *m/z* calcd for [C₂₁H₂₈ClNO₄ + H]⁺, 394.1785; found 394.1883.

2.12 (S)-1-(5-chloro-1-methyl-1H-indol-2-yl)hexan-1-ol (7)

To a stirred solution of (*S*)-BINOL (503 mg, 1.757 mmol) in tBuOMe (52 mL) was added Ti(O-*i*Pr)₄ (3.5 mL, 11.945 mmol) dropwise at rt, and the resulting red solution was stirred for 30 min. In another flask, containing a solution of BDMAEE (5.3 mL, 27.813) in tBuOMe (52 mL) was added freshly prepared pentyl magnesium bromide (27.094 mmol in 20 mL THF) at 0 °C. The resulting cloudy suspension was stirred at 0 °C for 35 min. The mixture from the former flask was transferred slowly into the latter at –15 °C. The reaction mixture was allowed to warm to rt and then stirred for 1 h. After that, 5-chloro-1-methyl-1H-indole-2-carbaldehyde (**1**) (1.553 g, 8.020 mmol) in THF (20 mL) was added dropwise at –15 °C over a period of 5 min. The reaction mixture was allowed to warm to rt and stirred for 36 h. The reaction was quenched by adding aqueous NH₄Cl solution (60 mL). The two layers were separated, the aqueous layer was extracted with EtOAc (3 × 20 mL), and the combined organic extracts were washed with brine (60 mL), dried over Na₂SO₄, filtered, and the solvents removed under reduced pressure. The brown viscous crude product was purified using silica gel column chromatography (10% EtOAc/hexane) to afford the product as a yellow solid (1.027 g, 48%). (See the experimental of the racemic compound **2** for the spectral properties). Using chiral HPLC, the %*S* enantiomer was determined to be 97.1. Recrystallization using hexane-methanol improved the enantiomeric purity to 100%.

2.13. (S)-2-(1-((tert-butyldimethylsilyl)oxy)hexyl)-5-chloro-1-methyl-1H-indole (8)

The procedure was identical to that described for 2-(1-((tert-butyldimethylsilyl)oxy)hexyl)-5-chloro-1-methyl-1H-indole (**4**).

2.14. (S)-5-(5-chloro-2-((S)-1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid (αS-OH-S230)

To a stirred solution of (*S*)-2-(1-((tert-butyldimethylsilyl)oxy)hexyl)-5-chloro-1-methyl-1H-indole (**8**) (47 mg, 0.124 mmol) in CH₂Cl₂ (8 mL) was added methyl (*R*)-5-chloro-3-methyl-5-oxopentanoate (27 mg, 0.151 mmol) in CH₂Cl₂ (1.1 mL). The solution was cooled to 0 °C, and Me₂AlCl (0.14 mL, 1.0 M solution in hexanes) was added over 15 min. The reaction mixture was then stirred at 0 °C for 1 h and then at rt for a further 24 h. Aqueous NaHCO₃ (5 mL) was added and the aqueous layer was extracted with EtOAc (3 × 5 mL). The combined organic extracts were washed with brine (10 mL), dried over Na₂SO₄, concentrated to dryness, and the product was purified using silica gel column chromatography (2% EtOAc/hexane) to afford a yellow viscous oil containing the acylated product along with some inseparable impurities. The mixture, which was used as is without further purification, was dissolved in CH₃CN (7 mL), followed by the addition of HF-Pyridine (0.18 mL, 30% pyridine, 70% HF) at 0 °C. The reaction mixture was stirred at rt for 30 min. Aqueous NaHCO₃ (4 mL) was added and the aqueous layer was extracted with EtOAc (3 × 4 mL) and the organic extract dried over Na₂SO₄. After removal of the solvents *in vacuo* the crude product was passed through a short silica gel column and the combined filtrate was concentrated to obtain a yellow viscous oil that was subjected to ester hydrolysis under basic conditions. The yellow viscous oil was dissolved in THF (2 mL) and H₂O (0.5 mL). To the stirred solution was added LiOH.H₂O (24 mg, 0.572 mmol) and a drop of MeOH. The reaction mixture was stirred at rt for 16 h. H₂O (10 mL) was added and the

aqueous layer was extracted with EtOAc (3×4 mL), and the organic extract dried over Na_2SO_4 , concentrated to dryness, and the product was purified using silica gel column chromatography (10% MeOH/ CH_2Cl_2) to obtain the final product as a yellow viscous oil, 8.6 mg, 18% over three steps). HRMS (ESI) m/z calcd for $[\text{C}_{21}\text{H}_{28}\text{ClNO}_4 + \text{H}]^+$, 394.1785; found 394.1892. The diastomeric ratio was determined to be 81/19 using chiral HPLC.

2.15. (R)-1-(5-chloro-1-methyl-1H-indol-2-yl)hexan-1-ol (10)

The procedure was identical to that described for (S)-1-(5-chloro-1-methyl-1H-indol-2-yl)hexan-1-ol (7) except (R)-BINOL was used instead of (S)-BINOL.

2.16. (S)-5-(5-chloro-2-((R)-1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid (α R-OH-S230)

The procedure was identical to that described for (S)-5-(5-chloro-2-((S)-1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid (α (S)-OH-S230).

3. Results

3.1. Identification of α -hydroxy metabolites of 230

We previously detected two isomeric forms of a plasma metabolite of **230** (metabolites **d** and **e** in Fig. 2A) with retention times between those of **230** and ω 2-OH-**230**. Mass spectral evidence suggested that these isomers might be formed by hydroxylation of the alkyl side chain α , or possibly β , to the indole. However, we were unable to conclusively identify these isomers because of the lack of an authentic synthetic standard for comparison. To address the hypothesis that metabolites **d** and **e** are two stereoisomers of α -OH-**230** we prepared a mixture of the 4 possible diastereomers of this compound² as shown in Fig. 3. The α -OH center was introduced using a Grignard reaction between 5-chloro-1-methyl-1H-indole-2-carbaldehyde (**1**) and pentyl magnesium bromide (**2**). Because the free α -OH group was not tolerated in a Friedel-Craft's reaction to introduce the 5-oxo-valerate side chain, it was first protected by conversion to a silyl ether (OTBDMS) derivative. RP-HPLC of synthetic α -OH-**230** gave two peaks, labeled **x** and **y** in Fig. 2B, which had retention times identical to those of plasma-derived **d** and **e** (Fig. 2C) when chromatographed under the same conditions. Furthermore, **d** and **e** cochromatographed with **x** and **y** when equal amounts of these substances were mixed together Fig. 2D). We previously showed [4] that **d** and **e** have identical mass spectra, with $[\text{M}-\text{H}]^-$ ions consistent with monohydroxy metabolites of **230** and MS^2 fragmentation patterns showing a single major ion at m/z 292 corresponding to loss of the alkyl side chain, which was consistent with the MS^3 fragmentation pattern of this ion. In the present study we examined the mass spectral fragmentation of **x** and **y** derived from authentic α -OH-**230**. Both **x** and **y** had $[\text{M}-\text{H}]^-$ ions at m/z 392.1633, compared to the theoretical value of m/z 392.1629 expected for α -OH-**230** (mass accuracy, 1.0 ppm) and had identical MS^2 spectra (Fig. 2E), which contained a single major ion at m/z 292 (loss of alkyl group and a smaller ion at m/z 274 (loss of H_2O). The MS^3 profile resulting from fragmentation of the ion at m/z 292 (Fig. 2F) displayed major ions at m/z 274 (loss of H_2O),

²“ α ” is used to designate the carbon of the hexyl side chain adjacent to the indole moiety, whereas “ ω 2” is used to designate the penultimate carbon of the hexyl side chain.

248 (loss of CO₂), 206 (loss of CH₂=CH-CH₂-CO₂H due to a McLafferty rearrangement), and 180. Both the MS² and MS³ spectra of **x** and **y** are identical to those that we previously reported for **d** and **e** [4], confirming the identities of these metabolites as α-OH-**230** diastereomers.

As it contains two chiral carbons, we would expect synthetic α-OH-**230** to be a 1:1:1:1 mixture of four diastereomers. As pairs of enantiomers are unlikely to be separated by RP- or NP- HPLC, we would assume that product **x** is a mixture of either the αS-OH,S-Me and αR-OH,R-Me diastereomers or alternatively the αS-OH,R-Me and αR-OH,S-Me diastereomers, and vice versa for product **y**. To investigate their antagonist potencies, we purified all four diastereomers by a combination of NP- and chiral HPLC. NP-HPLC of α-OH-**230** gave 2 peaks, **x** and **y**, in the same elution order as with RP-HPLC (Fig. 4A). Product **x** was separated into two diastereomers, **x1** and **x2**, by chiral HPLC (Fig. 4B). Chiral HPLC also separated product **y** into two diastereomers, **y1** and **y2** (Fig. 4C). The effects of the above four diastereomers on 5-oxo-ETE-induced calcium mobilization are shown in Fig. 4D. Only **y1** had appreciable antagonist activity with an IC₅₀ of 1.6 ± 0.3 μM, over 100 times higher than that of **S230** (IC₅₀, 13 ± 2 nM in these experiments). The other three α-OH-**230** diastereomers had only very weak effects, inhibiting calcium mobilization by between 4 and 9% at a concentration of 10 μM.

3.2. Identification of plasma metabolites of **S230** in cynomolgus monkeys

To investigate the chirality of metabolites **d** and **e** and the time course for their formation we conducted *in vivo* experiments in cynomolgus monkeys. Because nearly all of the antagonist activity of **230** resides in the S enantiomer, we focused on **S230**, which was administered to three monkeys by oral gavage at a dose of 30 mg/kg. Blood samples were obtained after 1, 4, 8, 12, and 24 h and **S230** and its metabolites were extracted from plasma and analyzed by RP-HPLC. Fig. 5A shows the profile of **S230** metabolites in plasma after 8 h (note that a shallower gradient was used for this chromatogram compared to that shown in Fig. 2A). A substantial amount of **e** was formed from **S230**, but in contrast to racemic **230**, very little **d** was detected. Since the chiral methyl group of the substrate is in the S configuration, this suggests that the α-hydroxylation reaction is stereospecific, with the alkyl hydroxyl group being almost exclusively in either the R or S configuration. Metabolite **e** should therefore be either αS-OH-**S230** or αR-OH-**S230**. These diastereomers should be separable by RP-HPLC, but authentic standards would be required to distinguish between them.

In addition to the two ω2-oxidized metabolites that we had previously identified [4] and α-OH-**S230**, we observed several other major metabolites of **S230**, including **a** and **b**, which were considerably more polar than ω2-OH-**S230** (Fig. 5A). A minor product (**c**) was also observed after 8 h, but was much more prominent 1 h after administration of **S230** (inset to Fig. 5A). The polar metabolite **b** has a UV spectrum almost identical to that of metabolite **e**, with a maximum at 312 nm, compared to about 305 nm for **230** and its ω2-oxidation products (Fig. 5B), suggesting that it might also have an alkyl hydroxyl group α to the indole. The mass spectrum of **b** revealed an [M-H]⁻ ion at m/z 408.1571, compared to the theoretical value of m/z 408.1578 expected for a dihydroxy metabolite of **230** (mass accuracy, 1.7 ppm). The MS² fragmentation pattern of **b** (Fig. 5D) is very similar to that of

authentic α -OH-**230** (Fig. 2E) except that the ion formed by loss of H₂O was observed at m/z 390 instead of 374, consistent with the presence of an additional hydroxyl group. The MS³ fragmentation profile of the major ion in Fig. 5D at m/z 292 (Fig. 5E) formed by loss of the alkyl side chain was virtually identical to that shown in Fig. 2F for α -OH-**230**. From these data we conclude that **b** is a hydroxy metabolite of α -OH-**230**, most likely α,ω -diOH-**230**.

Two additional major metabolites of **S230** were also detected: **c**, which was prominent only at 1 h, and **a**, which was detected at all time points investigated. The UV spectra of **a** and **c** (Fig. 5C) are virtually identical to that of **S230**, indicating that they do not have a hydroxyl group on the alkyl side chain α to the indole. The mass spectrum of metabolite **c** had an [M-H]⁻ ion at m/z 552.1957, compared to the theoretical value of m/z 552.2000 expected for **230** glucuronide (mass accuracy, 7.8 ppm). The MS² fragmentation pattern for **c** had a single major ion at m/z 376 due to the characteristic loss of m/z 176 from a glucuronide (Fig. 5F). The MS³ spectrum of the latter ion (Fig. 5G) was virtually identical to the MS² spectrum of **230** (Fig. 5K) with major ions at 358 (loss of H₂O), 332 (loss of CO₂), 316 (loss of CH₂C(OH)₂ due to a McLafferty rearrangement), 290 (loss of CH₂=CH-CH₂-CO₂H due to a different McLafferty rearrangement), and 248 (loss of the acyl side chain).

Metabolite **a** was also identified as a glucuronide. It had an [M-H]⁻ ion at m/z 568.1950, identical to the theoretical value expected for a glucuronide of a monohydroxy metabolite of **230**. The MS² spectrum of **a** (Fig. 5H) had a single ion at m/z 392, consistent with the loss of glucuronide (m/z 176). The MS³ fragmentation pattern of this ion was virtually identical to that of authentic ω 2-OH-**230** (Fig. 5J), prepared as previously described [4], with ions at m/z 374 (loss of H₂O), 348 (loss of CO₂), 332 (loss of CH₂C(OH)₂ due to a McLafferty rearrangement), 330 (loss of H₂O and CO₂), 306 (loss of CH₂=CH-CH₂-CO₂H due to a different McLafferty rearrangement), 264 (loss of acyl side chain), and 246 (loss of the acyl side chain + H₂O). These results indicate that **a** is the glucuronide derivative of an ω -hydroxy metabolite of **S230**, probably ω 2-OH-**S230** glucuronide.

3.3. Pharmacokinetics of **S230** and its metabolites

S230 and its metabolites were quantitated by RP-HPLC using an isoheptyl analog of **S230**, which has a longer t_R than **S230**, as an internal standard. The identities of all compounds quantitated were confirmed by LC-MS/MS as well as by cochromatography when the authentic standards were available.

S230 rapidly appeared in the blood, reaching a maximal concentration of 32 μ M within 1 h and then declining to about 11% of this level by 4 h and to 5% by 8 h (Fig. 6A). Although they are quite high, these concentrations are lower than those that we previously reported for racemic **230** [4], also shown in Fig. 6A for comparison. In contrast, a biphasic pattern was observed with α -OH-**S230**, which reached maximal levels by 1 h, declined, and then rose again at 8 h (Fig. 6B). As would be expected, the concentration of the dihydroxy metabolite of **S230** rose more slowly, reaching a maximum by 8 h and then declining. ω 2-OH-**S230** increased to maximal levels by 4 h and then declined, whereas the maximal level of its oxidation product ω 2-oxo-**S230** was observed at 8 h (Fig. 6C). The glucuronide metabolites

of **S230** both peaked at 1 h and then declined (Fig. 6D). In contrast to **S230** glucuronide, which dropped to very low levels by 4 h, the time course for ω 2-OH-**S230** glucuronide was biphasic, with a second maximum being observed at 8 h.

3.4. Chiral synthesis of α -hydroxy diastereomers of **S230**

To determine the chirality of the α -hydroxyl group in metabolite **e** we synthesized both α S-OH-**S230** and α R-OH-**S230** using a previously reported method for the stereospecific addition of Grignard reagents to aromatic aldehydes [19]. Accordingly, (S)-BINOL-mediated enantioselective addition of pentyl magnesium bromide (**2**) to the prochiral aldehyde **1** yielded the α -hydroxy compound, 97.1% of which was the S enantiomer **7** (Fig. 7). The small amount of R-enantiomer was completely removed after recrystallization as demonstrated by chiral HPLC (Fig. 8B) using conditions that completely separate the R and S enantiomers (Fig. 8A). The absolute configuration of **7** was confirmed using single crystal x-ray diffractometry (Fig. 8C). The α -OH group of **7** was protected using TBDMS to obtain **8**. Friedel-Craft's acylation of **8** using the chiral acyl side chain **9** [11], followed by removal of the TBDMS with HF-pyridine and ester hydrolysis under basic conditions, afforded the desired compound α S-OH-**S230** with a chiral purity of 78% as determined by chiral HPLC.

The α (R)-hydroxy compound, α R-OH-**S230**, was prepared in a similar fashion as shown in Fig. 9. The enantioselective addition of the Grignard reagent **2** to the aldehyde **1** in the presence of (R)-BINOL afforded the α -hydroxy compound, 97.3% of which was the R enantiomer **10**. Compound **10** was then treated similarly to the S-enantiomer **7** to yield the final product α R-OH-**S230** with a chiral purity of 72%.

3.5. Identification of metabolite **e** as α S-OH-**S230**

To determine the configuration of the α -hydroxy metabolite **e** we compared its chromatographic properties to those of the synthetic compounds α S-OH-**S230** and α R-OH-**S230**. Initial studies revealed that with RP-HPLC the α R-OH diastereomer (labeled "RS" in Fig. 10A) has a shorter retention time than the α S-OH diastereomer (labeled "SS" in the figure). The t_R of metabolite **e** under the same conditions was identical to that of α S-OH-**S230** (Fig. 10B) and this was confirmed by cochromatography of **e** with a mixture of the two synthetic α -hydroxy compounds (Fig. 10C). There appeared to be a very small peak (**d**) corresponding to the elution position of α R-OH-**S230** (Fig. 10B). We measured the area of this peak at all time points and in all cases it amounted to about 3% of the area of metabolite **e** (Fig. 10G).

Similar experiments were performed using chiral HPLC. In this case, the t_R of α S-OH-**S230** was shorter than that of the corresponding α R-OH diastereomer (Fig. 10D). The t_R of **e** (Fig. 10E) was identical to that of α S-OH-**S230** and the two compounds cochromatographed with one another (Fig. 10F). Furthermore, we analyzed metabolite **e**, purified by RP-HPLC using an acetonitrile gradient, by chiral HPLC at all time points investigated and did not detect any other diastereomers (data not shown). Therefore, we can conclude that **e** is identical to α S-OH-**S230**. Metabolite **d** derived from **S230** is therefore probably identical to α R-OH-**S230**, although the amount detected is too small to be certain of this.

4. Discussion

In our previous studies we identified racemic **230** as a potent 5-oxo-EET antagonist [8] that rapidly appears in the blood following oral administration to monkeys [4]. By preparing a series of potential metabolites of **230** by total chemical synthesis we identified ω 2-oxidation of the hexyl side chain as a major metabolic pathway for this compound. However, we also observed a number of additional metabolites, including a pair of isomeric compounds that displayed a bathochromic shift in their UV spectra compared to **230** (305 nm $\rightarrow$ 311 nm), suggestive of a modification close to the indole chromophore. This was supported by mass spectral evidence showing that these metabolites possess an additional hydroxyl group in the alkyl side chain α or possibly β to the indole, but we were unable to confirm their identities due to the lack of authentic standards.

For the reasons discussed above we hypothesized that the isomeric metabolites **d** and **e** absorbing at 311 nm are a mixture of isomers of α -OH-**230**, which has 2 chiral carbons and therefore 4 possible diastereomers. We initially developed a procedure for the synthesis of a mixture of all four diastereomers. RP-HPLC of this mixture gave two peaks that cochromatographed with our novel metabolites and had identical MS² and MS³ spectra, confirming the presence of the hydroxyl group on the alkyl side chain α to the indole.

We expected that addition of a hydroxyl group at the ω -end of the hexyl group of **230** would reduce antagonist activity, consistent with the loss of agonist activity following ω 1-hydroxylation of 5-oxo-EET [20], and this proved to be the case [4]. However, it is more difficult to predict the effect of addition of a hydroxyl group α to the indole, which would be equivalent to the ω 6-position of 5-oxo-EET. 5-Oxo-EET can be hydroxylated in the ω 6-position by 15-lipoxygenase, resulting in the formation of 5-oxo-15S-HETE [21], which, although not as potent as 5-oxo-EET, is an OXE receptor agonist [5, 22]. In contrast, 5-oxo-EET can be hydroxylated in the ω 9-position by platelet 12-lipoxygenase to give 5-oxo-12S-HETE, which was the first OXE receptor antagonist to be recognized [23]. It was therefore important to examine the antagonist activities of the four synthetic α -OH-**230** diastereomers, which were purified by a combination of normal-phase and chiral HPLC. Interestingly, only one of these diastereomers, subsequently identified as α S-OH-**S230** (Table 1), displayed significant antagonist activity (IC₅₀, 1.6 μ M), being about 100 times less potent than **S230** in inhibiting 5-oxo-EET-induced calcium mobilization. This is similar to the IC₅₀ for ω 2-hydroxy-**230** (1.4 μ M) and a bit higher than that for ω 1-hydroxy-**230** (0.27 μ M) [4].

Because an S-methyl group is required for appreciable antagonist activity it could be assumed that the active α -hydroxy metabolite of **230** has an S-methyl group. Of the three inactive diastereomers two would have an R-methyl group and would therefore be expected to have little or no activity. However, the remaining inactive diastereomer would have an S-methyl group and either an R or an S α -hydroxyl group, indicating that only one of these configurations is compatible with antagonist activity. The impact of the chirality of the α -hydroxyl group could possibly be due to an effect on the orientation of the alkyl group, which we have shown to be very important for interaction with the OXE receptor [24]. There could also be hydrogen bonding between the α -hydroxyl group and the oxo group, which would likely be affected by its chirality.

For further studies on the pharmacokinetics and metabolism of **230** we focused on the active S-enantiomer (**S230**), which is over 400 times more potent than the R-enantiomer (**R230**) [8, 9]. To provide the gram-amounts of antagonist that would be required for this and subsequent *in vivo* studies we developed a novel procedure using the chiral reagent (S)-Tol-BINAP for the synthesis of the chiral acyl side chain of **S230** [11]. This permitted the synthesis of substantial amounts of this compound with high (>98%) enantiomeric purity.

The pharmacokinetic profile of **S230** is quite similar to that which we previously observed for racemic **230** [4] except that the plasma concentrations were lower at all time points, peaking at 32 μ M after 1 h, compared to 87 μ M for racemic **230**. This could be explained, at least in part, by differences in the rates of metabolism or intestinal uptake between **S230** and **R230**, consistent with our finding of higher plasma levels of the R enantiomer following oral administration of racemic **230** [4].

Analysis of the plasma metabolites of **S230** by RP-HPLC revealed a number of peaks that we had not previously identified (*a*, *b*, and *c*), and only one significant peak (*e*) for α -OH-**S230** in contrast to the two peaks (*d* and *e*) observed with racemic **230**. Since *e* is derived from **S230** it could be either α S-OH-**S230** or α R-OH-**S230**, which should be separable by RP-HPLC, as they are not mirror images of one another. This suggests that the α -hydroxylation reaction is stereospecific. To test this hypothesis, and to determine whether the α -hydroxy metabolite of **S230** in plasma is the one with antagonist activity, we prepared both of the above diastereomers using (S)-BINOL and (R)-BINOL and confirmed the absolute chirality of the products by X-ray diffractometry. Comparison of the chromatographic properties of the resulting authentic standards clearly indicated that both metabolite *e* derived from **S230** and the α -hydroxy-**230** diastereoisomer with antagonist activity are identical to α S-OH-**S230**. It is interesting that this highly stereoselective hydroxylation reaction results in the formation of the only diastereomer with significant antagonist activity.

The site at which the α -hydroxylation reaction occurs in the monkey is not clear. In our previous studies we did not detect α -OH-**230** following incubation of racemic **230** with monkey liver microsomes in the presence of NADPH, which instead converted this compound almost exclusively to ω 2- and to a much lesser extent, ω 1- oxidation products [4]. We have subsequently examined microsomes from other sources, including kidney, intestine, and lungs but did not detect α -hydroxylation activity in the presence of NADPH (data not shown). Neither did we detect such products as a result of incubating **230** with either plasma or intact leukocytes. One possibility might be that this reaction is catalyzed by bacteria in the gastrointestinal tract.

Although benzylic hydroxylation is quite well known [25, 26] and is an important step in the formation of noradrenaline, there are few examples in the literature of α -hydroxylation of alkylindoles. The bacterial enzyme indolyl-3-alkane α -hydroxylase, isolated from *Pseudomonas*, has been shown to catalyze α -hydroxylation of the side chain in the 3-position of tryptophan [27, 28]. This is not a direct hydroxylation, but rather proceeds by the initial abstraction of 2 hydrogens, one on the indole nitrogen, and the other on the side chain carbon adjacent to the indole ring. This results in the formation of a 3-alkylidene indoline

intermediate that undergoes the 1,4 addition of H₂O to give the α -hydroxy product [29]. However, 2-alkylindoles such as **S230** would not be a substrate for such a series of reactions.

In addition to α S-OH-**S230** we identified several other novel metabolites of **S230**. One of these (metabolite **b**) was formed by a combination of α - and ω -oxidation (Fig. 11). Although we cannot be certain of the location of the second hydroxyl group, it is highly likely that it is in the ω 2-position, because ω 2-hydroxylation is a major pathway for the metabolism of **S230**. The resulting product, α S, ω 2-dihydroxy-**S230** could be formed from either α S-OH-**S230** or ω 2-OH-**S230**.

We also detected glucuronide metabolites of **S230** and hydroxy-**S230**, presumably ω 2-OH-**S230**. The **S230** metabolite would clearly be an acyl glucuronide, as this compound does not have a free hydroxyl group. In contrast, ω 2-OH-**S230** could be converted to either an acyl or an ether glucuronide, or to both. At all time points later than 1 h, the concentrations of ω 2-OH-**S230** glucuronide(s) were considerably higher than those of **S230** glucuronide, even when the plasma concentrations of **S230** were much higher than its ω 2-hydroxy metabolite, which might be consistent with the glucuronide being in a different (i.e. ether) position, resulting in a more stable metabolite.

Overall, the combined concentrations of **S230** metabolites remained between 2 and 3 μ M over the first 8 h and then slowly declined, in contrast to **S230**, which declined fairly rapidly after reaching high initial levels by 1 h. By 8 h, the combined amounts of **S230** metabolites exceeded that of **S230** itself. Nevertheless, its concentration at this time was still greater than 1.5 μ M, over 100 times higher than its IC₅₀ (13 nM) for inhibition of 5-oxo-ETE-induced calcium mobilization *in vitro*. Multiple dosing with **S230** (e.g. every 8 h) may be required to achieve therapeutic levels over a period of 24 h in future *in vivo* experiments.

In conclusion, we have identified a novel stereoselective α -hydroxylation pathway for the metabolism of a 2-alkylindole antagonist in primates. The α S-hydroxy product of this pathway still retains some antagonist activity, albeit considerably reduced, in contrast to the corresponding α R-hydroxy compound, which is virtually inactive. Thus **S230** is metabolized *in vivo* by a combination of α -hydroxylation, ω 2-hydroxylation and glucuronidation. In spite of its metabolism by these pathways, plasma levels of **S230** well in excess of its *in vitro* IC₅₀ value were maintained for 8 h. These results raise the possibility that either **S230** itself or a related compound modified to resist hydroxylation, might be a useful therapeutic agent in eosinophilic disorders such as asthma. Studies are currently underway in primate models of allergic disease to test this hypothesis.

Acknowledgments

This work was supported by the Canadian Institutes of Health Research (WSP: Grants MOP-6254 and PP2-133388), the American Asthma Foundation (JR: Grant 12-0049), and the National Heart, Lung, and Blood Institute (JR: Grant R01HL081873) and by AmorChem (Montreal, QC). The Meakins-Christie Laboratories-MUHC-RI are supported in part by a Centre grant from Le Fond de la Recherche en Santé du Québec as well as by the J. T. Costello Memorial Research Fund. JR also wishes to acknowledge the National Science Foundation for the AMX-360 (Grant CHE-90-13145) and Bruker 400 MHz (Grant CHE-03-42251) NMR instruments. DV and IS were supported by the Natural Sciences and Engineering Research Council of Canada (Grant RGPIN/435814-2103). IS also wishes to acknowledge the Centre for Biological Applications of Mass Spectrometry at Concordia University for PhD scholarship funding. The content is solely the responsibility of the authors and does

not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

Abbreviations

5-HETE	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid
5-oxo-ETE	5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid
230	5-(5-chloro-2-hexyl-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoate
S230	(S)-5-(5-chloro-2-hexyl-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoate
αS-OH-S230	(S)-5-(5-chloro-2-((S)-1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoate
ω2-OH-S230	(3S)-5-(5-chloro-2-(5-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoate
BDMAEE	bis[2-(<i>N,N</i> -dimethylamino)ethyl] ether
DART	direct analysis in real time
HF	Hydrofluoric acid
HRMS	high-resolution mass spectra
MeOH	methanol
(S)-BINOL	(<i>S</i>)-(-)-1,1'-bi(2-naphthol)
TBDMSCI	<i>tert</i> -butyldimethylchlorosilane
THF	tetrahydrofuran
TMS	tetramethylsilane

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Figure 1.
Inhibition of 5-oxo-EETE-induced eosinophil activation by S230.

Figure 2. Cochromatography of metabolites *d* and *e* from plasma with synthetic α -hydroxy-230 diastereomers

A: Plasma from a monkey obtained 4 h after oral administration of racemic **230** (30 mg/kg) was extracted as described in Materials and Methods and an aliquot was analyzed by RP-HPLC on a Kinetex ODS-silica column using a 30 to 65% gradient of MeCN in H₂O over 30 min. **B–D:** RP-HPLC of synthetic racemic α -hydroxy-**230** (**B**), metabolites *d* and *e* from plasma (**C**) and a mixture of plasma metabolites and synthetic racemic α -hydroxy-**230** (**D**) using a 35 to 56% gradient of MeCN in H₂O over 42 min. **E:** MS² fragmentation of the [M-H][−] ion at *m/z* 392 of synthetic α -OH-**230** (peak *y*). **F:** MS³ fragmentation of the ion at *m/z* 292 shown in panel E. * Ion formed due to a McLafferty rearrangement.

Figure 3.

Procedure for the synthesis of 5-(5-chloro-2-(1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid (α -OH-230).

Figure 4. Separation and antagonist activity of diastereomers of synthetic racemic α -OH-230

A: NP-HPLC of synthetic α -OH-230 was performed as described in Materials and Methods.

B: Peak *x* from panel A was subjected to chiral-HPLC on a Cellulose-2 column as described in Materials and Methods with a mobile phase of hexane/EtOH/HOAc (85:15:0.1).

C: Peak *y* from panel E was subjected to chiral-HPLC on a Cellulose-2 column with a mobile phase of hexane/EtOH/HOAc (93:7:0.1).

D: The effects of S230 and diastereomers *x1* ($\blacktriangle$), *x2* ($\triangle$), *y1* ($\bullet$) and *y2* ($\circ$), purified as shown in panels A to C, on 5-oxo-EET-induced calcium mobilization in human neutrophils were determined as described in Materials and Methods.

Figure 5. Identification of plasma metabolites of S230 by RP-HPLC, UV absorbance and mass spectrometry

A: Plasma was prepared from a blood sample taken from a cynomolgus monkey 8 h after oral administration of **S230** (30 mg/kg). After solid-phase extraction the sample was analyzed by RP-HPLC using a Kinetex ODS-silica column with a gradient between 35 and 66% MeCN in H₂O over 62 min. **B:** UV spectra of **230** and its major plasma metabolites formed by oxidation of the hexyl side chain. **C:** UV spectra of glucuronide metabolites **a** and **c** of **230**. **D:** MS² fragmentation of the [M-H]⁻ ion at m/z 408 for plasma metabolite **b**. **E:** MS³ fragmentation of the ion at m/z 292 for metabolite **b** from panel D. **F:** MS² fragmentation of the [M-H]⁻ ion at m/z 552 for metabolite **c**. **G:** MS³ fragmentation of the ion at m/z 376 from metabolite **c** shown in panel F. **H:** MS² fragmentation of the [M-H]⁻ ion at m/z 568 for metabolite **a**. **I:** MS³ fragmentation of the ion at m/z 392 from metabolite **a** shown in panel G. **J:** MS² fragmentation of synthetic ω 2-OH-230 for comparison with the spectrum shown in panel I. **K:** MS² fragmentation of **230** for comparison with the spectrum shown in panel G. * Ion formed due to a McLafferty rearrangement.

Figure 6. Levels of S230 and its major metabolites in plasma

Plasma was prepared from blood taken from cynomolgus monkeys at various times after oral administration of **S230** (30 mg/kg). After solid-phase extraction, components were analyzed as illustrated in Fig. 5 except that a gradient of MeOH (60 to 80% over 60 min) in H₂O was used (see Materials and Methods for further details). Plasma levels are shown for **A: S230** (▲) and racemic **230** (△; data from ref 4), **B: α-OH-S230** (●) and α,ω2-diOH-S230 (○), **C: ω2-OH-S230** (●) and ω2-oxo-S230, and **D: S230 glucuronide** (●; Gluc) and ω2-OH-S230 glucuronide (○; ω2-OH-G). The scales for the Y-axes in panels B, C, and D are identical.

Figure 7.

Procedure for the synthesis of (S)-5-(5-chloro-2-((S)-1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid ($\alpha(S)$ -OH-S230).

Figure 8. Chiral HPLC and X-ray crystal structure of 7

Chiral HPLC of the racemic version of **7** (**A**) and crystallized synthetic **7** (S-enantiomer) (**B**) on a Cellulose-2 column using hexane/MeOH/HOAc (98:2:0.1) as the mobile phase at a flow rate of 1 ml/min and a column temperature of 45 °C. **C**: X-ray crystal structure of **7**. Thermal ellipsoids are set at the 50% probability level.

Figure 9.

Procedure for the synthesis of (S)-5-(5-chloro-2-((R)-1-hydroxyhexyl)-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid ($\alpha(R)$ -OH-S230).

Figure 10. Cochromatography of metabolite *e* with α S-OH-S230

A–C: Synthetic α -OH-S230 diastereomers and metabolite *e* isolated from plasma were analyzed by RP-HPLC using a Kinetex ODS column with a mobile phase consisting of a gradient between 45 and 55% MeCN in H₂O over 30 min with a flow rate of 1 ml/min and a column temperature of 35 °C. **A:** a mixture of synthetic α S-OH-S230 (SS) and α R-OH-S230 (RS). **B:** Metabolite *e* purified from plasma from a monkey following administration of **230** (30 mg/kg). The arrow shows the elution position of α R-OH-S230 (RS), run separately. **C:** Cochromatography metabolite *e* with a mixture of the synthetic SS and RS isomers of α -OH-S230. **D–F:** Chiral HPLC of the above compounds on a Cellulose-2 column with a mobile phase of hexane/EtOH/HOAc (93:7:0.1) and a flow rate and column temperature of 1 ml/min and 45 °C, respectively. **D:** a mixture of synthetic α S-OH-S230 (SS) and α R-OH-S230 (RS); **E:** Metabolite *e* purified from plasma. **F:** Cochromatography metabolite *e* with a mixture of the synthetic SS and RS isomers of α -OH-S230. The elution positions of α R-OH-R230 (RR), and α S-OH-R230 (SR), run separately, are shown by arrows. **G:** Levels of metabolites *d* and *e* in plasma following the administration of **S230** (30 mg/kg) by oral gavage.

Figure 11. *In vivo* metabolism of S230 in monkeys

Metabolites shown in red were identified for the first time in the present study (i.e. the two α-OH metabolites and the two glucuronides). The broken arrows indicate that αS,ω2-diOH-S230 could potentially be formed from either ω2-OH-S230 or αS-OH-S230. The glucuronate in ω2-OH-S230 glucuronide could be attached to either the carboxyl group or the hydroxyl group. * It is highly likely that the hydroxyl group is in the ω2-position in **a** and **b**, but this has not been conclusively proven.

Table 1

Synthetic and biological α -hydroxy metabolites of **230**. The IC₅₀ values were determined from the data shown in Fig. 3D. The IC₅₀ value for **S230** in these experiments was 13 ± 2 nM.

Synthetic diastereomer	Structure	Plasma metabolite	IC ₅₀ (μ M)
<i>x1</i>	α S-OH- R230		$\gg 10$
<i>x2</i>	α R-OH- S230	<i>d</i> (?)	$\gg 10$
<i>y1</i>	α S-OH- S230	<i>e</i>	1.6 ± 0.3
<i>y2</i>	α R-OH- R230		$\gg 10$