Inhibition of Leukotriene and Thromboxane Biosynthesis by a 9-(4-Chlorophenyl) Analogue of Arachidonic Acid

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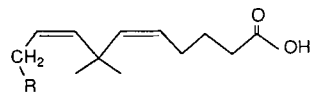
Abstract □ The synthesis of 9-(4-chlorophenyl)-7,7-dimethyl-5(*Z*), 8-nonadienoic acid (**7**) and its methyl ester **6**, and their effects on arachidonic acid metabolism in vitro are described. The IC_{50} values of 19.6 and 20.6 μM were observed for inhibition of leukotriene synthesis in human granulocytes for **6** and **7**, respectively. Additionally, the compounds inhibited thromboxane B_2 (TxB_2) synthesis, with respective IC_{50} values of 6.1 and 20 μM, while producing pronounced 3–8-fold increases in PGE_2 synthesis in human mononuclear cells. Increased PGE_2 synthesis may have reflected shunting of free arachidonic acid substrate at the thromboxane synthetase and endoperoxide E isomerase branchpoint of arachidonic acid metabolism.

The identification of the components of the slow-reacting substance of anaphylaxis (SRS-A) as leukotrienes and the elucidation of the biosynthetic pathway for leukotrienes via the lipoxygenation of arachidonic acid have prompted research into the design of specific inhibitors of the lipoxygenase enzymes (for reviews see Goetzl et al.[1] and Bach[2]). Such an agent might be of value in the therapy of diseases in which these potent lipid mediators have been implicated, such as asthma, psoriasis, allergic disorders, myocardial ischemia, and inflammation. The enzyme 5-lipoxygenase controls the entry of free arachidonic acid into the oxidative pathways which can lead to the formation of the peptidoleukotrienes (LTC_4, LTD_4, and LTE_4) 5,12-diHETEs and LTB_4, and lipoxins, as shown in Scheme I. Substrate recognition and subsequent catalysis require a 1,4-diene system that is 5–9 carbons removed from the carboxylic acid group in polyenoic acid substrates such as 5,8,11-eicosatrienoic acid, 5,8,11,14-eicosatetraenoic acid (arachidonic acid), and 5,8,11,14,17-eicosapentenoic acid.[3,4] In 5-lipoxygenation of arachidonic acid, the reaction appears to proceed via the coordinate addition of oxygen across the 5,6-double bond, with a double-bond shift from 5,6- to 6,7-positions to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE).[5] The reaction requires the abstraction of a proton at position 7 to complete the double-bond shifts. Based on this rationale, Pfister and Krishna Murthy,[6] Perchonock et al.,[7] and Achroyd et al.[8] synthesized several 7,7-dimethyl analogues of arachidonic acid to prevent the double-bond shift requisite for formation of 5-HPETE. The resulting compounds (**1**, **2**, and **3**) functioned as weak inhibitors or stimulators of leukotriene synthesis in intact cells in vitro, apparently through a competitive interaction with 5-lipoxygenase.



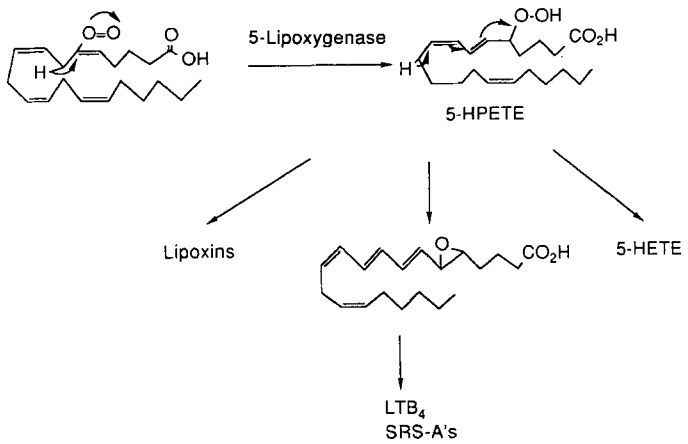
1, R= $CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}(CH_2)_4CH_3$

2, R= $CH{=}CH(CH_2)_7CH_3$

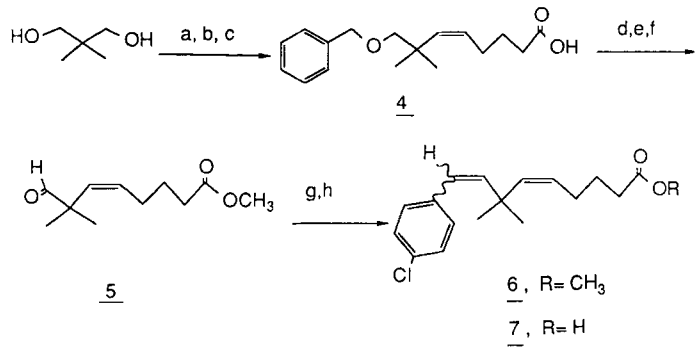
3, R= $(CH_2)_9CH_3$

Their results prompted us to report our findings on the syntheses and inhibitory activities of 9-(4-chlorophenyl)-7,7-dimethyl-5(*Z*), 8-nonadienoic acid (**7**) and its methyl ester (**6**). We speculated that the aryl group could isosterically replace the C-10 to C-20 diene segments of arachidonic acid. This may circumvent oxidative metabolism by 12- and 15-lipoxygenase enzymes which are known to reside in the same cells which contain both arachidonate substrate and 5-lipoxygenase enzymes.

The preparation of 9-(4-chlorophenyl)-7,7-dimethyl-5(*Z*), 8-nonadienoic acid was as follows (Scheme II). The key intermediate aldehyde **5** was prepared in a manner similar to that



Scheme I



Reagents:

a. $NaH/PhCH_2Cl$; b.PDC; c. $Ph_3P{=}CH(CH_2)_3CO_2^-Na^+$; d. Na/NH_3

e. CH_2N_2 ; f.PDC ; g. $Ph_3P{=}CHC_6H_4Cl$; h. $K_2CO_3/CH_3OH/H_2O$

Scheme II

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described by Achroyd et al.[8] 2,2-Dimethyl-1,3-propanediol was converted to the hydroxyl monobenzyl ether, which was oxidized by pyridinium dichromate (PDC) to the aldehyde. Immediate treatment with the ylid formed from (4-carboxybutyl)triphenylphosphonium bromide gave 7,7-dimethyl-8-(phenylmethoxy)-5(*Z*)-octenoic acid (4). Debenzylation with sodium in liquid ammonia and conversion to the methyl ester afforded the alcohol which was oxidized by PDC to the aldehyde 5. Wittig reaction with the ylid formed from (4-chlorobenzyl)triphenylphosphonium chloride gave methyl 9-(4-chlorophenyl)-7,7-dimethyl-5(*Z*), 8-(*E*/*Z*) nonadienoate (6). The *E*/*Z* isomers were unseparable by column chromatography on silica gel. The ratio of 8-(*E*/*Z*) isomers in the mixture was determined to be 1:2.7 by GC and ^{1}H NMR analyses. The ^{1}H NMR (360 MHz) showed two doublets (J = 12 Hz) at 5.75 and 6.38 δ that were attributed to C-8 and C-9 vinyl protons of the 8-*Z* isomer. The 8-*E* isomer exhibited a singlet at 6.30 δ that was attributed to the two vinyl protons at C-8 and C-9. Carbonate catalyzed hydrolysis gave the acid 7. The biological testing was done on the *E*/*Z* mixture.

Experimental Section

Elemental analyses were done by Atlantic Microanalysis, Inc., Atlanta, GA. The ^{1}H NMR measurements were obtained on a Varian Associates EM-360, EM-390 or Bruker 360 MHz spectrometer, and shift values are reported in δ downfield from tetramethylsilane as the internal standard. Infrared spectra were recorded on a Perkin Elmer 521 spectrophotometer. Mass spectra were recorded on a Finnigan 3300 spectrometer.

7,7-Dimethyl-8-(phenylmethoxy)-5(*Z*)-octenoic Acid—To a stirred mixture of 29.0 g (1.2 mol) of sodium hydride and 200 mL of dry dimethylformamide (DMF), under an atmosphere of nitrogen at 0 °C, was added a solution of 125 g (1.20 mol) of 2,2-dimethyl-1,3-propandiol in 100 mL of dry DMF in a dropwise manner. The resulting reaction mixture was stirred at room temperature overnight and then 139 mL (1.20 mol) of benzyl chloride was added. After stirring for 12 h, the resulting reaction solution was poured into 300 mL of an ice-water mixture and the aqueous solution was extracted three times with ethyl ether. The organic layers were combined and washed with saturated solution and then dried over Na_2SO_4. Removal of solvent gave a brown oil which was distilled through a 20-cm vigreux column to obtain 133.7 g (54.7%) of 2,2-dimethyl-3-(phenylmethoxy)propanol, bp 85–87 °C/0.02 mmHg, as a colorless liquid; ^{1}H NMR ($CDCl_3$): δ 0.90 (s, 6H), 3.3 (s, 2H), 2.9 (t, OH), 3.40 (d, J = 5 Hz, 2H), 4.45 (s, 2H), and 7.22 ppm (s, 5H).

In a dry three-necked flask, equipped with a mechanical stirrer and an addition funnel, was placed 280 g (0.74 mol) of pyridinium dichromate and 1000 mL of dry CH_2Cl_2. The mixture was vigorously stirred and a solution of 43.73 g (0.225 mol) of 2,2-dimethyl-3-(phenylmethoxy)propanol in 80 mL of CH_2Cl_2 was added in one portion. After stirring for 4 d, the dark brown reaction mixture was poured into 2000 mL of ethyl ether and the resultant mixture was passed through a dry silica gel column to obtain a colorless solution. Removal of the solvent gave a residue which was distilled through a 20-cm vigreux column to obtain 35.2 g (80.5% yield) of 2,2-dimethyl-3-(phenylmethoxy)propanol as a colorless liquid, bp 72–75 °C/0.02 mmHg; ^{1}H NMR ($CDCl_3$): δ 1.15 (s, 6H), 3.45 (s, 2H), 4.50 (s, 2H), 7.25 (s, 5H), and 9.50 ppm (s, 1H).

Into a dry three-necked flask, equipped with an addition funnel, a gas bubbler, and a magnetic stirrer, which was under an atmosphere of N_2, was placed 25.57 g (0.54 mol) of 50% oily sodium hydride. The oil was removed by washing with hexane. Dimethyl sulfoxide (DMSO; 270 mL, freshly distilled over CaH_2) was introduced, and then the flask was heated (82 °C) in an oil bath until the gas evolution ceased. The obtained greenish solution was cooled to room temperature and a solution of 120 g (0.27 mol) of (4-carboxybutyl)triphenylphosphonium bromide in 340 mL of DMSO was added in a dropwise manner. After the addition, a solution of 42.77 g (0.222 mol) of 2,2-dimethyl-3-(phenylmethoxy)propanol in 50 mL of DMSO was added, and stirring at room temperature was continued overnight. The reaction mixture was poured into ice water and acidified with 3 M HCl. The aqueous solution was extracted with three 300-mL portions of ethyl ether. The combined ether solution was washed successively with small portions of 2.8 M NH_4OH solution until the obtained aqueous solution was basic. The aqueous washings were combined and acidified with 3 M HCl. The acidified aqueous solution was extracted with three 100-mL portions of ethyl ether. The obtained organic solution was washed once with NaCl solution and then dried over magnesium sulfate. Removal of solvent on a rotary evaporator under reduced pressure gave an oily residue. The residue was distilled through a Kugelrohr distillation apparatus to give 56.81 g (93% yield) of the title product as a colorless oil, bp 151–161 °C/0.02 mmHg; ^{1}H NMR ($CDCl_3$): δ 1.11 (s, 6H), 1.55–1.9 (m, 2H), 2.08–2.5 (m, 4H), 3.2 (s, 2H), 4.5 (s, 2H), 5.2–5.5 (m, 2H), and 7.31 ppm (s, 5H); IR (neat): 1711 cm^{-1}.

Anal.—Calc. for $C_{17}H_{24}O_3$: C, 73.88; H, 8.75. Found: C, 73.21; H, 8.59.

Methyl 9-(4-chlorophenyl)-7,7-dimethyl-5(*Z*), 8-nonadienoate—In a dry flask, equipped with a mechanical stirrer, a dry-ice condenser, and a gas bubbler, was introduced 100 mL of liquid NH_3 and then 8.67 g of 7,7-dimethyl-8-(phenylmethoxy)-5(*Z*)-octenoic acid. Sodium metal in small portions was added until the blue color of the reaction mixture was persistent. The reaction was then quenched by addition of NH_4Cl; NH_3 was removed by bubbling with N_2 gas. The residue was dissolved in water and acidified with 6 M HCl. The aqueous solution was extracted with ethyl ether. The organic solution was washed with a water and NaCl solution and dried over $MgSO_4$. To the solution was added excess CH_2N_2 in ethyl ether solution and the solvent was removed to give a residue. The residue was distilled to yield 5.60 g (96%) of methyl 7,7-dimethyl-8-hydroxy-5(*Z*)-octenoate, bp 90 °C/0.05 mmHg; MS (CI): *m/e* 201 (M + 1); ^{1}H NMR ($CDCl_3$): δ 1.10 (s, 6H), 1.5–2.5 (m, 6H), 3.35 (d, 2H), 3.65 (s, 3H), and 5.2–5.40 ppm (m, 2H).

In a three-necked flask, equipped with a mechanical stirrer and an addition funnel, was placed 24 g (0.069 mol) of pyridinium dichromate and 120 mL of CH_2Cl_2. The mixture was stirred vigorously and a solution of 6.73 g (0.034 mol) of methyl 7,7-dimethyl-8-hydroxy-5(*Z*)-octenoate in 20 mL of CH_2Cl_2 was added in one portion. The reaction mixture was stirred continuously for 24 h, and then diluted with 200 mL of ethyl ether. The resulting mixture was filtered through a dry silica gel column to obtain a colorless filtrate. The residue, after removal of solvent, was distilled to give 5.20 g (77%) of methyl 7,7-dimethyl-8-oxo-5(*Z*)-octenoate, bp 75–82 °C/0.02 mmHg; ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 6H), 1.4–2.4 (m, 6H), 3.65 (s, 3H), 5.1–5.6 (m, 2H), and 9.40 ppm (s, 1H).

In a dry three-necked flask, equipped with an addition funnel and a mechanical stirrer under an atmosphere of N_2, was placed 120 mL of tetrahydrofuran (THF), 9.5 g (0.0224 mol) of (4-chlorobenzyl)triphenylphosphonium chloride (Alfa), and 4.1 g (0.0224 mol) of sodium hexamethyldisilazane (Petrarch Systems, Inc.). The mixture was stirred at room temperature for 0.5 h, and then a solution of 3.8 g (0.019 mol) of methyl 7,7-dimethyl-8-oxo-5(*Z*)octenoate in 200 mL of THF was added in a dropwise manner. The reaction mixture was stirred at room temperature for 12 h and then at 70 °C for 4 h. The resulting mixture was allowed to cool to room temperature and then was poured into an ice-water mixture. The aqueous solution was acidified with 1 M HCl and extracted three times with ether. The organic solution was washed with a water, 5% $NaHCO_3$, and NaCl solution, and dried over Na_2SO_4. The residue, after removal of solvent, was purified by flash-column chromatography on silica gel (hexane:EtOAc, 200:1 to 16:1, v/v) to give 4.50 g of the title compound (73% yield) as a mixture of 8-*Z* and 8-*E* isomers in a ratio of 2.7 to 1 as determined by GC; ^{1}H NMR ($CDCl_3$): δ 1.20 and 1.28 (s, 6H), 1.4–1.9 (m, 2H), 1.95–2.50 (m, 4H), 3.60 and 3.68 (s, 3H), 4.86–5.58 (m, 2H), 5.75 (d, J = 12 Hz, 0.72 H), 6.28 (d, J = 12 Hz, 0.72 H) assigned to two olefinic protons of the 8-*Z* isomer, and 6.30 ppm (s, 0.56 H) assigned to two olefinic protons of 8-*E* isomer; IR ($CHCl_3$): 1735.5 cm^{-1}.

Anal.—Calc. for $C_{18}H_{23}ClO_2$: C, 70.46; H, 7.56; Cl, 11.55. Found: C, 70.51; H, 7.58; Cl, 11.61.

9-(4-Chlorophenyl)-7,7-dimethyl-5(*Z*), 8-nonadienoic Acid—A mixture of 2.83 g of methyl 9-(4-chlorophenyl)-7,7-dimethyl-5(*Z*), 8-nonadienoate, 5.2 g of potassium carbonate, 60 mL of methanol, and 15 mL of water was stirred at room temperature for 24 h. Methanol was removed and the residue was diluted with 50 mL of water and extracted one time with ether to remove the unreacted starting material. The aqueous solution was acidified with 6 M HCl and then extracted with two times ethyl ether. The organic layer was washed with NaCl solution and dried over $MgSO_4$. Removal of solvent

yielded 2.40 g of the title compound as an oil; ^{1}H NMR ($CDCl_3$): δ 1.2 and 1.28 (s, 6H), 1.4–1.9 (m, 2H), 1.95–2.50 (m, 6H), 4.86–5.58 (m, 2H), 5.75 (d, J = 12 Hz, 0.72 H), 6.28 (d, J = 12 Hz, 0.72 H), and 6.30 ppm (s, 0.56 H); IR ($CHCl_3$): 1709.0 cm^{-1}.

Anal.—Calc. for $C_{17}H_{21}ClO_2$: C, 69.73; H, 7.23; Cl, 12.11. Found: C, 69.61; H, 7.26; Cl, 12.12.

Human Granulocyte Assay for Lipoxygenase Products (HGALP)—Synthesis of lipoxygenase products from [^{3}H]arachidonic acid precursor was studied in a granulocyte-enriched fraction from human blood by a modification of the procedure described by Aharony et al.[9] Briefly, a granulocyte-enriched fraction was prepared from freshly drawn human blood by standard techniques using ficoll hypaque. Cells were prelabeled with [^{3}H]arachidonic acid (20 μCi per 2 mL of cell suspension) by incubation at 37 °C for 30 min. The labeled cells were washed and resuspended in an incubation buffer consisting of 14 μM indomethacin in minimum essential medium at a final concentration of 1×10^7 cells/mL. Cell suspensions (1.0 mL) were incubated for 5 min in the presence or absence of test compounds delivered in 5 μL of DMSO. The calcium-dependent metabolism of [^{3}H]arachidonic acid was stimulated by adding 10 μM calcium ionophore A23187. After 5 min at 37 °C, incubations were stopped by adding 1 mL of ice-cold phosphate buffered saline, and cells were pelleted by centrifugation. Supernatants were passed over C_{18} Sep Pak cartridges which were washed with 1 mL of distilled water. Lipoxygenase metabolites eluted from the columns in two 1-mL volumes of methanol. In separate experiments, the radioactivity eluted under these conditions was identified as predominately 5,12-diHETEs, including LTB_4, by HPLC analysis. The eluates were collected in scintillation vials and counted in 10 mL of aqueous counting scintillant (ACS) to quantify the synthesis of radiolabeled lipoxygenase metabolites.

Values were normalized to DMSO vehicle controls and expressed as percent inhibition; IC_{50} values were estimated from the linear regression analysis of log concentration versus response curves.

Measurement of PGE_2 and Thromboxane B_2 (TxB_2) in a Human Mononuclear Cell-Enriched Fraction—A mononuclear cell fraction was prepared from human blood by standard methods using ficoll hypaque. Cells were incubated with and without experimental drugs and stimulated to produce cyclo-oxygenase products with 10 μM calcium ionophore A23187, using the incubation conditions described above. The supernatants were assayed for PGE_2 and TxB_2 using radioimmunoassay kits purchased from Seragen, Boston, MA. Values for inhibition of prostanoid synthesis were calculated as described above.

Immunologically Mediated Contraction of the Guinea Pig Lung Parenchymal Strip (IMCPS)—A modification of the procedure described by Forsberg and Sorenby[10] was used to measure the immunologically evoked release of leukotrienes in an airway smooth muscle preparation. Male Hartley guinea pigs (Hazelton Dutchland) weighing 200–250 g were immunized by the subcutaneous administration of 1.0 mg of chicken egg albumin plus 11×10^9 cells of *Bordetella pertussis* (Mass. Public Health Biologics Laboratories) three to four weeks prior to experiments. Animals were sacrificed and heart and lungs removed en bloc, and peripheral lung strips were prepared. Lung strips were suspended in a 10 mL of siliconized, glass-isolated organ bath containing oxygenated Krebs buffer. Strips were attached to a Grass FT03 isometric force displacement transducer under an initial load of 1 g, and equilibrated for 45–60 min. Chlorpheniramine (10 μM) and indomethacin (100 μM) were added to eliminate the contribution of histamine and prostaglandins to the contractile response. Tissues originating from the same animal were matched so that one tissue served as a vehicle control. Test drug was added and the tissues were incubated an additional 30 min. Egg albumin (0.1 μg/mL) was added to each bath and the contractile response was measured as milligrams of developed load. Values were normalized to vehicle controls and expressed as percent inhibition.

Results and Discussion

Compounds 6 and 7 demonstrated comparable inhibition of arachidonic acid metabolism via the 5-lipoxygenase pathway in intact human granulocytes (HGALP assay), with calculated IC_{50} values of 19.6 and 20.6 μM, respectively (Table I). Eicosatetraynoic acid (ETYA), a known irreversible inhibitor of 5-lipoxygenase,[11] exhibited an IC_{50} of 3.9 μM in the same experiment. Compounds 6 and 7 produced >90% inhibition of [^{3}H]arachidonic acid metabolism at the highest tested concentration of 100 μM. In our functional assay of 5-lipoxygenase activity in airway smooth muscle, the free acid 7 demonstrated greater activity (80% inhibition at 100 μM) than the methyl ester 6 (20% inhibition at 100 μM) in inhibiting the antigen-induced contraction of the lung parenchymal strip (IMCPS). This difference in activity was interpreted to be due to the greater solubility of the free acid, since the aqueous buffer of the isolated tissue bath requires that the compounds be sufficiently soluble to diffuse through the bath to reach the sites of action in the suspended tissues. In the HGALP assay, the high concentration of cells in a small volume (1×10^7 cell per mL) requires less aqueous solubility of compounds to reach the cells. Hence, equivalent intrinsic activity for 6 and 7 was observed in this assay. Compound 7 compared favorably with the reference inhibitor ETYA in the IMCPS assay. Thus, in two separate assays, the 4-chlorophenyl-substituted analogues of arachidonic acid functioned as inhibitors of arachidonic acid metabolism via the 5-lipoxygenase pathway. To determine the specificity of this inhibitory activity, the effects of 6 and 7 on the cyclooxygenase pathway of arachidonic acid metabolism were assessed by determining their effect on PGE_2 and TxB_2 synthesis in human mononuclear cells stimulated by calcium ionophore A23187. Both compounds inhibited the synthesis of TxB_2 (Table II). Compound 7 exhibited an IC_{50} of 20 μM, and 6 was slightly more potent with an IC_{50} of 6.1 μM. Both compounds were substantially less potent than the reference cyclo-oxygenase inhibitor indomethacin, which produced an IC_{50} of 0.018 μM. In contrast to the inhibition of TxB_2 synthesis, the synthesis of PGE_2 was markedly enhanced by both compounds in the same human mononuclear cell preparation stimulated to produce prostinoids by treatment with the calcium ionophore A23187. This pattern of inhibition of TxB_2 synthesis with concurrent increased PGE_2 synthesis was interpreted as reflecting selective inhibition of thromboxane synthetase. With the thromboxane synthetase pathway blocked, more free arachidonic acid would be available to enter into the PGE_2 branch of the prostinoid synthetic pathway in human mononuclear cells. A similar phenomenon has been reported previously with the specific thrombox-

Table I—Effects of Compounds in Human Granulocyte Assay for Lipoxygenase Products (HGALP) and Immunologically Mediated Contraction of Parenchymal Strip (IMCPS) Tests for Lipoxygenase Function

Compound	HGALP		IMCPS
	IC_{50}, μM (95% confidence limits)	% Inhibition at 100 μM	% Inhibition at 100 μM[a]
6	19.6 (11.1–39.9)	93	20 ± 7
7	20.6 (11.3–44.0)	91	80 ± 18
ETYA[b]	3.9 (2.2–60)	96	62 ± 4

[a] Mean ± SD. [b] Eicosatetraynoic acid.

Table II—Effects of Compounds on PGE_2 and Thromboxane B_2 (TxB_2) Synthesis in Human Mononuclear Cells

Compound	TxB_2 Synthesis	PGE_2 Synthesis
	IC_{50}, μM (95% confidence limits)	% Stimulation at 100 μM (Mean ± SD)
6	6.1 (3.6–10)	378 ± 87
7	20 (14–30)	805 ± 100
Indomethacin	0.018 (0.014–0.024)	0.0061 (0.0051–0.0080)[a]

[a] Inhibition, IC_{50} (μM, 95% confidence limits).

ane synthetase inhibitor dazoxiben.[12,13] In contrast, the reference cyclo-oxygenase inhibitor indomethacin inhibited both PGE_2 and TxB_2 synthesis.

Conclusions

Replacement of the undecadienyl chain at the 9-position of 7,7-dimethyl analogues of arachidonic acid with a 4-chlorophenyl group produced inhibitors of the lipoxygenase pathway and thromboxane synthetase. These findings demonstrate that aromatic moieties may replace the dienoic "tail" of arachidonic acid analogues giving substrate analogues which exhibit significant potency as inhibitors of arachidonic acid metabolism. This approach may allow the design of more stable and specific inhibitors of arachidonic acid metabolism.

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