

## Inhibition of Leukotriene C and Leukotriene D Biosynthesis\*

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Lars Örnning and Sven Hammarström

From the Department of Chemistry, Karolinska Institutet, S-10401 Stockholm 60, Sweden

Leukotriene C<sub>4</sub> and leukotriene D<sub>4</sub> are slow reacting substances produced by mouse mastocytoma cells and rat basophilic leukemia cells, respectively. Serine-borate complex, an inhibitor of glutamyl transpeptidase (EC 2.3.2.2), prevented the formation of the biologically more potent leukotriene D<sub>4</sub> and caused accumulation of leukotriene C<sub>4</sub> in rat basophilic leukemia cell suspensions. 5,8,11-Eicosatriynoic acid, a lipoxygenase inhibitor, prevented the biosynthesis of leukotriene C<sub>4</sub> (ID<sub>50</sub>, 5 μM) by mastocytoma cells. The results demonstrate that γ-glutamyl transpeptidase is involved in the biosynthesis of leukotriene D<sub>4</sub> and suggest that leukotriene C<sub>4</sub> is formed as an intermediate.

"Slow reacting substance of anaphylaxis" (SRS-A) is a chemical mediator of immediate hypersensitivity (1). Recently, the structures of several compounds belonging to this category have been determined (2-6). The term "leukotriene" has been introduced as a trivial name for the new substances (7, 8).<sup>1</sup> Leukotriene C<sub>4</sub>, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid, is a slow reacting substance (SRS) produced by mouse mastocytoma cells (2-4). The proposed pathway of biosynthesis (Fig. 1, Refs. 2-5) involves formation of 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) (12) and 5(S)-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid (10), leukotriene (LT) A<sub>4</sub> from arachidonic acid and conjugation of leukotriene A<sub>4</sub> with glutathione. Leukotriene D<sub>4</sub>, 5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid, is a more potent SRS<sup>2</sup> produced by rat basophilic leukemia (RBL-1) cells (5). An identical product was obtained from leukotriene C<sub>4</sub> by enzymatic elimination of the γ-glutamyl residue by kidney γ-glutamyl transpeptidase.

This report shows that a transition state inhibitor of glutamyl transpeptidase (EC 2.3.2.2) (13) prevents leukotriene D<sub>4</sub> formation and leads to accumulation of leukotriene C<sub>4</sub> in

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<sup>1</sup> A dihydroxy-triene metabolite of arachidonic acid (9) and an epoxy-triene intermediate in the formation of this compound (10) were first described in leukocytes. These compounds were subsequently named leukotriene A<sub>4</sub> and leukotriene B<sub>4</sub>, respectively (7, 8, 11), and it was postulated that leukotriene A<sub>4</sub> is an intermediate in leukotriene C<sub>4</sub> biosynthesis (2).

<sup>2</sup> The abbreviations used are: SRS, slow reacting substance; HPLC, high performance liquid chromatography; RBL-1, rat basophilic leukemia cells; SRS-A, slow reacting substance of anaphylaxis.

RBL-1 cells. In addition, leukotriene C<sub>4</sub> biosynthesis by mouse mastocytoma cells was inhibited by a selective inhibitor of lipoxygenase (14).

## EXPERIMENTAL PROCEDURES

**Materials**—Ionophore A23187 was kindly provided by R. Hamill of Eli Lilly, the SRS antagonist FPL 55712 was provided by P. Sheard of Fisons Pharmaceuticals Ltd. (Loughborough, England), soybean lipoxygenase (EC 1.13.11.12) type I and glutamyl transpeptidase (EC 2.3.2.2) from porcine kidney were purchased from Sigma, RPMI 1640 medium, newborn calf serum, amphotericin B, penicillin G, and streptomycin were from GIBCO-Europe (Glasgow, Scotland).

**RBL-1 Cells**—The cells were grown in suspension culture (5).

**Mouse Mastocytoma Cells**—The cells (CXBGABMCT-1) were propagated as ascites tumors in syngenic mice (2).

**Incubation Conditions**—Cells were suspended to 10<sup>7</sup>/ml in incubation buffer (150 mM NaCl/3.7 mM KCl/3.0 mM Na<sub>2</sub>HPO<sub>4</sub>/3.5 mM KH<sub>2</sub>PO<sub>4</sub>/0.9 mM CaCl<sub>2</sub>/5.6 mM dextrose, adjusted to pH 7.0 with NaOH). RBL-1 cells were preincubated for 2 min at 37°C with L-serine-borate complex (20 mM; Ref. 13) and with L-cysteine (10 mM) for an additional 2-min period. Ionophore A23187 (20 μM) and arachidonic acid (16 μM) were added and as an ethanol solution, and the incubation was continued for 20 min. Mastocytoma cells were preincubated for 15 min with 5,8,11-eicosatriynoic acid (added as ethanol solutions), and the incubations were continued after additions of L-cysteine and A23187 as described above. After removal of the cells by centrifugation (2500 × g, 5 min) supernatants were made 80% (v/v) in ethanol.

**Purification of Leukotrienes C<sub>4</sub> and D<sub>4</sub>**—A purification scheme consisting of treatment with NaOH and chromatography on Amberlite XAD-8 and silicic acid followed by reverse phase high performance liquid chromatography (see below) was used (2, 5).

**High Performance Liquid Chromatography (HPLC)**—Determinations of leukotriene conversions by γ-glutamyl transpeptidase (Figs. 2 and 3) were performed with a column of C<sub>18</sub> nucleosil (250 × 4.6 mm) and with methanol/water (65:35, v/v) at 1 ml/min as mobile phase. Co-injections of leukotrienes formed in the present experiments with previously characterized leukotrienes (Fig. 4) were performed using the same conditions. In experiments with RBL-1 cells, methanol/ethyl acetate (1:1, v/v) and methanol eluates from silicic acid chromatographies were subjected to HPLC on a column of C<sub>18</sub> Polygosil (500 × 10 mm) eluted with methanol/water (7:3, v/v) at 4.5 ml/min (Fig. 5). Experiments with mastocytoma cells were analyzed in the same way, except that the methanol/water ratio was 65:35 (v/v) (Fig. 6). All solvents for HPLC also contained 0.1% acetic acid and were adjusted to pH 5.4 with NH<sub>4</sub>OH. For additional details, see Refs. 2 and 5.

**Incubations with Soybean Lipoxygenase and Bioassay on Guinea Pig Ileum**—These procedures were performed as described (2, 5).

**Determination of γ-Glutamyl Transpeptidase**—Reaction mixtures (1 ml) contained: L-serine-borate complex (0 to 50 mM) and enzyme (0.2 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.5/10 mM MgCl<sub>2</sub>. L-γ-Glutamyl-p-nitroanilide (5.6 mM) was added, followed after 3 min at 37°C by 2 ml of 1.5 M acetic acid. The light absorption at 410 nm was determined (15). Alternatively, leukotriene C<sub>4</sub> (4.8 μM) dissolved in Tris/MgCl<sub>2</sub> buffer, L-serine-borate (0 to 60 mM), and enzyme (0.1 mg/ml; total volume, 0.1 ml) were incubated at 37°C for 0 to 20 min (30 min). Reactions were terminated by addition of 1 volume of 0.75% acetic acid in methanol. Conversions of leukotriene C<sub>4</sub> to leukotriene D<sub>4</sub> were determined by HPLC (see above).

For experiments with RBL cells, the washed cells were suspended to 10<sup>7</sup>/ml in either 0.1 M Tris-HCl/10 mM MgCl<sub>2</sub>/44 mM NaCl, pH 8.6, or in 0.1 M Tris-HCl/10 mM MgCl<sub>2</sub>/15 mM L-serine-borate complex/14 mM NaCl, pH 8.6, and 1-ml samples were preincubated for 2 min at 37°C. L-γ-Glutamyl-p-nitroanilide (5.6 mM) was added followed after 2 or 10 min by 2 ml of 1.5 M acetic acid. The mixtures were centrifuged at 35,000 × g for 15 min prior to determination of the light absorption at 410 nm. For blank samples, acetic acid was added prior to the substrate.

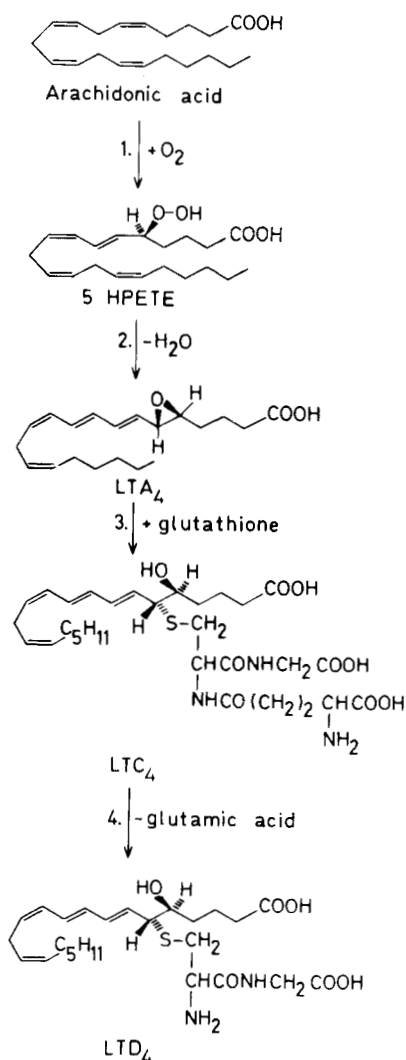


FIG. 1. Proposed scheme of biosynthesis of leukotrienes (LT) C<sub>4</sub> and D<sub>4</sub>. Enzymes: 1, lipoxygenase; 2, dehydrase; 3, glutathione-S-transferase; 4,  $\gamma$ -glutamyl transpeptidase. Intermediates: 5-HPETE, 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; leukotriene (LT) A<sub>4</sub>, 5(S)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid.

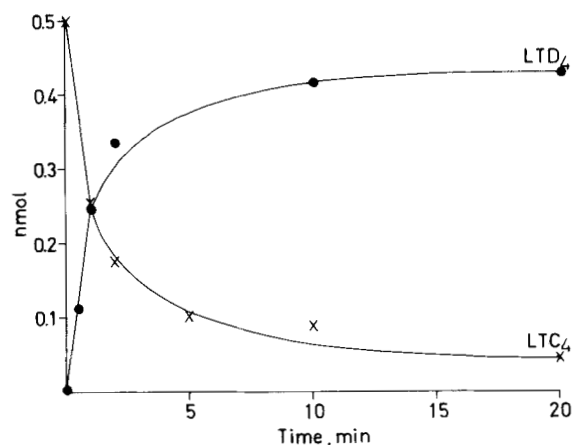


FIG. 2. Time course of the conversion of leukotriene (LT) C<sub>4</sub> to LTD<sub>4</sub> by porcine kidney  $\gamma$ -glutamyl transpeptidase. Conditions: LTC<sub>4</sub> (4.8  $\mu$ M) and partially purified enzyme (0.1 mg/ml) were incubated at 37°C in 0.1 M Tris-HCl buffer, pH 8.5/10 mM MgCl<sub>2</sub>. Reactions were terminated by addition of 1 volume of 0.75% acetic acid in methanol and substrate and product concentrations were determined by HPLC (for conditions, see the text).

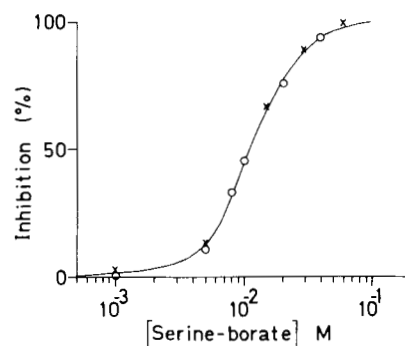


FIG. 3. Inhibition of  $\gamma$ -glutamyl transpeptidase by L-serine-borate complex (10). Substrates: L- $\gamma$ -glutamyl-*p*-nitroanilide, 5.6 mM (O), and leukotriene C<sub>4</sub>, 4.8  $\mu$ M (x). Serine-borate was added prior to addition of substrates. Other conditions for incubations and the determination of products are described in the text.

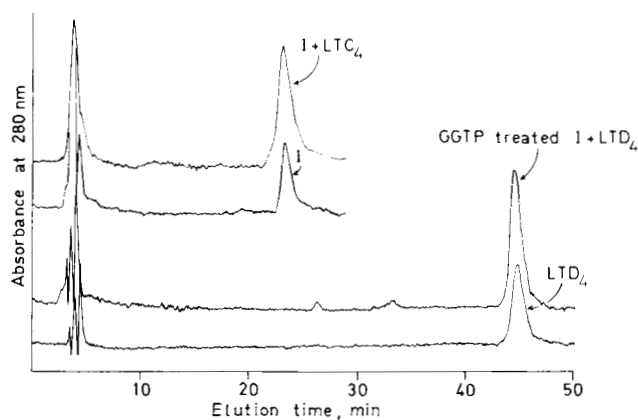


FIG. 4. Identity of Compound I depicted in Fig. 5, produced by RBL-1 cells in the presence of serine-borate, and leukotriene C<sub>4</sub>. The upper curves show HPLC chromatograms of Compound I and of Compound I plus an equal amount of leukotriene C<sub>4</sub>. The lower curves show HPLC chromatograms of Compound I, treated with  $\gamma$ -glutamyl transpeptidase, plus an equal amount of leukotriene D<sub>4</sub> and of leukotriene D<sub>4</sub> alone. Conditions for HPLC are described in the text.

## RESULTS

**Inhibition by L-Serine-Borate of Leukotriene C<sub>4</sub> to Leukotriene D<sub>4</sub> Conversion**—Fig. 2 shows the time course of leukotriene C<sub>4</sub> to leukotriene D<sub>4</sub> conversion catalyzed by porcine kidney  $\gamma$ -glutamyl transpeptidase. Under the conditions used, product formation increased linearly with time for the first minute. After 20 min, the extent of the reaction was 90%.

Fig. 3 shows dose inhibition curves for the formation of *p*-nitroaniline from L- $\gamma$ -glutamyl-*p*-nitroanilide (O) and the formation of leukotriene D<sub>4</sub> from leukotriene C<sub>4</sub> (x) when using L-serine-borate complex as an inhibitor (13). The curves are identical and the concentration for 50% inhibition is 11 mM.

**Inhibition of Leukotriene D<sub>4</sub> Formation in RBL-1 Cells**—Fig. 5 shows high performance liquid chromatograms of material obtained from 1.15  $\times 10^9$  RBL-1 cells incubated in the presence or absence of L-serine-borate complex. The methanol/ethyl acetate (1:1, v/v, dotted lines) and the methanol eluates (solid lines) were analyzed. A slow reacting substance produced by RBL-1 cells and eluting after 46 min in this HPLC system was recently identified (leukotriene D<sub>4</sub>, Ref. 5). The component formed in the present experiments with an elution time of 45 min, co-chromatographed with leukotriene D<sub>4</sub>, and showed the same ultraviolet spectrum and spectral shift upon treatment with soybean lipoxygenase as leukotriene

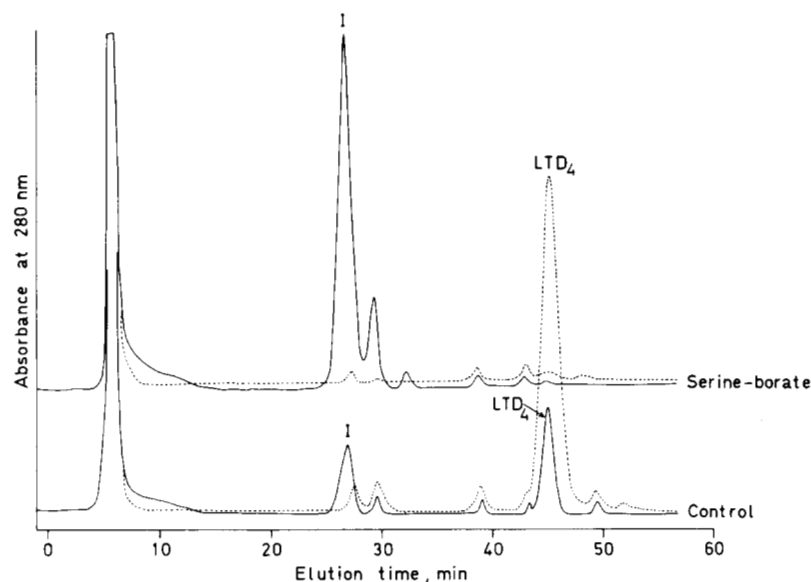


FIG. 5. Inhibition of leukotriene D<sub>4</sub> biosynthesis in RBL-1 cells by L-serine·borate complex. The figure shows high performance liquid chromatograms of methanol/ethyl acetate (1:1, v/v) (----) and methanol eluates (—) from silicic acid chromatographies. The upper curves show chromatograms of material from RBL-1 cells treated with ionophore A23187 plus arachidonic acid and L-cysteine after addition of serine·borate (20 mM), and the lower curves show chromatograms from the corresponding experiment without serine·borate.

D<sub>4</sub>. The formation of this substance was inhibited by serine·borate.

The amounts of another component (I) with an elution time of 27 min increased when leukotriene D<sub>4</sub> formation was inhibited. Compound I co-chromatographed with leukotriene C<sub>4</sub> on HPLC (Fig. 4, upper tracing).  $\gamma$ -Glutamyl transpeptidase converted Compound I to a less polar derivative which co-chromatographed with leukotriene D<sub>4</sub> (Fig. 4, lower tracings). The ultraviolet spectrum of Compound I, the spectral change observed upon treatment with soybean lipoxygenase, and the biological effects on isolated guinea pig ileum were identical with the corresponding properties of leukotriene C<sub>4</sub>. Based on these results, Compound I was identified as leukotriene C<sub>4</sub>.

In the presence of serine·borate, the formation of leukotriene C<sub>4</sub> increased from 3 to 15 nmol, whereas the biosynthesis of leukotriene D<sub>4</sub> decreased from 20 nmol to 1 (these values refer to the experiment described in the legend to Fig. 5).

**$\gamma$ -Glutamyl Transpeptidase Activity in RBL-1 Cells—**When RBL-1 cells ( $10^7$ /ml) were incubated with L- $\gamma$ -glutamyl-*p*-nitroanilide (5.6 mM), *p*-nitroaniline was formed linearly with time for 10 min (concentrations after 2 and 10 min were 216 and 975  $\mu$ M). In the presence of 15 mM L-serine·borate complex, product formation was decreased by 58% after 2 min and by 44% after 10 min. The same concentration of inhibitor reduced the activity of kidney  $\gamma$ -glutamyl transpeptidase by 67% (3 min incubation, Fig. 3).

**Inhibition of Leukotriene C<sub>4</sub> Biosynthesis—**Leukotriene C<sub>4</sub> biosynthesis induced by ionophore A23187 and L-cysteine in mastocytoma cells was inhibited by 5,8,11-eicosatriynoic acid. In one experiment,  $1.5 \times 10^9$  cells incubated in the absence of acetylenic acid produced 20 nmol of leukotriene C<sub>4</sub> as determined by HPLC. The product measured co-chromatographed with previously characterized leukotriene C<sub>4</sub> (2–4) and showed the same ultraviolet spectrum and spectral change upon treatment with soybean lipoxygenase as leukotriene C<sub>4</sub>. The amounts of this product decreased when cells were incubated with 5,8,11-eicosatriynoic acid for 15 min prior to addition of cysteine and ionophore. Fig. 6 shows a dose inhibition curve (three experiments were performed and gave similar results). Under the conditions used, the acetylenic acid did not reduce the viability of the cells as determined by trypan blue exclusion before addition of ionophore and before centrifugation at the end of incubations.

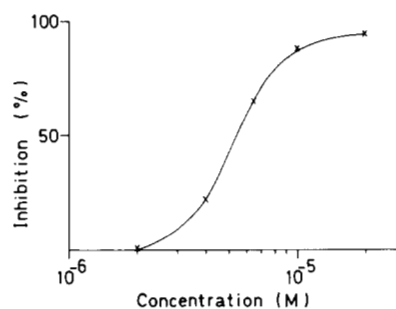


FIG. 6. Inhibition of leukotriene C<sub>4</sub> biosynthesis by 5,8,11-eicosatriynoic acid. Murine mastocytoma cells ( $1.5 \times 10^9$ /experimental point) were incubated with 5,8,11-eicosatriynoic acid (0 to 20  $\mu$ M) for 15 min at 37°C. Cysteine and ionophore A23187 were added and the incubation was continued for 20 min. After a short centrifugation, ethanol was added to the supernatant and leukotriene C<sub>4</sub> was isolated by chromatography on Amberlite XAD-8 and SilicAR CC-7 followed by HPLC. Conditions for HPLC are described in the text.

## DISCUSSION

Leukotrienes C<sub>4</sub> and D<sub>4</sub> are derivatives of arachidonic acid containing hydroxyl and peptide substituents and a conjugated triene chromophore. At pico- or nanomolar concentrations, these compounds produce contractions of intestinal (2–5), respiratory,<sup>3</sup> and vascular smooth muscle<sup>4</sup> and increase capillary permeability.<sup>3</sup> Leukotriene D<sub>4</sub> is more potent than leukotriene C<sub>4</sub> in some of these systems (5).<sup>3</sup>

The proposed biosynthetic pathway of leukotrienes C<sub>4</sub> and D<sub>4</sub> (Fig. 1, Ref. 2 to 5) involves several enzymes, *viz.* 1) lipoxygenase, 2) dehydrase (leukotriene A synthetase), 3) glutathione-S-transferase, and 4)  $\gamma$ -glutamyl transpeptidase. Thus, multiple points should exist for the inhibition and/or modulation of leukotriene biosynthesis.

$\gamma$ -Glutamyl transpeptidase activity in RBL cells was demonstrated by the ability to convert L- $\gamma$ -glutamyl-*p*-nitroanilide to *p*-nitroaniline and the inhibition of this reaction by L-serine·borate. The effects of this inhibitor on ionophore A23187-stimulated RBL-1 cells (prevention of leukotriene D<sub>4</sub> and potentiation of leukotriene C<sub>4</sub> formation) demonstrate in-

<sup>3</sup> P. Hedqvist, S. E. Dahlén, L. Gustavsson, S. Hammarström, and B. Samuelsson (1980) *Acta Physiol. Scand.*, in press.

<sup>4</sup> H. von Holst, unpublished observation.

volvement of  $\gamma$ -glutamyl transpeptidase in leukotriene D<sub>4</sub> biosynthesis and indicate that leukotriene C<sub>4</sub> is an intermediate.

5,8,11-Eicosatriynoic acid is a selective (with respect to cyclooxygenase) inhibitor of platelet lipoxygenase (14). The present results show that this compound inhibits leukotriene C<sub>4</sub> biosynthesis and that the ID<sub>50</sub> (5.2  $\mu$ M) is lower than the ID<sub>50</sub> for inhibition of 12-hydroxy-5,8,10,14-eicosatetraenoic acid formation in platelets (24  $\mu$ M, Ref. 14).

Inhibitors of leukotriene biosynthesis may be useful for investigations regarding the physiological and pathophysiological roles of these compounds and may also find use as drugs for the treatment of atopic diseases.

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