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Contractile activities of structural analogs of leukotrienes C and D: Role of the polar substituents

(slow reacting substance of anaphylaxis/leukotriene receptor)

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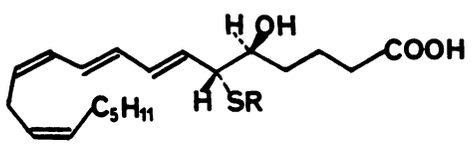
ABSTRACT **Twenty-three structural analogs of the leukotriene components of slow reacting substance of anaphylaxis (SRS-A), in which the polar regions of the leukotriene were systematically modified, were tested for their contractile activities on guinea pig pulmonary parenchymal strips and guinea pig ileum. The structural modifications allowed evaluation of the separate contributions of the four polar units in the C-1 to C-6 region of the SRS-A leukotrienes to smooth muscle spasmogenic activity. The free NH_2-terminal amino group of the *S*-linked peptide was necessary for full activity, and its deletion or substitution reduced activity by more than one but less than two orders of magnitude. A similar level of importance was apparent for the free glycine carboxyl group. In contrast, a free eicosanoid carboxyl at C-1 is not required for full activity on the airway and for substantial activity on the ileum. A role for the C-5 hydroxyl is indicated by the inactivity of the one available 5-desoxy analog. Nucleophilic, divalent sulfur is not critical to leukotriene D (LTD) activity, in that one sulfoxide had substantial function. The conformational relationship between the eicosanoid and peptide moieties of LTD is of considerable importance in that epimers at the C-5 or C-6 position were less active than LTD by more than two orders of magnitude. Several lines of evidence suggest that the relative geometrical arrangement of the C_{20} chain and the peptide unit is important to activity, consistent with the existence of a true receptor for LTD.**

Each of the leukotriene constituents of slow reacting substance of anaphylaxis (SRS-A)—namely, 5-(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid (leukotriene C; LTC) and the 6-*S*-cysteinylglycyl (LTD) and 6-*S*-cysteinyl (LTE) analogs (Fig. 1) (1–6)—has significant biological potency as a nonvascular smooth muscle spasmogen for guinea pig pulmonary parenchymal strips and guinea pig ileum (6, 7). The likely role of these leukotrienes in immediate hypersensitivity reactions and the desirability of devising chemical agents that can function as antagonists prompted a study of the activity of synthetic leukotriene analogs chosen to provide structural information regarding the nature of the recognition site(s). A study of 16 leukotriene analogs, in which the principal alteration of structure was in the "ω" portion of the eicosanoid chain (as opposed to the "carboxylic" end) revealed that, although a long hydrophobic chain is required for high activity, the degree of unsaturation and the geometry of the ω end are not very critical (8). We now report the comparative contractile activities of a series of 23 additional SRS-A analogs in which the polar regions of the leukotriene system have been systematically modified. These variations are of five types: change in the peptide or amino acid substituent at C-6 of the eicosanoid chain, modification in the carboxylic groups of the eicosanoid and peptide moieties, removal or modification of free amino in the peptide section, oxidation of sulfur attached to C-6, and change in absolute chirality at C-5 or C-6. The results obtained allow an assessment of the structural discrimination of the recognition–function response and the condition of the ionizable leukotriene functional groups during binding.



LTC R = Glutathione
LTD R = Cys gly
LTE R = Cys

FIG. 1. Formulas for leukotrienes C, D, and E.

MATERIALS AND METHODS

Synthesis of Leukotriene Analogs. The order of presentation in this section of the synthetic analogs is similar to that used in Table 1; the entry number from the table is indicated for each analog. Analogs in Table 1 are named relative to the parent leukotriene (LTC, LTD, or LTE). Leukotrienes C and D were prepared as described (2, 9). They and all the other analogs used in this study were characterized by ultraviolet absorption; all compounds possessing the 7,9,11-triene system exhibited an ultraviolet maximum at 281.5–282 nm with shoulders at about 272 and 292 nm (in water or methanol solution except as noted). Each leukotriene analog was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) as described (2), and only purified, chromatographically homogeneous compounds were employed in the biological measurements. Retention volume (R_V) data refer to a Waters Associates C_{18} μ-Bondapak RP-HPLC column eluted with methanol/water,

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Abbreviations: SRS-A, slow reacting substance of anaphylaxis; LTC, leukotriene C; LTD, leukotriene D; LTE, leukotriene E; pen, penicillamine (β, β-dimethylcysteine); RP-HPLC, reversed-phase high-performance liquid chromatography; R_V, retention volume; LTA, leukotriene A; EC_{50}, amount required to give 50% of maximum response.

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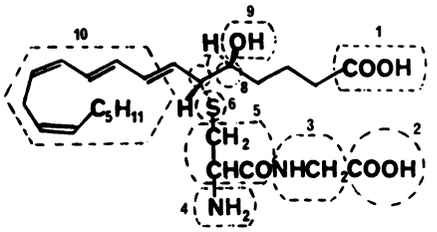
Table 1. Contractile activities of leukotriene analogs; activity ratios to LTD on guinea pig tissues

Entry	Compound	Structural variation	Pulmonary parenchymal strip, EC_{50} ratio*	Ileum dose ratio†
1	LTD	None	1	1
				
2	LTD monoamide (C-1)	1	1	0.25
3	LTD bisamide (C-1, Gly)	1,2	<0.001	<0.002
4	Deamino LTD bisamide (C-1, Gly)	1,2,4	<0.001	<0.002
5	LTD monoamide (Gly)	2	0.10	ND
6	LTD monodimethylamide (Gly)	2	0.006	<0.002
7	D-Ala‡ LTD	3	0.11	0.33
8	L-Ala‡ LTD	3	0.09	0.05
9	Pro‡ LTD	3	0.03	0.03
10	Glu‡ LTD	3	0.02	0.02
11	Val‡ LTD	3	0.008	0.01
12	Homocys§ LTD	5	0.26	0.20
13	D-Pen§ LTD	5,6	<0.001	<0.002
14	D-Cys§ LTD	6	0.11	0.02
15	D-Pen LTE	5,6	<0.001	0.005
16	Deamino LTD	4	0.05	0.20
17	*N*-Acetyl-LTD	4	0.03	ND
18	*N*-Acetyl-LTE	4	0.04	0.04
19	LTD-sulfoxide, isomer 1	6	0.10	0.10
20	LTD-sulfoxide, isomer 2	6	<0.001	<0.002
21	6-Epi-LTD	7	0.004	0.005
22	6-Epi-LTC	7	0.005	0.002
23	5-Epi-LTD	8	0.004	0.005
24	5-Dehydroxy-9-12,14,15-hexahydro LTD	9,10	<0.001	<0.002

ND, not determined.
* Analog/LTD EC_{50} ratio; EC_{50} of LTD = 0.6 nM.
† Analog/LTD ratio; LTD reference dose = 0.4 nM.
‡ This amino acid replaced Gly of LTD.
§ This amino acid replaced L-Cys of LTD.

65:35 (vol/vol), containing 0.1% acetic acid buffered to pH 5.6 with ammonium hydroxide. All reactions involving leukotrienes and all storage of leukotrienes were under an atmosphere of argon.

LTD C-1 monoamide (Table 1, entry 2) was synthesized by reaction of *N*-trifluoroacetyl LTD dimethyl ester (9) with 15 M ammonium hydroxide/dimethyoxyethane, 1:1 (vol/vol) at 0°C for 1 hr followed by purification by RP-HPLC and cleavage of the remaining ester and *N*-trifluoroacetyl groups (0.1 M potassium carbonate in aqueous methanol at 23°C for 12 hr). A small amount of hydroquinone was used in this and other deprotection steps. Final purification was conducted with RP-HPLC; R_V = 11.0 (9.3 for LTD).

LTD glycine monoamide (LTD glycinamide) (entry 5) was prepared from LTA and *N*-trifluoroacetylcysteinylglycine amide by the procedure for coupling and deprotection as described (2); R_V = 12.9.

LTD bisamide (entry 3) was prepared from LTD glycine monoamide by reaction with anhydrous ammonia/ammonium chloride at 25°C for 24 hr; R_V = 7.1.

Deamino LTD (entry 4) bisamide was prepared by LTA methyl ester coupling with 2-mercaptoproponylglycine methyl ester and subsequent reaction with ammonia/ammonium chloride as described; R_V = 9.5.

Deamino LTD (entry 16) was prepared from the intermediate diester of the foregoing synthesis by the usual saponification; R_V = 9.9.

LTD glycine monodimethylamide (LTD dimethylglycinamide) (entry 6) was prepared by coupling of LTA and *N*-trifluorocysteinylglycine dimethylamide (2) and deprotection as described; R_V = 12.9.

The various dipeptide analogs of LTD (entries 7–14) were prepared similarly from LTA by coupling of the *N*-trifluoroacetyl methyl ester of the free sulfhydryl dipeptide in methanolic triethylamine (2), followed by deprotection (2) to cleave *N*-trifluoroacetyl and methyl ester groups. R_V values of LTD analogs in which glycine was replaced were D-alanine = 10.9, L-alanine = 10.4, proline = 12.3, glutamic acid = 7.6, and valine = 12.7; R_V values of LTD analogs in which L-cysteine was replaced were homocysteine = 0.48, D-penicillamine (pen; β, β-dimethylcysteine) = 11.4, and D-cysteine = 7.6.

The D-pen analog of LTE (entry 15) was synthesized from LTA and *N*-trifluoroacetyl-D-pen methyl ester as for LTE (6); R_V = 13.0.

N-Acetyl-LTD and -LTE (entries 17 and 18) were prepared by reaction of acetic anhydride with LTD or LTE in methanol/water at 0°C.

Reaction of LTD (40 μg/ml) with 20 equivalents of sodium periodate at pH 6.8 in water containing 20% ethanol at 23°C for 6 hr afforded two diastereomeric sulfoxides (entries 19 and 20), each having an ultraviolet maximum at 1.5-nm-higher wavelength than that of LTD: isomer 2 (more polar) R_V = 6.0; isomer 1 (less polar) R_V = 6.5.

6-Epi-LTD (entry 21) and 6-epi-LTC (entry 22) were made as described elsewhere (10); R_V of 6-epi-LTD = 10.7.

5-Epi-LTD (entry 23) was derived from the coupling of the racemic *cis* epoxide corresponding to LTA with glutathione (forming a mixture of 5- and 6-epi-LTC), cleavage with γ-glutamyl transpeptidase and RP-HPLC separation of the resulting mixture of 5- and 6-epi-LTD; R_V of 5-epi-LTD = 11.5.

5-Dehydroxy-9-12,14,15-hexahydro-LTD (entry 24) (together with the C-6 epimer) was synthesized by coupling methyl 6-tosyloxy-eicosa-*cis*-11,12-enoate with *N*-trifluoroacetylcysteinylglycine methyl ester followed by deprotection by base (2).

Biological Measurements. Pulmonary parenchymal strips were prepared for the recording of isometric contractile activity (11) at an initial tension of 1.0 g and allowed to relax to a baseline tension of approximately 250 mg. A concentration–effect relationship for histamine (obtained as diphosphate from Sigma) was then determined for each tissue, and assays for activity of leukotriene analogs were carried out on those tissues that had achieved net active tensions of at least 12 mg and 225 mg after exposure to 0.1 and 100 μM histamine solutions, respectively. The tissues to be used were washed three times with saline and allowed to return to their baseline tensions. A concentration–effect curve was determined 30–90 min later with LTC, LTD, or a structural analog used as the contractile agonist. Each structural analog was assessed for contractile activity on 4–16 guinea pig pulmonary parenchymal strips. Tachyphylaxis of the pulmonary parenchymal strips prevented concentration–effect testing of more than one compound on the same tissue strip. Data were expressed as a percentage of the active tension elicited by 100 μM histamine for each tissue. For the 17 active compounds, the molar concentration of naturally-occurring leukotriene or analog required to achieve a response equal in magnitude to 50% of the 100 μM histamine response (EC_{50}) was determined by interpolation from the concentration-effect relationship for each tissue. The substantial variability among parenchymal tissues in the magnitude of the response at low concentrations (0.1–10 pM) of LTD and the lesser variability at the EC_{50} (8) form the basis for the use of the EC_{50} as the major index of comparison among LTC (EC_{50} = 10 nM), LTD (EC_{50} = 0.6 nM), and the more active of the analogs. Comparison of the EC_{50} of the analog to that of LTD yielded an EC_{50} ratio, and the quantities of analogs available allowed ratios as low as 0.001 to be determined; inactive analogs are described by activity ratios of less than 0.001. Whenever an analog presented a two-phase dose–response curve similar to that of LTD (6, 7), it was recorded.

The guinea pig ileum bioassay was prepared as described (3). The response of the ileum to LTD was linear in the concentration range of 0.2–0.8 nM and corresponded to responses in the histamine concentration range from 5–20 ng/ml (40–160 nM). Because LTC effects on the ileum persisted in a slowly decreasing degree, despite repeated washing, this compound was not utilized for standardization of responses. The responses to 0.2, 0.4, and 0.8 nM LTD were assayed before and after testing each analog on an ileum preparation. Ileum preparations did not show tachyphylaxis, and, thus, each tissue preparation was used to test up to four compounds. Each analog was assessed on at least two ileum preparations and available quantities of the analogs allowed activity ratios versus LTD as low as 0.002 to be accurately determined.

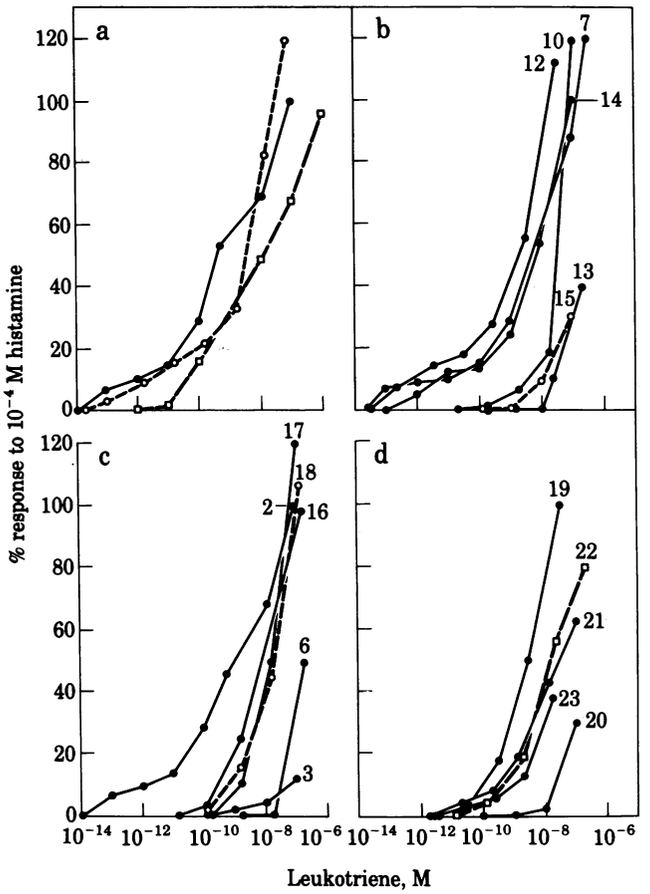


FIG. 2. Dose-response characteristics of selected analogs on guinea pig pulmonary parenchymal strips. (*a*) LTC (□---□), LTD (●——●), and LTE (○----○). (*b*, *c*, *d*) Selected analogs with numbers corresponding to those in Table 1.

RESULTS

The mean contractile effects of LTC, LTD, and LTE on guinea pig pulmonary parenchymal strips are shown in Fig. 2*a* for reference to the actions of structural analogs. The low-slope and high-slope portions of the LTD and LTE dose–response curves are apparent. The activities of the various leukotriene analogs in both pulmonary parenchymal strip and ileum bioassays are summarized in Table 1.

Peptide Modification. Replacement of an amino acid in the peptide part of LTD or LTE by another amino acid, as studied for nine synthetic analogs (Table 1, entries 7–15), generally resulted in a 1–3 orders of magnitude decrement in contractile activity; none of the analogs exhibited enhanced activity compared to the natural leukotriene parent. The analogs in which the glycine unit of LTD was replaced by D-alanine or L-alanine (entries 7 and 8) or those in which the L-cysteine unit was replaced by D-cysteine or L-homocysteine (entries 14 and 12) still showed substantial activity. More dramatic attenuation of activity was observed upon replacement of glycine by valine (entry 11), glutamic acid (entry 10), or proline (entry 9). The substitution of pen for cysteine in either LTD (entry 13) or LTE (entry 15) resulted in a loss of activity by 3 orders of magnitude or more (Fig. 2*b*).

Alteration of the Peptide and Eicosanoid Carboxyls. LTD monoamide (glycinamide) (entry 5) and the corresponding dimethylamide (entry 6) were each less active than LTD on the pulmonary parenchyma, with EC_{50} ratios of 0.10 and 0.006, respectively. In contrast, the eicosanoid (C-1) monamide of LTD (entry 2) was equiactive with LTD on the pulmonary parenchymal strip (Fig. 2*c*) and substantially active on the ileum with a dose ratio of 0.25. LTD bisamide (entry 3) was virtually inactive on the pulmonary parenchymal strips and ileum.

Loss or Alteration of the Free Amino Residue. *N*-acetyl-LTE, *N*-acetyl-LTD, and deamino LTD (entries 18, 17 and 16) each exhibited a significant loss of activity relative to LTD, with EC_{50} ratios of 0.04, 0.03, and 0.05, respectively (Fig. 2*c*). *N*-acetyl-LTE retained 31% of activity relative to LTE on the pulmonary strip and 57% on the ileum. Deamino LTD had a dose ratio of 0.20 for the ileum relative to LTD, whereas deamino LTD bisamide, and LTD bisamide (entries 4 and 3) were essentially inactive.

Oxidation Level at the C-6-Linked Sulfur. The two sulfoxides of LTD differed greatly from one another in spasmogenic activities for either assay tissue. Whereas the less polar sulfoxide (isomer 1, entry 19) was 10% as active as LTD on the pulmonary parenchymal strip and on the ileum, the more polar sulfoxide (isomer 2, entry 20) was less than 0.1% as active on both assays (Fig. 2*d*).

Chirality at C-5 and C-6. The 5(*S*)-hydroxy-6(*S*)-cysteinylglycyl isomer of LTD (6-epi-LTD, entry 21) and 5(*R*)-hydroxy-6(*R*)-cysteinylglycyl isomer of LTD (5-epi-LTD, entry 23) each had EC_{50} ratios of 0.004 on the pulmonary parenchymal strips, and retained a low-slope initial response phase (Fig. 2*d*). An equivalent loss of activity relative to LTD was apparent on the ileum assay. The 6-epimer of LTC (entry 22) retained substantial activity (8–10%) on both assays relative to LTC. Whereas 5-hydroxyl-9,12,14,15-hexahydro-LTD had an EC_{50} of 0.019 on the pulmonary parenchymal strip and a dose ratio of 0.005 on the ileum (8), removal of the 5-hydroxyl group yielded an analog with no measureable activity on either assay (entry 24).

DISCUSSION

We have shown that the hydrophobic ω portion of an SRS-A leukotriene is necessary for contractile agonist activity on nonvascular smooth muscle. With the present set of analogs, we now address some of the structural requirements at the polar part of the molecule that determine its potency as a spasmogen. LTC, LTD, and LTE have in common four polar substituents, each of which might be critical to the biological activity: a free amino group on the NH_2-terminal amino acid, a carboxylic function at C-1 of the eicosanoid backbone, a carboxylic function at the COOH-terminal amino acid, and a C-5-linked hydroxyl group. Each of these substituents was assessed in a preliminary way by use of the present set of analogs, the activities of which varied over 3 orders of magnitude.

As the absence of the NH_2-terminal amino group (deamino LTD) or its *N*-acetylation in LTD or LTE reduced the EC_{50} ratio to 0.03–0.05 on the pulmonary parenchymal strip and the dose ratio to 0.04–0.20 on the ileum, the free amino group contributes to full activity. The loss in activity for LTD is less than 2 orders of magnitude, a finding consistent with the observation that *N*-acetylating the amino group of LTE decreased the biological activity, relative to LTE, by less than one-half order of magnitude on either assay. A similar level of importance may be assigned to the glycine carboxyl as indicated by the reduction of agonist function for the pulmonary parenchymal strips to an EC_{50} of 0.10 for the LTD glycine monoamide. The LTD glycine dimethylamide had an even greater loss of activity relative to LTD on both assays. In contrast, as evidenced by LTD C-1 amide, a free carboxyl group at C-1 is not required for full activity on the pulmonary parenchymal strip and substantial activity on the ileum. Lastly, in comparison with an analog differing only in the presence of the C-5 hydroxyl group, the lack of activity in both assays for 5-dehydroxy-9-12,14,15-hexahydro LTD implies a critical function for the 5-hydroxyl group.

The functional studies suggest that for maximum action at the recognition site of the pulmonary parenchyma, it is not necessary for the C-1 carboxyl function to be ionized (i.e., to carboxylate). The same may be true for the peptidic carboxyl, which could serve as a proton donor in hydrogen bonding. The relatively low activity of the LTD glycine dimethylamide would be expected if such hydrogen bonding were important to function. No evidence for an obligatory ammonium charge in the peptide part of LTD was found in these studies. The crucial role of the C-5 hydroxyl group is suggested by the one 5-desoxy analog that was studied, but further exploration of this point is required. One interesting possibility is that a δ-lactone (C-1 carboxyl group to C-5 hydroxyl group) may play a part in the expression of biological activity of the SRS-As. This possibility is consistent with the observed activity of the C-1 monoamide of LTD because the same δ-lactone would be formed from this analog or LTD.

Nucleophilic, divalent sulfur is not critical to substantial LTD function, suggesting that C-5 to C-6 epi-sulfonium ion formation is not an important biochemical part of the recognition or binding process. The large difference in activities of the two diastereomeric LTD sulfoxides and the large fall in bioactivity when D-pen (but not D-cysteine or L-homocysteine) is substituted for L-cysteine in LTD argue that the conformational relationship between the eicosanoid and peptide moieties of LTD may be of considerable importance to contractile activity. This is also indicated by the greater than 2-orders-of-magnitude decrement in activity for 5- and 6-epi-LTD. It is noteworthy that the activity of 5-epi,6-epi-LTE has been reported as 50% of that of LTE (12). The precise nature of the dipeptide subunit of LTD is not decisive because the length of the peptide substituent, its size, and the chirality of the Cys unit can each be varied markedly with a 1- to <3-orders-of-magnitude loss of biological activity. It is possible that certain portions of the peptide part of LTD are not specifically bound to the site of recognition but instead interact with surrounding water molecules.

The evidence that small geometrical and structural changes can significantly decrease LTD activity provides evidence for the existence of a receptor (13) for LTD SRS-A. The exceedingly high potency of LTD in eliciting an effect on guinea pig pulmonary parenchymal strips at concentrations as low as 0.1 pM provides an additional argument in favor of a specific receptor (8). The other criteria, saturability and a dose-dependent relationship of binding to the functional response, have yet to be addressed. Based upon these data and those in a previous communication (8), we hypothesize that the pulmonary parenchyma of the guinea pig may possess a true receptor for LTD, which is comprised of both (*i*) a loose hydrophobic binding site, accepting the ω portion of the eicosanoid; and (*ii*) a complex active site, accommodating the C-1 carboxyl group in the nonionized form, the C-5 hydroxyl group, and a peptide part having a nonprotonated amino function. It would seem that the stereo relationship between the eicosanoid and peptide portions is not rigidly set but that one or more optimal relative arrangements exist.

The foundation is now in place for a more intimate probing of the structure–activity relationships for SRS-As and a better understanding of their binding and molecular function.

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