Thymosin-like Peptides as Potential Immunostimulants. Synthesis via the Polymeric-Reagent Method[1]

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This paper reports our attempt at designing new immunostimulating peptides which are chemically related to the bioactive peptides thymosin α_1 and thymopentin. Three peptides were synthesized, Asp-Leu-Lys-Glu-Arg-Lys-Asp-Val-Tyr (**3**), Arg-Lys-Asp-Val-Tyr-Glu-Glu-Ala-Glu-Asn (**2**), and Asp-Leu-Lys-Glu-Arg-Lys-Asp-Val-Tyr-Glu-Glu-Ala-Glu-Asn (**1**), each of which contains the thymopentin sequence and portions of the bioactive sequence of thymosin α_1. Peptides **1–3** were assembled from selected blocked fragments that were synthesized by the polymeric-reagent method, using PHBT (polystyrene-bound 1-hydroxybenzotriazole) as the activating polymer. The ability of peptides **1–3** to enhance the activation (RNA synthesis) and proliferation (DNA synthesis) of human T lymphocytes was determined. In comparison to thymosin α_1, thymosin α_1(15–28), and thymopentin, peptides **1–3** did not show significant enhancement of these processes.

Earlier reports[2] have indicated the effectiveness of a variety of natural peptides, isolated from the thymus gland, as biological response modifiers specifically for use in immunodeficiency diseases and cancer. The complete sequences of such active peptides as thymopoietin, thymosins α_1, α_{11}, β_4, and β_9, and FTS (isolated from porcine serum) are known.[3-8]

Of these natural peptides, thymosin α_1 ($T\alpha_1$) and a pentapeptide segment of thymopoietin, thymopentin, have been the most studied and probably have the greatest diversity of biological activity. $T\alpha_1$ is one of the major peptides in the thymus extract thymosin fraction 5. This latter preparation has already been shown to be of use, in vivo, in humans with immunodeficiency diseases and selected cancers.[9-11] $T\alpha_1$ is known to have a role in immunoregulation and has been reported to enhance immune function in vivo and in vitro.[5,12-15] It has the ability to stimulate the secretion of certain lymphokines such as α and γ interferons, T-cell growth factor, and migration inhibitory factor.[15,16]

$T\alpha_1$ is a polypeptide containing 28 residues; however, several laboratories have shown that it is the C-terminal region of this peptide wherein most of the activity resides. Birr and co-workers[17] have reported that $T\alpha_1$ fragments 20–26, 25–27, and 25–28 have about 90% of the stimulatory activity of the parent molecule in a mixed-lymphocyte culture (MLC) assay. Felix and co-workers[18] examined a series of amino-terminal fragments (1–3, 4–10, 1–10, and 1–14) and carboxy-terminal fragments (27–28, 26–28, 25–28, 23–28, 22–28, 21–28, 20–28, 19–28, 18–28, 15–28, and 11–28) of $T\alpha_1$ using an in vivo immunosuppressed mouse bioassay. They found that fragments 15–28 and 18–28 were as potent in this infection assay as $T\alpha_1$, while fragment 21–28 had 75% and fragments 23–28 and 25–28 had 50% of the activity of the parent. The amino terminal fragments had little or no activity.

On the other hand, studies on bovine thymopoietin, an immunologically active 49 amino acid polypeptide,[3] have shown that a pentapeptide segment, thymopentin (residues 32–36), was able to retain the biological activity of the parent.[19-21] This is also true for human thymopoietin (48 amino acid polypeptide); the two pentapeptide active sites are identical.[22] Thymopentin has regulatory effects on the immune system of animals (e.g. induction of early T-cell differentiation), immunonormalizing activity in experimental animals who have undergone thymectomy, and has, and is, being explored as a therapeutic immunoregulatory agent in man.[23]

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	15 20 25
Thymosin α1 (15-28)	Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn
	32 36
Thymopoietin (32-36) (Thymopentin)	Arg-Lys-Asp-Val-Tyr
Tetradecapeptide 1	Asp-Leu-Lys-Glu-Arg-Lys-Asp-Val-Tyr-Glu-Glu-Ala-Glu-Asn
Decapeptide 2	Arg-Lys-Asp-Val-Tyr-Glu-Glu-Ala-Glu-Asn
Nonapeptide 3	Asp-Leu-Lys-Glu-Arg-Lys-Asp-Val-Tyr

Figure 1. Comparison of synthetic peptides **1–3** with $T\alpha_1$(15–28) and thymopentin.

Structure–activity studies[23] of thymopentin showed that a number of amino acid substitutions are allowed; however, there was an absolute requirement for Arg to be at position 1 and Asp to be at position 3 in order to retain biological activity. In another study,[24] substitutions that would retain thymopentin's biological activity while enhancing its enzymatic stability were evaluated. It was found that N-terminal acetylation and C-terminal amidation increased the resistance to proteolytic degradation by exopeptidases.

It has been suggested[10] that the individual thymic hormones do not by themselves elicit all of the biological functions of the thymus but rather that they each act on different subpopulations of T-cells in the maturation process, and acting in concert, they carry out the hormonal function of the thymus. Therefore, in order to design peptides with immunostimulating properties greater than that of the individual natural thymic hormones, and yet be of relatively short length, we thought that one should combine into one molecule the biologically active sequences of two separate molecules; the so-called[25] "symbiotic approach to drug design." Inasmuch as the amino acid sequence of thymopentin is similar to the sequence 19–23 of $T\alpha_1$, it was felt that substituting thymopentin for the latter sequence (see Figure 1) would yield a new peptide, **1**, with greater biological activity than either $T\alpha_1$(15–28) or thymopentin alone. Since various C-terminal fragments of $T\alpha_1$ also have biological activity, we decided to prepare decapeptide **2** and nonapeptide **3** with thymopentin again being substituted for sequence 19–23 of $T\alpha_1$.

Chemistry

For the preparation of peptides **1–3** we have chosen to use the polymeric-reagent method of peptide synthesis.[26] This method is considered to be a hybrid of both the solution- and solid-phase methods of peptide synthesis and combines some of the advantages inherent in both. We have had considerable experience using macroporous polystyrene-bound 1-hydroxybenzotriazole (PHBT) in the synthesis of $T\alpha_1$(15–28)[27] and in the preparation of linear tetrapeptides used as precursors to cyclic tetrapeptides.[28] In the synthesis of peptides wherein there are several re-

Scheme I

Compound (% yield)	
	Boc-Tyr(2Br-Z)-OH
	↓ 1. Cs_2CO_3 2. phenacyl bromide
11 (81)	Boc-Tyr(2Br-Z)-OPa
	↓ 1. TFA 2. Boc-Val-PHBT
12 (73)	Boc-Val-Tyr(2Br-Z)-OPa
	↓ 1. TFA 2. Boc-Asp(OcHex)-PHBT
13 (86)	Boc-Asp(OcHex)-Val-Tyr(2Br-Z)-OPA
	↓ 1. TFA 2. Boc-Lys(2Cl-Z)-PHBT
14 (88)	Boc-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-OPa
	↓ 1. TFA 2. Boc-Arg(Tos)-PHBT
4 (74)	Boc-Arg(Tos)-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-OPa

Scheme II

Compound (% yield)	
	H-Asn-OBzl
	↓ Boc-Glu(OcHex)-PHBT
15 (87)	Boc-Glu(OcHex)-Asn-OBzl
	↓ 1. TFA 2. Boc-Ala-PHBT
16 (96)	Boc-Ala-Glu(OcHex)-Asn-OBzl
	↓ 1. TFA 2. Boc-Glu(OcHex)-PHBT
17 (77)	Boc-Glu(OcHex)-Ala-Glu(OcHex)-Asn-OBzl
	↓ 1. TFA 2. Boc-Glu(OcHex)-PHBT
5 (51)	Boc-Glu(OcHex)-Glu(OcHex)-Ala-Glu(OcHex)-Asn-OBzl

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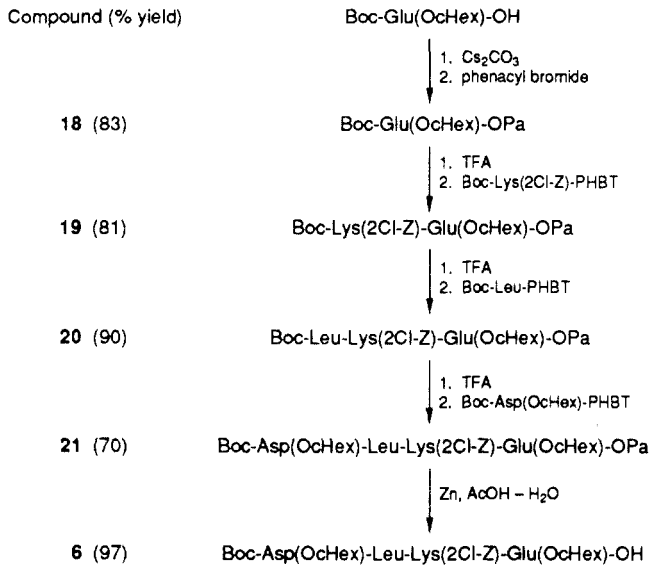
Scheme III



peating units of the same amino acid, such being the case in peptide **1**, the polymeric-reagent method is particularly advantageous. This is because the repeating unit, e.g. Glu, can be activated once by esterifying it to PHBT and then used from stock each time the repeating unit appears in the sequence.

The strategy we chose was to build the 14-residue peptide **1** from three smaller suitably blocked fragments, namely pentapeptides **4** and **5** and tetrapeptide **6**. These three fragments also allowed the construction of the desired peptides **2** and **3**. The preparation of the central pentapeptide **4** (see Scheme I) was planned so that a selective cleavage of the blocking groups would unveil either the N-terminal amine or the C-terminal carboxyl and as such could be used in the synthesis of all three peptides, **1**, **2**, or **3**.

With use of the stepwise method, with each of the properly blocked amino acids activated by esterification to PHBT, the fully blocked pentapeptide **4** was obtained in an overall yield of 33% (Scheme I). Scheme II depicts the synthetic route to pentapeptide **5**. The overall yield of **5**, via PHBT esters, was 33%. Tetrapeptide **6** was synthesized as shown in Scheme III in an overall yield of 41%, the OPa protecting group being selectively removed in excellent yield.

Having all the desired fragments in hand allowed us to assemble the three fully protected peptides **8**, **9** and **10**, which are the precursors to the final peptides **2**, **1**, and **3**, respectively (see Scheme IV). Selective removal[29] of the phenacyl (OPa) group from **4** gave the free acid **7**. The latter was condensed, by the DCC/HOBT method described by Felix et al.[30], with the free amine obtained by the selective removal of the Boc group from **5**, affording the decapeptide **8** in a 61% yield. Cleavage of the amino-terminal blocking group from **8** followed by coupling with **6**, as above, gave blocked tetradecapeptide **9** (yield 69%). Without further purification, **9** was deblocked with anhydrous hydrogen fluoride (HF) to the desired peptide **1** and then converted to the acetate form via ion exchange (yield 49%, 90% pure by HPLC). Tetradecapeptide **1** was

Scheme IV

further purified by semipreparative HPLC to greater than 99% purity.

Peptide **6** was coupled with the free amine derived from **4** (see Scheme IV) with diisopropylcarbodiimide (DIC)/HOBT, furnishing blocked nonapeptide **10** in 92% yield. The OPa group of **10** was selectively removed,[29] the resulting acid was treated with HF to remove the remaining blocking groups, and nonapeptide **3** was then purified to homogeneity by HPLC. In a similar way, decapeptide **2** was obtained directly from its blocked counterpart **8** by HF cleavage and HPLC purification.

The molecular weights of peptides **1–3** were checked by liquid secondary ion mass spectrometry (LSIMS). The amino acid sequences of peptides **1–3** were verified by high-performance tandem mass spectrometry.[31] Collision-induced decomposition tandem mass spectra (CID MS/MS) were obtained for the MH^+ ions of peptides **1–3**; in each MS/MS spectra the product ions corresponded to those predicted for the amino acid sequences of these peptides.[32]

Biological Testing

The immunological activity of peptides can be examined by their ability to modulate immune cell function. Several parameters can be examined such as cell activation, proliferation, and production of soluble factors. Enhancement of activation and proliferation in the presence of test peptides are good preliminary indicators of peptide biological activity when used with suboptimal concentrations of lectins such as phytohemagglutinin (PHA).

As can be seen in Figures 2 and 3, $T\alpha_1$, $T\alpha_1(15–28)$, and thymopentin (TP5) significantly enhanced DNA and RNA synthesis of human T lymphocytes. This enhancement was observed whether the cells were stimulated with a polyclonal activator such as PHA or a specific signal against the T3 receptor with OKT3 monoclonal antibody. In contrast, tetradecapeptide **1** (TDP), decapeptide **2** (DP), and nonapeptide **3** (NP) did not show significant en-

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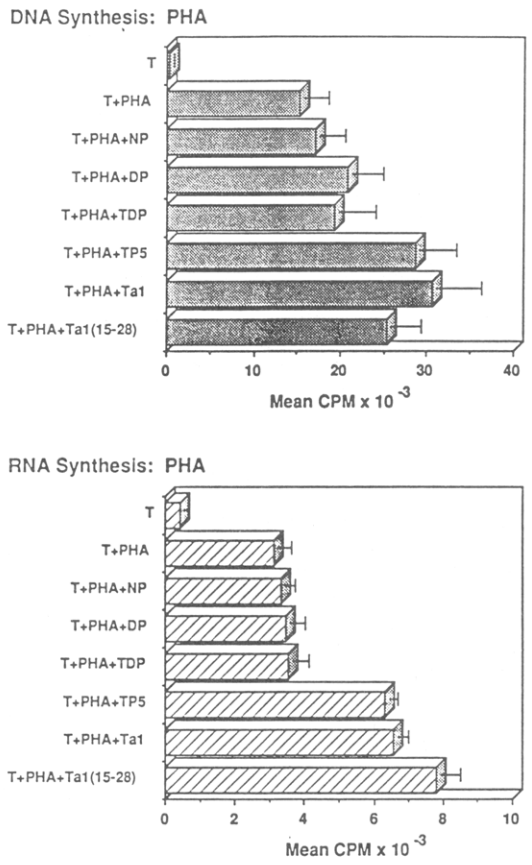

Figure 2. Effect of various peptides on DNA synthesis (proliferation) and RNA synthesis (activation) of human T lymphocytes stimulated with PHA. The data presented show the mitogenic activity of cells incubated with 1 μg/mL of the various peptides, an optimal concentration for Tα_1, Tα_1(15–28), and TP5. Enhancement of DNA and RNA syntheses in the presence of Tα_1, Tα_1(15–28), and TP5 was significant to $P < 0.005$. NP = nonapeptide **3**; DP = decapeptide **2**; TDP = tetradecapeptide **1**. The error bars represent ±SEM.

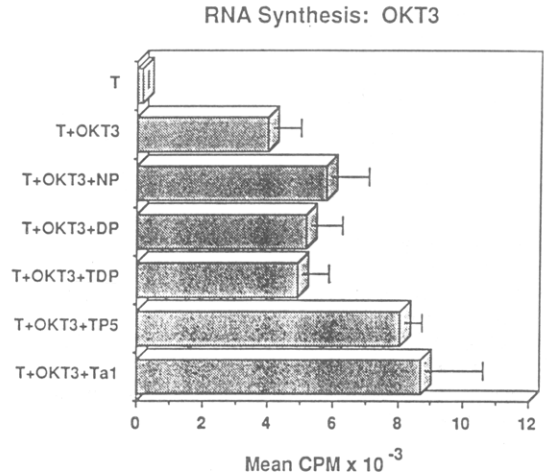

Figure 3. Effect of various peptides on RNA synthesis (activation of human T lymphocytes stimulated with a monoclonal antibody raised against the T3 receptor (OKT3). The mitogenic activity was obtained from cells incubated with 1.56 ng/mL of OKT3 and various peptides at 1 μg/mL. Enhancement of RNA synthesis in the presence of Tα_1 and TP5 was significant to $P \leq 0.05$. NP = nonapeptide **3**; DP = decapeptide **2**; TDP = tetradecapeptide **1**. The error bars represent ±SEM.

hancement of these processes.

Conclusions

The synthesis and basic screening data for peptides **1–3** are reported. Apparently, the substitution of thymopentin for residues 19–23 in Tα_1 fragments **1–3** does not yield peptides with greater biological activity than their individual components, namely Tα_1(15–28) and thymopentin. Since both Tα_1(15–28) and thymopentin individually cause significant enhancement of DNA and RNA synthesis in human T lymphocytes, it appears that the poor activity of peptides **1–3** might be due to the fact that either thymopentin can not substitute for residues 19–23 of Tα_1 or when attached to other amino acid residues at the N terminus (e.g. peptide **3**), the C terminus (e.g. peptide **2**), or both termini (e.g. peptide **1**) its biological activity is reduced. In any case, the "symbiotic approach to drug design" has not been borne out in this study.

Experimental Section

All of the blocked amino acids were of the L configuration and were purchased from either Vega Biochemicals or Bachem, Inc. Symbols and abbreviations generally follow the IUPAC–IUB recommendations as published in the *Int. J. Pept. Protein Res.* **1984**, *24*, app. 9–37. In addition, the following abbreviations are used: 2Br-Z, 2-bromobenzyloxycarbonyl; 2Cl-Z, 2-chlorobenzyloxycarbonyl; cHex, cyclohexyl. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. HPLC analyses and purifications were performed with a Waters Associates system, monitoring at both 254 and 214 nm, using Vydac C_{18}, 5-μm particle size, 300-Å pore size columns, 0.46 × 15 cm (column 1) or 1.0 × 25 cm (column 2). With use of linear conditions, solvent A was 2% CH_3CN–0.1% TFA and solvent B was 60% CH_3CN–0.1% TFA. The columns were protected with a Vydac guard cartridge containing the same packing, but of 10-μm particle size. Peak separations were analyzed with a Hitachi Model D-2000 Chromato-Integrator. Thin-layer chromatography (TLC) was carried out with silica gel on aluminum (EM Reagents), while preparative TLC was carried out on Analtech silica gel GF plates (1000 μm thick). Column chromatography was performed with Merck silica gel, 70–230 mesh. Optical rotations were determined on a Perkin-Elmer Model 241 automatic Polarimeter. All evaporations were performed in vacuo on a rotary evaporator. Organic solutions that had been previously extracted with aqueous solutions were dried with anhydrous Na_2SO_4 prior to evaporation. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Amino acid analyses were obtained at Carnegie-Mellon University on a Durrum D-50 instrument or at the University of Pittsburgh on a Beckman Model 6300 analyzer, from samples that had been hydrolyzed for 24 h with 6 N HCl at 110 °C in evacuated tubes. The dimethylformamide (DMF) used was purified by drying over KOH overnight and then distilling in vacuo from ninhydrin. The trifluoroacetic acid (TFA) used was redistilled. Dicyclohexylcarbodiimide (DCC) and diisopropylethylamine (DIEA) were obtained from Chemical Dynamics; the latter was distilled from ninhydrin. Methylene chloride (CH_2Cl_2) was distilled from anhydrous Na_2CO_3. The PHBT was a gift from Dr. Abraham Patchnornik, The Weizmann Institute of Science, Rehovot, Israel. The esterification of PHBT by each protected amino acid was carried out as previously described.[27] Couplings using PHBT were done by shaking, rather than stirring, the polymers with a Tekmar VXR shaker. All filtrations and washings of the PHBT polymers were carried out in an apparatus similar to that described by Stern et al.[33] The apparatus for conducting the liquid HF cleavages was constructed as previously described.[34] The HF was dried prior to use by distilling it into a vessel which contained CoF_3, and then it was distilled from there into the reaction vessel. The petroleum ether used had bp 30–60 °C. In most cases the intermediate blocked peptides were obtained analytically pure by preparative TLC followed by crystallization, unless stated otherwise. The Boc groups were routinely removed by stirring the peptide with neat TFA at room temperature for 10 min; after evaporation of the TFA the product was solidified

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with anhydrous ether and then dried in vacuo over P_2O_5 and KOH pellets, unless stated otherwise. The progress of all peptide coupling reactions was followed by TLC.

Boc-Tyr(2Br-Z)-OPa (11). Boc-Tyr(2Br-Z)-OH (2.00 g, 4.05 mmol) was dissolved in EtOH (12 mL), H_2O (3 mL) was added, and then the mixture was titrated to a pH of 7.0 (pH meter) with an aqueous solution of Cs_2CO_3 (Alfa Inorganics, 0.82 g, 2.52 mmol, in 3.3 mL of H_2O). The solution was evaporated and the residue was triturated with benzene (20 mL) and evaporated. Evaporation two more times from benzene and drying overnight in vacuo over P_2O_5 gave the dry cesium salt of Boc-Tyr(2Br-Z). The latter was dissolved in DMF (10 mL) and treated with a solution of freshly recrystallized phenacyl bromide (from petroleum ether, 0.81 g, 4.07 mmol) in DMF (10 mL). After the addition of another 5 mL of DMF, the suspension was stirred overnight at room temperature and concentrated to dryness. The residue was triturated with $CHCl_3$ (50 mL) and filtered to remove cesium salts. The organic layer was washed successively with 2 × 10 mL of cold 5% Na_2CO_3, 2 × 10 mL of cold 10% citric acid, 10 mL of H_2O, and 10 mL of brine, dried, and concentrated to a solid. Crystallization from ether–petroleum ether gave, in two crops, 2.02 g (81%) of **11**, mp 106–107 °C. The analytical sample gave mp 106–107 °C; $[\alpha]^{27}_D$ −1.4° (*c* 1.0, $CHCl_3$). Anal. ($C_{30}H_{30}NO_8Br$) C, H, N.

Boc-Val-Tyr-(2Br-Z)-OPa (12). (General Coupling Procedure). Phenacyl ester **11** (0.70 g, 1.14 mmol) was converted to its TFA salt. Boc-Val-PHBT[26] (2.50 g, 2.50 mmol) was mixed with CH_2Cl_2 (17 mL) and allowed to swell, and the excess CH_2Cl_2 was removed with a pipet. The TFA salt was transferred to the swelled polymer with CH_2Cl_2 (8 mL); this mixture was shaken for 10 min and then treated with DIEA (0.40 mL, 2.29 mmol). The mixture was shaken at room temperature for 21 h and filtered, and the polymer washed with 6 × 20 mL of CH_2Cl_2 (with shaking for 10 min with each wash). The combined organic solutions were evaporated; the residue was dissolved in $CHCl_3$ (60 mL), washed successively with 3 × 15 mL of cold 10% citric acid, 3 × 15 mL of cold 5% Na_2CO_3, 1 × 15 mL of H_2O, 1 × 15 mL of brine, and dried. Evaporation of the solvent left 0.72 g of solid, which was crystallized from EtOAc–petroleum ether, affording, in two crops, 0.59 g (72.8%) of **12**, mp 165–167 °C. The analytical sample gave mp 161–163°; $[\alpha]^{25}_D$ −4.3° (*c* 0.99, $CHCl_3$). Anal. ($C_{35}H_{39}N_2O_9Br$) C, H, N.

Boc-Asp(OcHex)-Val-Tyr(2Br-Z)-OPa (13). Dipeptide **12** (0.86 g, 1.21 mmol) was converted to its TFA salt, mixed with CH_2Cl_2 (10 mL) and Boc-Asp(OcHex)-PHBT (2.75 g, 2.40 mmol, prepared from Boc-Asp(OcHex)-OH, which was obtained by the method of Tam et al.[35]), and added along with another 10 mL of CH_2Cl_2. After 75 min another portion of DIEA (0.1 mL, 0.57 mmol) was added and the shaking was continued for a total of 2 h. Workup as described above gave 0.94 g (86%) of **13**, nearly pure according to TLC. The analytical sample gave mp 129–131 °C (EtOAc–petroleum ether); $[\alpha]^{27}_D$ −23.0° (*c* 1.0, $CHCl_3$). Anal. ($C_{45}H_{54}N_3O_{12}Br$) C, H, N.

Boc-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-OPa (14). Tripeptide **13** (0.89 g, 0.98 mmol) was converted to its TFA salt, dissolved in DMF (10 mL), Boc-Lys(2Cl-Z)-PHBT (2.44 g, 1.95 mmol) and DMF (5 mL) were then added, and the mixture was shaken for 5 min before the addition of DIEA (0.34 mL, 1.95 mmol). After 30 min the mixture was filtered and the polymer was washed four times with DMF. Evaporation of the combined DMF solutions and workup as above gave 1.04 g (87.8%) of **14**, which showed only trace amounts of impurities upon examination by TLC. The analytical sample gave mp 166–167 °C (EtOAc–petroleum ether); $[\alpha]^{25}_D$ −15.8° (*c* 1.01, $CHCl_3$). Anal. ($C_{59}H_{71}N_5O_{15}BrCl$) C, H, N.

Boc-Arg(Tos)-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-OPa (4). Tetrapeptide **14** (0.98 g, 0.81 mmol) was converted to its TFA salt, dissolved in DMF (10 mL), and then treated with Boc-Arg(Tos)-PHBT (2.42 g, 1.50 mmol) and another 5 mL of DMF. After shaking for 10 min, DIEA (0.26 mL, 1.49 mmol) was added. After 30 min another portion of DIEA (0.07 mL, 0.04 mmol) was added. The reaction appeared complete after 1.5 h and was worked up as described above for **14**, affording 1.11 g of **4**. TLC evaluation (7% MeOH–$CHCl_3$) indicated small impurities which were, for the most part, removed by crystallization from EtOAc–petroleum ether, yielding 0.91 g (74.1%) of **4**. This was not purified further but used as such in further reactions.

Boc-Arg(Tos)-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-OH (7). Pentapeptide **4** (0.37 g, 0.24 mmol) was dissolved in glacial AcOH (25 mL) and then treated with a solution of AcOH (6.5 mL) and H_2O (5.5 mL). Activated zinc powder[36] (0.90 g) was added and the mixture was stirred for 24 h at room temperature. After filtration, the zinc insolubles were stirred with a fresh portion (23 mL) of 15% H_2O–AcOH and again filtered. The combined filtrates were evaporated to a solid, which was stirred with a 2% solution of EDTA in H_2O (50 mL) for 15 min; the solid was collected by filtration and triturated with petroleum ether to remove acetophenone. This afforded **7** as a white solid (0.32 g, 94%), pure according to TLC (10% MeOH–$CHCl_3$ + 2% AcOH). An analytical sample of **7** was obtained by crystallization from AcOH–H_2O: mp 178–180 °C; $[\alpha]^{25}_D$ −12.7° (*c* 0.96, DMF). Amino acid analysis showed Asp 1.00, Val 1.01, Tyr 1.05, Lys 1.00, Arg 1.09. Anal. ($C_{64}H_{83}N_9O_{17}BrClS$) H, N; C: calcd 54.99; found 55.44.

Boc-Glu(OcHex)-Asn-OBzl (15). Asn-OBzl hydrochloride[26] (0.52 g, 2.00 mmol) was suspended in CH_2Cl_2 (14 mL) and treated with DIEA (0.78 mL, 4.47 mmol). Immediately after the base addition, Boc-Glu(OcHex)-PHBT (4.55 g, 3.14 mmol, prepared from Boc-Glu(OcHex)-OH, which was synthesized by the method of Tam et al.[35]) was added followed by 9 mL of CH_2Cl_2. After shaking of the mixture for 1 h, it was worked up as usual, affording 1.16 g of product. Crystallization from acetone–petroleum ether gave 0.93 g (86.9%) of **15** containing only trace impurities upon TLC (11% MeOH–$CHCl_3$); mp 138–140 °C. The analytical sample gave mp 139–141°; $[\alpha]^{23}_D$ + 11.8° (*c* 0.89, $CHCl_3$). Anal. ($C_{27}H_{39}N_3O_8$) C, H, N.

Boc-Ala-Glu(OcHex)-Asn-OBzl (16). Dipeptide **15** (0.65 g, 1.22 mmol) was selectively deblocked by stirring it with a solution of 50% TFA/CH_2Cl_2 (12 mL) for 45 min. After evaporation of the solvent and the usual workup with ether, the resulting TFA salt was mixed with CH_2Cl_2 (15 mL). Boc-Ala-PHBT (3.00 g, 2.97 mmol) was added followed by DIEA (0.54 mL, 3.08 mmol) and the mixture was shaken for 75 min. During this time another 0.27 mL (1.54 mmol) of DIEA was added. The usual workup afforded a pale yellow solid, which was crystallized from acetone–petroleum ether to give 0.71 g (95.9%) of **16**, contaminated with only very minor impurities according to TLC (10% MeOH/$CHCl_3$), mp 136–138 °C. The analytical sample gave mp 135–137°; $[\alpha]^{23}_D$ −14.0° (*c* 0.52, DMF). Anal. ($C_{30}H_{44}N_4O_9$) C, H, N.

Boc-Glu(OcHex)-Ala-Glu(OcHex)-Asn-OBzl (17). Tripeptide **16** (0.53 g, 0.87 mmol) was converted to its TFA salt. Boc-Glu(OcHex)-PHBT (1.72 g, 1.31 mmol) was swelled in DMF (6.5 mL) and then the TFA salt, dissolved in DMF (3.5 mL), was added to the polymer. After shaking for 15 min, DIEA (0.30 mL, 1.72 mmol) was added and the shaking continued for 4.5 h. After filtration, the beads were extracted with 4 × 15 mL of DMF and the combined organic layers were combined and evaporated to dryness. The residue was triturated with 10% citric acid (30 mL) and allowed to remain in a refrigerator overnight. The resulting solid was collected by filtration, washed with H_2O, stirred with cold 5% Na_2CO_3, washed with H_2O, stirred with cold 10% citric acid, filtered, and washed with H_2O. After drying in vacuo over P_2O_5, 0.55 g (77.0%) of **17** was obtained. This was considered to be pure enough (TLC, 10% MeOH–$CHCl_3$) to use in the next step without further purification.

Boc-Glu(OcHex)-Glu(OcHex)-Ala-Glu(OcHex)-Asn-OBzl (5). Tetrapeptide **17** (0.55 g, 0.67 mmol) was converted to its TFA salt. Boc-Glu(OcHex)-PHBT (1.57 g, 1.19 mmol) was swelled in DMF (8.0 mL) and then excess DMF removed with a pipet. Another 5 mL of DMF was added to the swelled polymer, the mixture was allowed to shake for 5 min, and the excess DMF was again removed with a pipet. To this polymer was added the TFA salt dissolved in DMF (5 mL), and the mixture was shaken for 15 min and then treated with DIEA (0.23 mL, 1.32 mmol). The reaction was terminated after 3 h and worked up as described above for **17**. The resulting crude **5** (0.63 g) was purified by column

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chromatography using silica gel packed as a slurry in 5% MeOH–$CHCl_3$ (40 g, 2.3 × 25 cm). The appropriate fractions were combined to give 0.35 g (50.7%) of **5**. An analytical sample of **5** was obtained by crystallization from EtOAc–petroleum ether; mp 170–174 °C; $[\alpha]^{23}_D$ −11.4° (*c* 0.86, DMF). Amino acid analysis showed Asp 1.00, Glu 3.05, Ala 1.03. Anal. ($C_{52}H_{78}N_6O_{15}$) C, H, N.

Boc-Glu(OcHex)-OPa (18). Boc-Glu(OcHex)-OH (1.00 g, 3.04 mmol) was converted to its phenacyl ester in essentially the same manner as that for **11**. Crystallization of the product from cyclohexane gave 1.13 g (82.9%) of **18**, mp 117–117.5 °C. The analytical sample gave mp 116–117 °C; $[\alpha]^{21}_D$ −24.9° (*c* 0.92, MeOH). Anal. ($C_{24}H_{33}NO_7$) C, H, N.

Boc-Lys(2Cl-Z)-Glu(OcHex)-OPa (19). Boc-Lys(2Cl-Z)-PHBT (3.77 g, 2.98 mmol) was swelled in CH_2Cl_2 (12 mL). The TFA salt (0.91 g, 1.97 mmol) derived from **18** was dissolved in CH_2Cl_2 (6 mL) and added to the swelled polymer. After 5 min, DIEA (0.69 mL, 3.96 mmol) was added and the shaking was continued for 3 h. Workup as described for **12** and crystallization from CCl_4 gave 1.20 g (81.4%) of **19**, mp 55–58 °C. Recrystallization gave the analytical sample; mp 61–63 °C; $[\alpha]^{21}_D$ −21.5° (*c* 1.1, MeOH). Anal. ($C_{38}H_{50}N_3O_{10}Cl$) C, H, N.

Boc-Leu-Lys(2Cl-Z)-Glu(OcHex)-OPa (20). Dipeptide **19** (1.20 g, 1.61 mmol) was converted to its TFA salt, dissolved in CH_2Cl_2 (17 mL), and added to Boc-Leu-PHBT (2.50 g, 2.55 mmol). After shaking for 10 min, DIEA (0.56 mL, 3.21 mmol) was added and the reaction was worked up, as usual, after 2 h. The resulting tripeptide **20** (1.24 g, 90.1%) was nearly pure according to TLC (4% MeOH–$CHCl_3$). The analytical sample gave mp 111–113 °C (CCl_4–petroleum ether); $[\alpha]^{23}_D$ −33.6° (*c* 0.81, $CHCl_3$). Anal. ($C_{44}H_{61}N_4O_{11}Cl$) C, H, N.

Boc-Asp(OcHex)-Leu-Lys(2Cl-Z)-Glu(OcHex)-OPa (21). Tripeptide **20** (0.37 g, 0.43 mmol) was converted to its TFA salt. Boc-Asp(OcHex)-PHBT (0.94 g, 0.82 mmol, see **13** above) was swelled in CH_2Cl_2 (5 mL) and then mixed with the TFA salt and CH_2Cl_2 (3 mL). After the addition of DIEA (0.17 mL, 0.98 mmol), the mixture was shaken for 25 h and then worked up as usual. The crude product was crystallized from EtOAc–petroleum ether and in two crops afforded 0.32 g (69.8%) of **21**, mp 131–133 °C. The analytical sample gave mp 130–131 °C; $[\alpha]^{25}_D$ −34.4° (*c* 0.8, $CHCl_3$). Anal. ($C_{54}H_{76}N_5O_{14}Cl$) C, H, N.

Boc-Asp(OcHex)-Leu-Lys(2Cl-Z)-Glu(OcHex)-OH (6). Tetrapeptide **21** (0.74 g, 0.70 mmol) was dissolved in glacial AcOH (59.5 mL) and while the mixture was stirred H_2O (10.5 mL) was added dropwise. Activated zinc powder[36] (2.0 g) was next added and the mixture was stirred overnight. The workup followed the method described above for the preparation of **7**. The resulting solid was crystallized from acetone–H_2O and then stirred with petroleum ether to remove traces of acetophenone, affording 0.64 g (97.1%) of **6**; mp 159–163 °C; amino acid analysis showed Asp 1.00, Lys 0.97, Leu 0.94, Glu 1.02.

Boc-Arg(Tos)-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-Glu(OcHex)-Glu(OcHex)-Ala-Glu(OcHex)-Asn-OBzl (8). The TFA salt derived from **5** (0.040 g, 0.038 mmol) was combined with **7** (0.059 g, 0.042 mmol) and HOBT hydrate (Aldrich, 0.017 g, 0.11 mmol) and dissolved in a solution of DMF (0.70 mL) containing *N*-methylmorpholine (purified by distillation from ninhydrin, 17 μL, 0.15 mmol). After stirring for 5 min in ice, DCC (0.013 g, 0.063 mmol) was added as a solution in DMSO (0.35 mL, distilled in vacuo from NaOH) and the clear solution was stirred in ice for 1 h and then for 24 h at room temperature. Another 5 μL (0.045 mmol) of *N*-methylmorpholine was added 3.5 h after the addition of the DCC. The reaction was terminated by filtering the mixture directly into H_2O, the aqueous mixture was stirred for 2 h, and the product was collected by filtration and dried in vacuo over P_2O_5. The solid was triturated with MeOH, collected by centrifugation, and crystallized from trifluoroethanol–H_2O to afford 0.053 g (60.5%) of **8**, nearly one spot on TLC (10% MeOH–$CHCl_3$ + 3% AcOH). Amino acid analysis showed Asp 2.00, Glu 3.10, Ala 0.98, Val 1.00, Tyr 1.10, Lys 1.02, Arg 1.10.

Arg-Lys-Asp-Val-Tyr-Glu-Glu-Ala-Glu-Asn (2). Liquid HF (8 mL) was allowed to distill into a reaction vessel which already contained decapeptide **8** (0.25 g, 0.11 mmol) and anisole (redistilled, 1.0 mL). After stirring at 0 °C for 40 min, the HF was evaporated, the residue was mixed with 6% AcOH–H_2O (40 mL), the anisole was removed by three extractions with ether, and the aqueous layer was lyophilized. The lyophilized powder was dissolved in H_2O (45 mL) and stirred with AG 1-X8 (acetate form, 8 g, Bio-Rad) for 5 h. The aqueous mixture was filtered, lyophilized, and purified via HPLC (column 2, 10–40% B in 50 min, 3 mL/min), affording **2** (0.10 g, 72.7%), which was greater than 94% pure according to analytical HPLC (column 1, 10–40% B in 40 min, 1 mL/min, t_R 11.9 min). For purposes of biological testing, **2** was purified further by HPLC to give a product of 98.2% purity. Amino acid analysis showed Asp 2.10, Glu 3.00, Ala 0.92, Val 0.90, Tyr 0.90, Lys 0.93, Arg 0.90. LSIMS (MH^+) calcd 1252.6, found 1252.6.

Boc-Asp(OcHex)-Leu-Lys(2Cl-Z)-Glu(OcHex)-Arg-(Tos)-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-Glu-(OcHex)-Glu(OcHex)-Ala-Glu(OcHex)-Asn-OBzl (9). Decapeptide **8** (0.12 g, 0.051 mmol) was converted to its TFA salt and combined with tetrapeptide **6** (0.061 g, 0.065 mmol), HOBT hydrate (Aldrich, 0.031 g, 0.20 mmol), DMSO (purified, 0.3 mL), and a solution of *N*-methylmorpholine (purified, 0.32 mmol) in DMF (1.4 mL). The solution was stirred in ice and then treated with a solution of DCC (0.031 g, 0.15 mmol) in DMSO (purified, 0.4 mL). Stirring was continued in ice for 80 min and thereafter at room temperature. After 26 h the DCU was removed by filtration and the filtrate was passed directly into H_2O (100 mL). The resulting mixture was stirred for 80 min and then excess NaCl was added. This caused the precipitate to float to the top and allowed easy collection by filtration. The dried precipitate was triturated twice with MeOH and collected by centrifugation, affording 0.11 g (68.6%) of **9**, which appeared mainly as one spot on TLC (12% MeOH–$CHCl_3$).

Asp-Leu-Lys-Glu-Arg-Lys-Asp-Val-Tyr-Glu-Glu-Ala-Glu-Asn (1). Tetradecapeptide **9** (0.09 g, 0.029 mmol) was cleaved with HF essentially as described above for **2**. The resulting crude product was purified on a column (0.9 × 8.7 cm) of Amberlite-IRA 400 (acetate form, 20–50 mesh); the column was developed successively with H_2O, 0.2 M AcOH, and finally 0.4 M AcOH. Lyophilization of the fractions gave **1** (0.028 g, 48%), which upon HPLC evaluation (column 1, 10–40% B in 40 min, 1 mL/min, t_R 17.6 min) was 90.4% pure. For analytical purposes, **1** was further purified by HPLC (column 2, 10–40% B, 40 min, 3 mL/min) to give a product of better than 99% purity. Amino acid analysis showed Asp 3.29, Glu 4.00, Ala 1.08, Val 0.89, Leu 1.08, Tyr 0.88, Lys 2.20, Arg 0.89. LSIMS (MH^+) calcd 1737.8, found 1737.8.

Boc-Asp(OcHex)-Leu-Lys(2Cl-Z)-Glu(OcHex)-Arg-(Tos)-Lys(2Cl-Z)-Asp(OcHex)-Val-Tyr(2Br-Z)-OPa (10). Pentapeptide **4** (0.059 g, 0.039 mmol) was converted to its TFA salt and dissolved in DMF (0.7 mL) along with HOBT hydrate (recrystallized, 0.017 g, 0.11 mmol), tetrapeptide **6** (0.039 g, 0.042 mmol), and *N*-methylmorpholine (17 μL, 0.15 mmol). After stirring in ice for 10 min, DIC (Aldrich, 0.008 g, 0.063 mmol) dissolved in DMSO (0.35 mL) was added and the solution was stirred in ice for 1 h and then for 6 h at room temperature. At this time *N*-methylmorpholine (15 μL) was again added and the solution was stirred for another 32 h. The resulting mixture was filtered directly into H_2O (60 mL), and the precipitate was collected by filtration, washed with H_2O and MeOH (2 × 2 mL) and dried over P_2O_5 to given nonapeptide **10** (0.084 g, 92.3%). The latter appeared as one spot on TLC (10% MeOH–$CHCl_3$ + 3% AcOH).

Asp-Leu-Lys-Glu-Arg-Lys-Asp-Val-Tyr (3). The phenacyl group in **10** (0.063 g, 0.027 mmol) was selectively removed by dissolving it in 15% H_2O–AcOH, adding activated zinc powder[36] (0.18 g), and stirring at room temperature for 8 h. After filtration and lyophilization the solid was washed with 2% disodium EDTA solution (2 × 10 mL) and H_2O and dried over P_2O_5 and NaOH pellets to give the free acid (0.058 g, 96%). This acid was cleaved with HF and worked up, including ion exchange, essentially as described above for **2**. Crude **3** was purified via HPLC (column 2, 10–20% B in 40 min, 3 mL/min, gradient curve 8, t_R 42.2 min) until it was obtained as 96.5% pure. Amino acid analysis showed Glu 1.00, Asp 2.18, Ala 0.91, Leu 0.93, Lys 1.89, Arg 0.90, Tyr 0.91. LSIMS (MH^+) calcd 1165.6, found 1165.6.

Mass Spectrometry. LSIMS were obtained for samples dissolved in 5% acetic acid–glycerol, with a MAT 731 instrument with a 7 keV Cs^+ primary beam. Accelerating voltage was 8 kV. CID MS/MS were obtained with a JEOL HX110/HX110 tandem

mass spectrometer (EBEB) with a 6 keV Xe^0 primary beam. Accelerating voltage was 10 kV. CID took place in a cell located between MS-1 and MS-2, which was held at 3 kV above ground, so that the CID energy was 7 kV. Helium was used as the collision gas, at a pressure sufficient to reduce the MH^+ abundance to 20% of its initial value.

Biological Studies. Thymic Hormones. Synthetic $T\alpha_1$ was endotoxin free and was provided by Alpha 1 Biomedicals, Inc., Washington, D.C., as a gift from Dr. A. L. Goldstein, Department of Biochemistry, The George Washington University School of Medicine and Health Sciences, Washington, D.C. Synthetic TP5 was purchased from Sigma Chemical Co.

Purification of Human T Lymphocytes. Human T lymphocytes were purified from whole blood as previously described.[15] In brief, peripheral blood mononuclear cells were incubated for 1 h at room temperature on plastic petri dishes in order to remove the macrophages. The T lymphocytes were then purified from the nonadherent cell population by rosetting for 1 h at 4 °C with neuraminidase-treated sheep erythrocytes. The rosetted T cells were separated from B cells on Ficoll-Hypaque gradients. The rosetted cells (pellet) were treated with a 0.8% solution of Tris-ammonium chloride, pH 7.4, to lyse the sheep erythrocytes. Following lysis, the T cells were washed well and examined for purity by using fluorescein-conjugated CD2, CD3 monoclonal antibodies for T cells (Ortho Diagnostic Systems, Raritan, NJ), CD20 for B cells (Coulter Corp., Hialeh, FL), and CD11 and CD14 for monocytes/macrophages (Becton-Dickinson, Mountain View, CA). The B cells were removed from the interface, washed well, and examined for purity as T lymphocytes. These purification procedures normally result in T lymphocytes of greater than 90% purity. For optimal response of T lymphocytes, 5–10% macrophages are added to T cell suspensions.

Effect of Peptides on T Lymphocytes. Human T cell cultures were incubated in microtiter plates at 37 °C and 5% CO_2 in a humidified atmosphere. The mixtures were composed of 2×10^5 T cells per well, 0.5% (v/v) PHA (GIBCO), and peptides at 1 μg/mL concentration, in a total volume of 200 μL. For RNA synthesis, 1 μCi of [^{3}H]uridine (ICN Radiochemicals) was added initially and the cultures were incubated for 18–24 h. In some experiments a specific activation signal anti-T3 receptor monoclonal antibody (OKT3, Ortho), at 1.56 ng/mL, was used instead of PHA. For DNA synthesis, the cultures were incubated for a total of 72 h with the final 16 h being in the presence of 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol, ICN Radiochemicals). Following incubation, the cells were harvested and counted in a liquid-scintillation counter. The degree of incorporation of radiolabeled uridine or thymidine was used as a measure of the effect of the peptides on the T lymphocytes.

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Registry No. 1, 123167-50-0; 2, 123167-51-1; 3, 123167-52-2; 4, 123167-53-3; BOC-deblocked 4·TFA, 123167-87-3; 5, 123167-54-4; BOC-deblocked 5·TFA, 123167-83-9; 6, 123167-55-5; 7, 123167-56-6; 8, 123167-57-7; BOC-deblocked 8·TFA, 123167-85-1; 9, 123167-58-8; 10, 123167-59-9; 10 (free acid), 123167-88-4; 11, 123167-60-2; BOC-deblocked 11·TFA, 123183-56-2; 12, 123167-61-3; BOC-deblocked 12·TFA, 123167-71-5; 13, 123167-62-4; BOC-deblocked 13·TFA, 123183-58-4; 14, 123183-53-9; BOC-deblocked 14·TFA, 123183-60-8; 15, 123167-63-5; BOC-deblocked 15·TFA, 123167-73-7; 16, 123167-64-6; BOC-deblocked 16·TFA, 123167-75-9; 17, 123167-65-7; BOC-deblocked 17·TFA, 123183-62-0; 18, 123167-66-8; BOC-deblocked 18·TFA, 123167-77-1; 19, 123183-54-0; BOC-deblocked 19·TFA, 12316.-79-3; 20, 123167-67-9; BOC-deblocked 20·TFA, 123167-81-7; 21, 123167-68-0; BOC-Tyr(2Br-Z)-OH, 47689-67-8; BOC-Tyr(2Br-Z)-OCs, 123167-69-1; $PhCOCH_2Br$, 70-11-1; H-Asn-OBzl·HCl, 69863-43-0; BOC-Glu(OcHex)-OH, 73821-97-3; thymosin, 61512-21-8.

2-Substituted-1-naphthols as Potent 5-Lipoxygenase Inhibitors with Topical Antiinflammatory Activity

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The synthesis, biological evaluation, and structure–activity relationships of a series of 1-naphthols bearing carbon substituents at the 2-position are described. These compounds are potent inhibitors of the 5-lipoxygenase from RBL-1 cells and also inhibit bovine seminal vesicle cyclooxygenase. Structure–activity relationships for these two enzymes are different, implying specific enzyme inhibition rather than a nonspecific antioxidant effect. 2-(Arylmethyl)-1-naphthols are among the most potent 5-lipoxygenase inhibitors reported (IC_{50} values generally 0.01–0.2 μM) and show excellent antiinflammatory potency in the mouse arachidonic acid ear edema model. To study the effects of structure on in vitro and in vivo activity, four general features of the molecules were varied: the 2-substituent, the 1-hydroxyl group, substitution on the naphthalene rings, and the 1,2-disubstituted naphthalene unit itself. 2-Benzyl-1-naphthol (**5a**, DuP 654) shows a very attractive profile of topical antiinflammatory activity and is currently in clinical trials as a topically applied antipsoriatic agent.

The enzyme 5-lipoxygenase (5-LO) catalyzes the first step in the oxidation of arachidonic acid to leukotrienes. Leukotrienes elicit a variety of biological responses such as smooth muscle contraction, increased vascular permeability, and leukocyte chemotaxis.[1,2] The importance of these phenomena to the inflammatory response implicates the leukotrienes in the pathology of a variety of inflammatory and allergic diseases. The widely used nonsteroidal antiinflammatory drugs such as indomethacin, which show

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