

## Fluorenylalkanoic and Benzoic Acids as Novel Inhibitors of Cell Adhesion Processes in Leukocytes

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A series of fluorene-9-ylalkanoic and alkylbenzoic acids was prepared as simplified analogues of a previously reported series of antiinflammatory agents which act to inhibit neutrophil recruitment into inflamed tissue. The previous compounds ("leumedins") contained (alkoxy-carbonyl)amino or benzoic acid moieties tethered to a fluorene ring. This functionality was replaced with simple structural elements. The new compounds were, in general, found to be more potent than the earlier series at inhibiting adherence of neutrophils to serum-coated wells or endothelial cells *in vitro*. Compound **9** was approximately 10-fold more potent than the previously reported FMOC-phenylalanine, of which it is an analogue. Similarly, compound **19** was superior in potency to its first generation progenitor, NPC 16570. The new compounds were shown to inhibit neutrophil adherence under conditions in which adherence is mediated by Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18); they thus appear to target  $\beta_2$ -integrins in their antiadhesion activity. These compounds provide a departure point for the further development of new cell adhesion inhibitors which should exhibit enhanced potency and a more selective mode of action.

### Introduction

The recruitment of leukocytes from the blood to sites of tissue insult, with subsequent phagocytosis of microorganisms and the release of reactive oxygen radicals and lysosomal enzymes, constitutes an inflammatory reaction that is a normal part of the body's defense system. However, inappropriate recruitment can result in a deleterious inflammatory response, resulting in tissue destruction. One of the most important aspects of leukocyte recruitment is adhesion between leukocytes and vascular endothelial cells (EC).<sup>1</sup> Current evidence indicates that at least two distinct classes of leukocyte adhesion molecules, selectins and integrins, are involved in leukocyte extravasation.<sup>2</sup> The selectins are responsible for slowing the flow of leukocytes through the postcapillary venules, resulting in a "rolling" phase. The integrins are subsequently activated and mediate a stronger adherence between the leukocytes and endothelium. Integrins are heterodimeric proteins; the  $\beta_2$ -integrins found on leukocytes share a common  $\beta$  subunit (CD18) associated with one of three  $\alpha$  subunits (CD11a, b, or c). Adhesion receptors on leukocytes interact with complementary ligands on the endothelium; the integrin adhesion receptors fluctuate between low- and high-avidity states for their respective counterligands.<sup>3</sup> On polymorphonuclear granulocytes (PMN, neutrophils), the  $\beta_2$ -integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) mediate adhesion to the endothelium via immunoglobulin superfamily members on the EC such as ICAM-1 (intercellular adhesion molecule-1). Disruption of these adhesion processes has the potential to be an excellent strategy for the development of new nonsteroidal antiinflammatory drugs (NSAIDs).

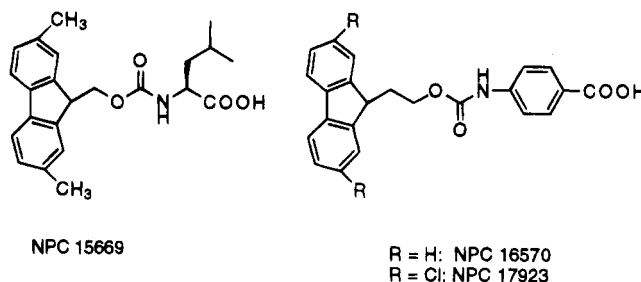


Figure 1

Previous reports from these laboratories have described a novel class of small molecule antiinflammatory agents ("leumedins",<sup>4</sup> exemplified by NPCs 15669,<sup>5</sup> 16570,<sup>6</sup> and 17923<sup>7</sup>; Figure 1) which act by inhibiting cell adhesion processes in neutrophils. As part of an ongoing program to develop more potent second generation inhibitors of integrin-mediated adhesion, we were interested in defining the structural elements necessary for leumedin activity. Herein we describe the synthesis and adhesion inhibition activity of a series of fluorenylalkanoic and benzoic acid compounds.

The first generation of leumedin compounds consisted of a fluorene ring connected to either an amino acid or *p*-aminobenzoic acid via a linker containing a carbamate or amide moiety. We prepared a series of simple fluorene-containing carboxylic acids to determine whether these structural elements were in fact necessary for activity. The compounds were assayed for their ability to inhibit the adhesion of neutrophils to serum or endothelial cells.

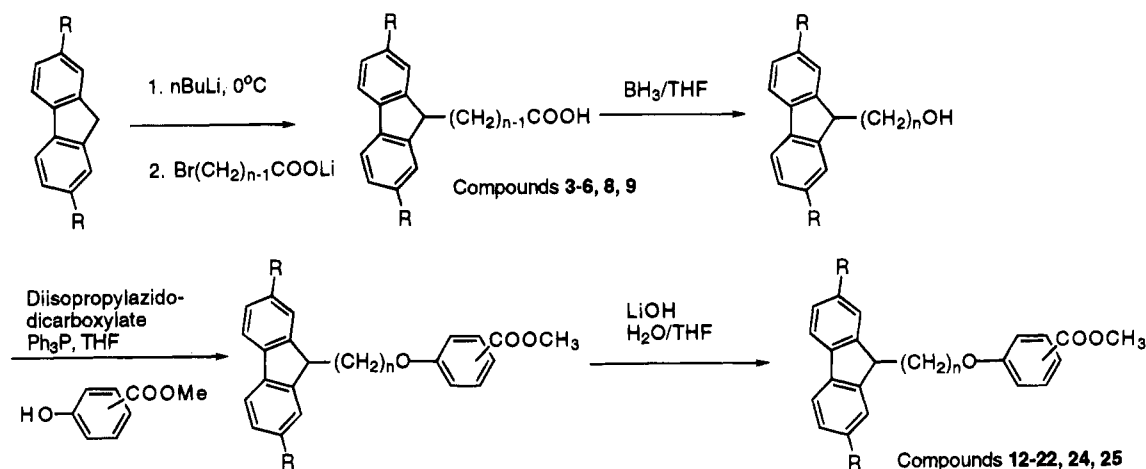
### Chemistry

The compounds in this study were synthesized as depicted in Schemes 1-3. Compounds 1 and 2 were

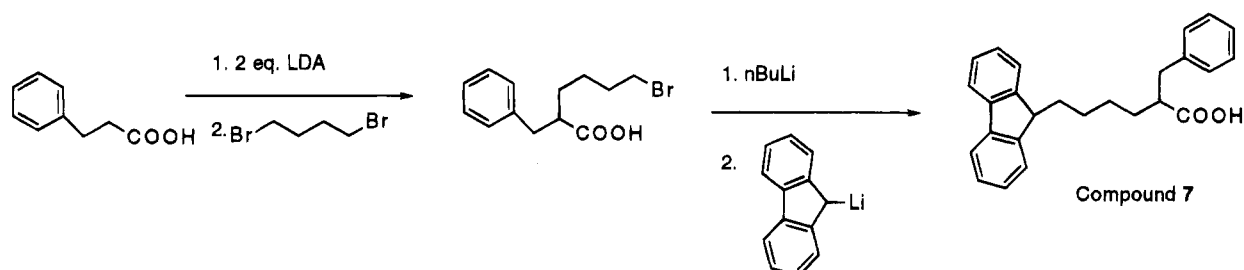
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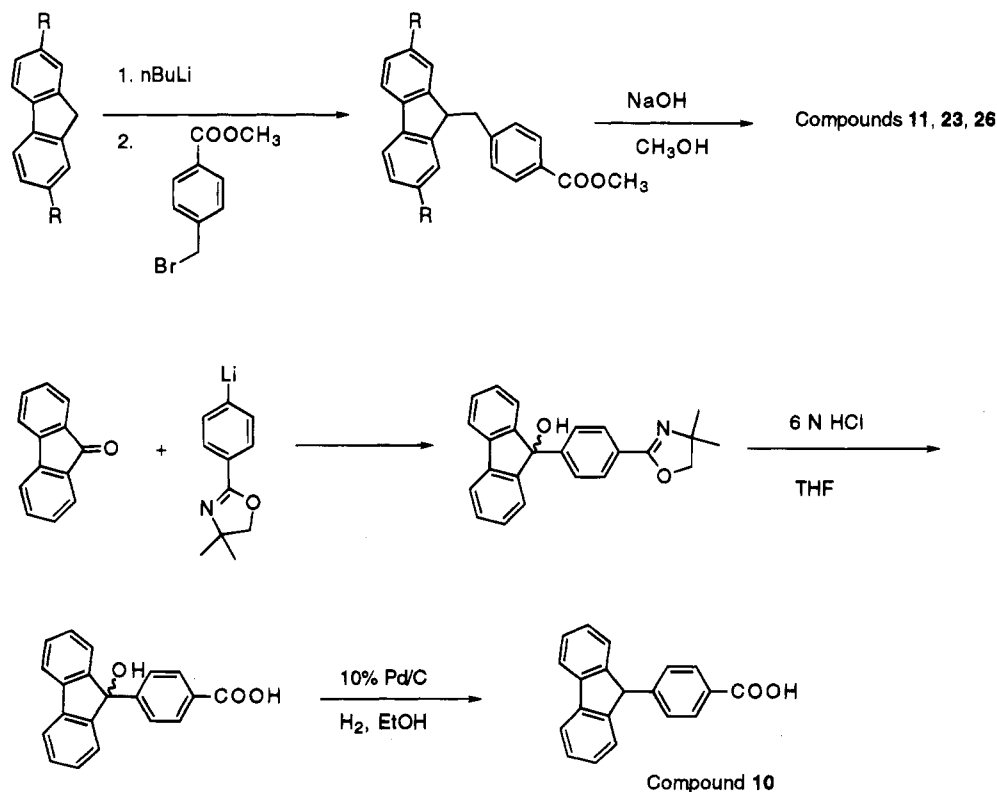
## Scheme 1



## Scheme 2

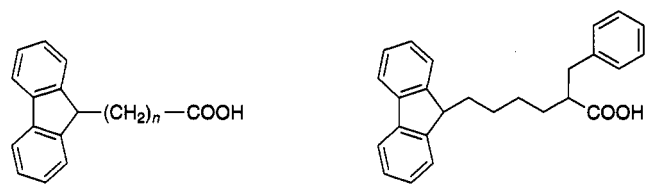


## Scheme 3



obtained commercially. Alkylation of 9-lithiofluorene, or its substituted analogues, with the lithium salts of various bromoalkanoic acids furnished the fluorenylalkanoic acids 3-6, 8, and 9 of Table 1 in a straightforward manner (Scheme 1). Reduction of the requisite carboxylic acids with diborane, followed by Mitsunobu coupling<sup>8</sup> of the obtained alcohols with methyl 2-, 3-, or

4-hydroxybenzoate, delivered fluorenylbenzoic esters which were hydrolyzed to the final products. Benzyl-substituted fluorenylalkanoic acid 7 was synthesized as shown in Scheme 2. Compound 10 was prepared via the addition of 2-(4-bromophenyl)-4,4-dimethyl-2-oxazoline to 9-fluorenone (Scheme 3), followed by hydrolysis and hydrogenolysis. Compounds 11, 23, and 26 were

**Table 1.** Physical Properties and Potencies of Fluorenylalkanoic Acids To Inhibit Adhesion of TNF- $\alpha$ -Activated Neutrophils to Serum-Coated Wells


compd	n	mp °C	formula	analysis <sup>a</sup>	IC <sub>50</sub> <sup>b</sup> (μM)
1	0	—	—	—	>200
2	1	—	—	—	>200
3	2	—	C <sub>16</sub> H <sub>14</sub> O <sub>2</sub>	C, H	>200
4	3	128–129	C <sub>17</sub> H <sub>16</sub> O <sub>2</sub>	C, H	97
5	4	34.5–36	C <sub>18</sub> H <sub>18</sub> O <sub>2</sub> ·0.15H <sub>2</sub> O	C, H	87.0 ± 7.2
6	5	59–60	C <sub>19</sub> H <sub>20</sub> O <sub>2</sub>	C, H	55.2 ± 5.1
7	7	84–85	C <sub>21</sub> H <sub>24</sub> O <sub>2</sub> ·0.25H <sub>2</sub> O	C, H	32.2 ± 6.9
8	9	76–77	C <sub>23</sub> H <sub>26</sub> O <sub>2</sub>	C, H	11.6 ± 1.2
9	—	107.5–108.5	C <sub>26</sub> H <sub>26</sub> O <sub>2</sub>	C, H	6.0 ± 0.8

<sup>a</sup> All elemental analyses gave results ± 0.4% of the theoretical values. <sup>b</sup> For each assay, seven dilutions of the compound were assayed in duplicate to generate IC<sub>50</sub> values. Values reported are the average of at least three separate experiments ± SEM.

prepared by alkylation of 9-lithiofluorenes with methyl 4-(bromomethyl)benzoate followed by hydrolysis (Scheme 3).

### In Vitro Assays

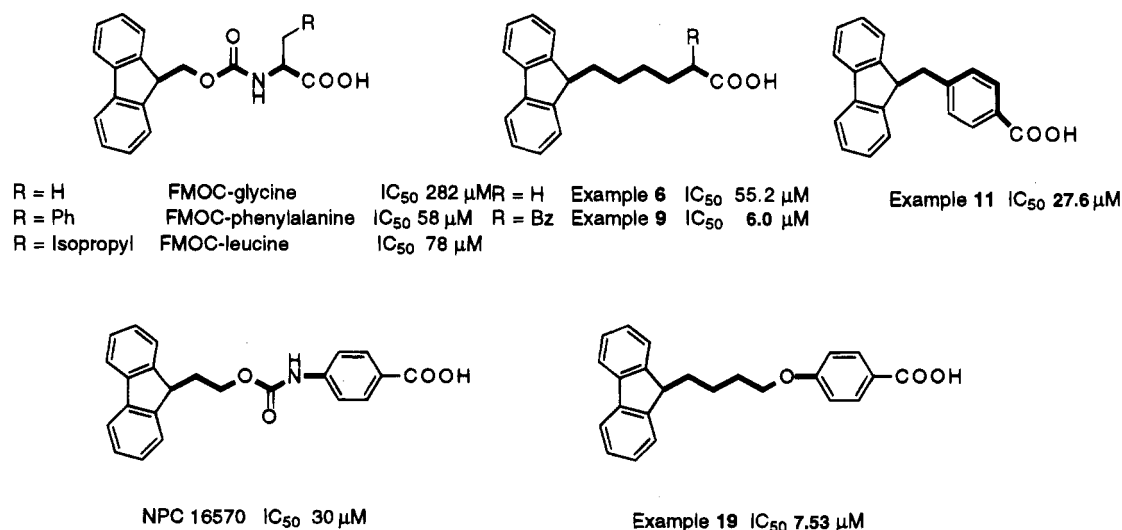
Compounds were evaluated in one or more of three adhesion assays; details of the assay protocols are given in the Experimental Section.<sup>9</sup> All compounds were examined for their ability to inhibit the adhesion of neutrophils activated by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to serum-coated plastic wells.<sup>10</sup> Adherence of TNF- $\alpha$ -treated PMN to serum was completely blocked by the anti-CD18 antibody TS1/18<sup>11</sup> and thus is mediated by  $\beta_2$ -integrins.<sup>10</sup> 50–80% of this adherence was blocked by saturating amounts of the anti-CD11b antibodies, MY904 and 44aach;<sup>11</sup> thus Mac-1 contributes to this process. Selected compounds were also tested in a similar assay using platelet activating factor (PAF) to activate the neutrophils. The anti-Mac-1 antibodies MY904 and 44aach (IC<sub>50</sub> = 5 μg/mL) completely blocked the adherence of PAF-activated PMN to serum-coated

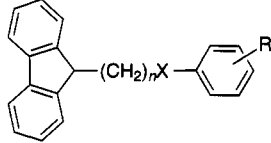
wells, indicating that in this case adherence is entirely mediated by Mac-1.<sup>10</sup> Hybridomas producing these antibodies were obtained from ATCC (Maryland). All antibodies were purified on Protein A sepharose prior to use.

Certain compounds were also evaluated for their ability to inhibit the adherence of activated neutrophils to endothelial cells. For this assay (referred to hereafter as the INF- $\gamma$  HUVEC assay), PMNs which had been activated by TNF- $\alpha$  were allowed to adhere to human umbilical vein endothelial cells (HUVECs) which had been activated with IFN- $\gamma$ .<sup>1</sup> We have demonstrated by fluorescence antibody labeling that the HUVECs treated with INF- $\gamma$  for 20 h express elevated levels of ICAM-1 and very little (*E*)-selectin (data not shown). We found that the adherence of PMNs to HUVECs under these conditions of activation was completely inhibitable by anti-CD18 antibodies and by a combination of antibodies directed against CD11a and CD11b (data not shown). Thus this assay allowed us to test the compounds for their ability to block adherence mediated by Mac-1/LFA-1 on PMNs and ICAM-1 on endothelial cells. Typically we found that the compounds inhibited adhesion completely.

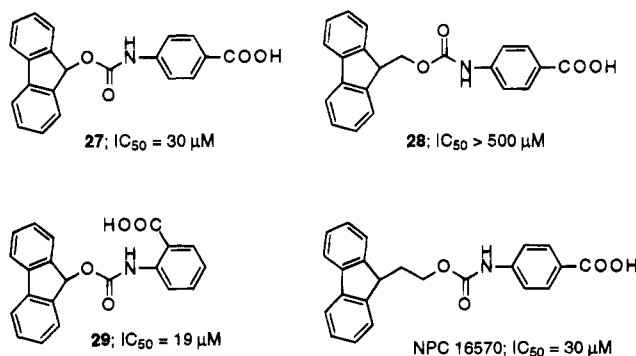
### Results and Discussion

The results in Table 1 indicate that simple fluorenylalkanoic acids are capable of inhibiting neutrophil adhesion to serum *in vitro*. The potency of these simpler compounds is comparable to the previously reported leumedins. Compound 6 (Table 1, IC<sub>50</sub> 55.2 μM) is an analogue of (fluorenylmethoxycarbonyl)glycine (IC<sub>50</sub> 282 μM) in that the carboxylic acid is connected to the fluorene ring by the same number of atoms (Figure 2). However, 6 is more potent. Adding a benzyl group  $\alpha$  to the carboxylate of 6 (compound 9) results in a 10-fold increase in potency. Again, the corresponding leumedin, (fluorenylmethoxycarbonyl)phenylalanine, is substantially less potent (IC<sub>50</sub> 58 μM). Compound 11 (Table 2) was prepared as an analogue of FMOC-leucine in which the entire carbamoyl amino acid moiety has been replaced with a benzoic acid group (Figure 2). The greatly enhanced potency of this compound, together with the above results, indicates that the carbamate linkage of the leumedins can be profitably replaced with

**Figure 2.** Analogy between first generation leumedins and new compounds.

**Table 2.** Physical Properties and Potencies of Fluorenylbenzoic Acids To Inhibit Adhesion of TNF- $\alpha$ -Activated Neutrophils to Serum-Coated Wells


compd	n	X	R	mp, °C	formula: anal. <sup>a</sup>	IC <sub>50</sub> (μM) <sup>a</sup>
10	0	—	4-COOH	>230 dec	C <sub>20</sub> H <sub>14</sub> O <sub>2</sub> ·0.25 H <sub>2</sub> O: C, H	81.6 ± 11.6
11	1	—	4-COOH	224–225	C <sub>21</sub> H <sub>16</sub> O <sub>2</sub> : C, H	27.6 ± 3.4
12	2	O	2-COOH	103–105	C <sub>22</sub> H <sub>18</sub> O <sub>3</sub> : C, H	73.3 ± 10.3
13	2	O	3-COOH	258–259	C <sub>22</sub> H <sub>18</sub> O <sub>3</sub> : C, H	31.0 ± 1.9
14	2	O	4-COOH	185–186	C <sub>22</sub> H <sub>18</sub> O <sub>3</sub> : C, H	14.0 ± 0.6
15	3	O	2-COOH	127–128	C <sub>22</sub> H <sub>20</sub> O <sub>3</sub> ·0.2H <sub>2</sub> O: C, H	55.4 ± 5.0
16	3	O	3-COOH	153–156	C <sub>23</sub> H <sub>20</sub> O <sub>3</sub>	13.8 ± 0.6
17	3	O	4-COOH	179–180.5	C <sub>22</sub> H <sub>20</sub> O <sub>3</sub> ·0.1H <sub>2</sub> O: C, H	15.6 ± 1.8
18	4	O	3-COOH	153–154.5	C <sub>24</sub> H <sub>22</sub> O <sub>3</sub> : C, H	6.6 ± 0.7
19	4	O	4-COOH	170–171.5	C <sub>24</sub> H <sub>22</sub> O <sub>3</sub> : C, H	7.53 ± 1.1
20	5	O	2-COOH	88.5–90	C <sub>25</sub> H <sub>24</sub> O <sub>3</sub> : C, H	15.8 ± 0.9
21	5	O	3-COOH	151.5–153.5	C <sub>25</sub> H <sub>24</sub> O <sub>3</sub> : C, H	5.23 ± 0.4
22	5	O	4-COOH	152–155	C <sub>25</sub> H <sub>24</sub> O <sub>3</sub> : C, H	7.13 ± 1.7

<sup>a</sup> See footnotes to Table 1.**Figure 3**

simple structural elements with an improvement in *in vitro* potency. Furthermore, it appears that increased lipophilicity may be favorable for activity.

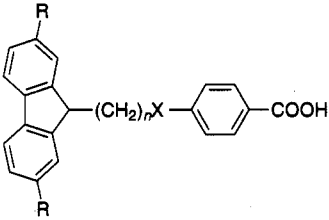
Encouraged by these results, we next investigated the replacement of the carbamate linkage in benzoic acid-containing leumedin, such as NPC 16570 and 17923, with simple linker chains. The results of this study are shown in Table 2. Again, replacement of the carbamate moiety of the original leumedin with simple carbon chains has increased the *in vitro* potency of the compounds. Compound 19, which is isosteric with NPC 16570 with respect to the length of the linker (Figure 2), is 4-fold more potent.

It appears from this study that the minimal structural elements necessary for inhibition of cell adhesion by these compounds are a lipophilic aromatic moiety and an acidic proton. Relatively weak activity by the shorter chain compounds, as well as variation in activity in the benzoic acid series from moving the carboxylate around the ring, may relate to a specific orientation of the carboxylic acid relative to the fluorene ring required for optimal activity. In this respect, it is interesting to note the changes in adhesion inhibition activity in the set of leumedin compounds shown in Figure 3. All of these compounds contain the conformationally restrictive carbamate functionality. Adding a single carbon to the linker of 27 results in an inactive compound, 28. (All IC<sub>50</sub>s are for inhibition of TNF- $\alpha$ -activated neutrophils to serum.) An additional methylene in the linker

restores the activity (29), as does moving the carboxylic acid from the *para* to the *ortho* position of the aromatic ring (30).

Adding lipophilic ring substituents to the leumedin compounds was previously shown to substantially enhance their *in vitro* activity.<sup>7</sup> In the present case, the results of ring substitution are not so salutatory. The dichloro analogues of compounds 11 and 19 (23 and 24, respectively; Table 3) are equipotent with the unsubstituted compounds. It is possible that hydrophobic effects are important for the activity of the compounds, with the log *P* values of 11 and 19 already lying near the optimum value. Exploration of this hypothesis by evaluation of the ability of various compounds to permeate cell membranes and calculation of log *P* values is currently underway. Interestingly, addition of *tert*-butyl groups to 11 abolished the activity (compound 25), whereas the *tert*-butyl analogue of 19 (26) was active. This may be due to the inability of the shorter-chain compound to accommodate the bulky groups in its interaction with the putative binding domain.

Certain of the new compounds were selected for a more extensive profile of their *in vitro* activity; these data are presented in Table 4. These compounds were evaluated for their ability to inhibit neutrophil adhesion to serum under conditions in which this adhesion is specifically mediated by the integrin CD11b/CD18 (Mac-1). In addition, the LD<sub>50</sub> values for the compounds in neutrophils and ECs were determined. The results indicate that representative members of this new class of cell adhesion inhibitors are capable of inhibiting  $\beta$ -integrin-mediated adhesion of neutrophils to endothelial cells at IC<sub>50</sub>s comparable to their potencies to block TNF- $\alpha$ - or PAF-activated neutrophil adhesion to serum-coated wells. These results are consistent with antagonism of  $\beta$ -integrin activity as the primary target for these compounds. Together with the observation that compounds 11 and 17, unlike NPCs 15669 and 17923, do not affect fMLP receptor binding,<sup>12</sup> these data suggest that the new compounds are more selective in their activity. Furthermore, several of the compounds exhibit a substantially improved therapeutic index relative to the first generation compounds. While the

**Table 3.** Physical Properties and Potencies of Ring-Substituted Fluorenylbenzoic Acids To Inhibit Adhesion of TNF- $\alpha$ -Activated Neutrophils to Serum-Coated Wells


compd	n	X	R	mp	formula: anal. <sup>a</sup>	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>
23	1	—	Cl	245–248	C <sub>21</sub> H <sub>14</sub> Cl <sub>2</sub> O <sub>2</sub> ·H <sub>2</sub> O: C, H, Cl	29.1 $\pm$ 2.5
24	4	O	Cl	170–171	C <sub>24</sub> H <sub>20</sub> Cl <sub>2</sub> O·0.5H <sub>2</sub> O: C, H	8.1 $\pm$ 2.7
25	1	—	<i>t</i> -Bu	249–253	C <sub>29</sub> H <sub>32</sub> O <sub>2</sub> ·0.25H <sub>2</sub> O: C, H	>200
26	4	O	<i>t</i> -Bu	159–160	C <sub>32</sub> H <sub>38</sub> O <sub>3</sub> : C, H	8.0 $\pm$ 0.8

<sup>a</sup> See footnotes to Table 1.**Table 4.** *In Vitro* Profile of Selected Compounds

compd	IC <sub>50</sub> , $\mu$ M			LD <sub>50</sub> , $\mu$ M <sup>b</sup>	
	TNF- $\alpha$ serum adhesion <sup>a</sup>	PAF serum adhesion <sup>a</sup>	IFN- $\gamma$ HUVEC adhesion	PMN	EC
9	6.0 $\pm$ 0.8	—	10.2 $\pm$ 2.5	79 (2)	72 (1)
11	27.6 $\pm$ 3.4	17.8 $\pm$ 2.9	91.2 $\pm$ 2.3	306 (2)	>1000 (3)
17	15.6 $\pm$ 1.8	36.4 $\pm$ 11.2	23.2 $\pm$ 6.6	281 (5)	>1000 (3)
19	7.53 $\pm$ 1.1	5.2 $\pm$ 4.2	19.8 $\pm$ 7.2	113 (3)	>1000 (2)
22	7.13 $\pm$ 1.7	11.7 $\pm$ 2.0	48.2 $\pm$ 17.8	295 (2)	>1000 (2)
NPC 15669	41.7 $\pm$ 7.4	31.7 $\pm$ 7.8	117 $\pm$ 17.2	343 (2)	354 (2)
NPC 17923	9.8 $\pm$ 1.4	17.8 $\pm$ 10.4	26.1 $\pm$ 2.4	62.5 (2)	82.3 (2)

<sup>a</sup> See footnote b, Table 1. <sup>b</sup> Values are the averages of IC<sub>50</sub>'s from the indicated number of separate determinations.

mechanism of action of these compounds has not yet been elucidated in detail, they do not appear to act by directly blocking ICAM-1/Mac-1 interactions. Mechanistic studies to date are consistent with a mechanism involving inhibition of signal pathways in leukocytes.<sup>10</sup>

Using these new cell adhesion inhibitors as departure points, we have developed several new classes of structure having improved toxicological profiles and potencies. Results of these studies, as well as studies regarding the mechanism of action of these compounds and their effects on leukocyte cellular function, will be the subject of future reports.

## Experimental Section

**General Experimental Methods.** Melting points are uncorrected. NMR spectra were recorded at 400 MHz in deuterated solvents. Elemental analyses were performed by Atlantic Microlabs of Atlanta, GA. Air-sensitive reactions were performed under an argon atmosphere in oven-dried glassware. Thin layer chromatography (TLC) was performed on 0.25 mm layers of Merck silica gel 60F-254 on glass backed plates. Plates were visualized by exposure to iodine vapor, viewing under UV light, or treatment with KMnO<sub>4</sub> dip. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). Solvents used for elution are noted for the specific examples. Solvent extracts were routinely dried over MgSO<sub>4</sub> and concentrated using a rotary evaporator.

Anhydrous solvents, starting materials and reagents were purchased from Aldrich Chemical Co. and used as obtained.

**General Procedure for the Synthesis of Fluorenylalkanoic Acids. Exemplified for 4-(9H-Fluoren-9-yl)butyric Acid (4).** A solution of fluorene (18.0 g; 0.108 mol) in THF (200 mL) was cooled to –78 °C and treated with 48.8 mL of a 2 M solution of *n*-butyllithium in hexanes (0.112 mol). The resulting mixture was stirred for 30 min and warmed to 0 °C. In a separate flask, a solution of 2-bromobutyric acid (15.0 g; 89.8 mmol) in 200 mL of THF was cooled to –78 °C and treated with 36 mL of 2.3 M *n*-butyllithium (82.8 mmol). After this solution was stirred at –78 °C for 30 min, the

solution of 9-lithiofluorene was added via canula, and the resulting mixture was stirred at –78 °C for 30 min and room temperature for 20 h. After cooling back to 0 °C, water (100 mL) was added, and the THF was removed under reduced pressure. NaOH (1 N, 500 mL) was added, and the solution was extracted with ether (2  $\times$  200 mL). The aqueous phase was made acidic (pH 2) with concentrated HCl and extracted with ethyl acetate; the organic layer was dried and concentrated to obtain 4 as a white solid (16.0 g; 70%): mp 128–129 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.39–1.49 (m, 2H), 2.05–2.12 (m, 2H), 2.25 (t, *J* = 7.42, 2H), 4.00 (t, *J* = 5.6, 1H), 7.24–7.38 (m, 4H), 7.49 (d, *J* = 7.2, 2H), 7.74 (d, *J* = 7.2, 2H). Anal. (C<sub>17</sub>H<sub>16</sub>O<sub>2</sub>) C, H.

**General Procedure for the Preparation of 9-Fluorenylalkanol. Exemplified for 4-(9H-Fluoren-9-yl)butanol.** A solution of acid 4 (5.0 g; 19.84 mmol) in THF (50 mL) was cooled to 0 °C and treated with 30 mL of a 1.0 M solution of diborane in THF. The resulting mixture was stirred at 0 °C for 30 min and at room temperature overnight. After cooling to 0 °C, 100 mL of 2 N NaOH was added with caution. After stirring for several hours, ether (100 mL) was added and the layers were separated. The organic phase was washed with brine, dried, concentrated, and passed through a silica gel column, eluting with 10% ethyl acetate in hexane, to obtain 4.12 g (88%) of the alcohol as a clear oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.22 (m, 2H), 1.49 (m, 2H), 2.07 (m, 2H), 3.48 (t, 2H), 4.04 (t, 1H), 7.31 (m, 4H), 7.50 (d, *J* = 7.2, 2H), 7.75 (d, *J* = 7.2, 2H).

**General Procedure for the Preparation of Methyl Benzoates by Mitsunobu Reaction. Exemplified for Methyl 4-[4-(9H-Fluoren-9-yl)butoxy]benzoate.** To a solution of the above alcohol (0.880 g; 3.69 mmol), methyl 4-hydroxybenzoate (0.674 g; 4.43 mmol), and triphenylphosphine (1.26 g; 4.80 mmol) in THF (20 mL) at room temperature was slowly added diisopropyl azodicarboxylate (1.05 g; 5.10 mmol). After stirring for 30 min, the reaction mixture was poured into ether (250 mL) and washed with water (150 mL). The organic layer was washed with brine, dried, and freed of solvent. The crude material was purified on a silica gel column, eluting with 5% ethyl acetate in hexane, to obtain 910 mg (66%) of the product as a clear oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.31 (m, 2H), 1.71 (m, 2H), 2.08 (m, 2H), 3.86 (s, 3H), 3.88 (m, 2H), 4.01 (t, 1H),

6.82 (d,  $J = 8.9$ , 2H), 7.28–7.38 (m, 4H), 7.50 (d,  $J = 7.26$ , 2H), 7.75 (d,  $J = 7.3$ , 2H), 7.95 (d,  $J = 11.6$ , 2H).

**General Procedure for the Hydrolysis of Methyl Benzoates to Final Products. Exemplified for 4-[2-(9H-Fluoren-9-yl)butoxy]benzoic Acid (19).** A solution of the above ester (870 mg; 2.34 mmol) in 40 mL of methanol containing 206 mg (5.14 mmol) of NaOH was heated to reflux for 12 h. The methanol was removed *in vacuo*, and the residue was partitioned between 1 N HCl and methylene chloride. The organic layer was dried and concentrated, and the white solid obtained was triturated with cold hexane and dried to obtain 600 mg (71%) of 19 as a white solid: mp 171–171.5 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.31 (m, 2H), 1.75 (m, 2H), 2.11 (m, 2H), 3.91 (t,  $J = 6.5$ , 2H), 4.01 (t,  $J = 5.6$ , 1H); 6.83–8.03 (m, 12H); IR ( $\text{CHCl}_3$ ) 3014, 2947, 1688, 1604, 1257  $\text{cm}^{-1}$ . Anal. ( $\text{C}_{24}\text{H}_{23}\text{O}_3$ ) C, H.

**Synthesis of 6-(9H-Fluoren-9-yl)-2-benzylhexanoic Acid (7).** A solution of phenylpropionic acid (5.0 g; 33.3 mmol) in 30 mL of THF was cooled to 0 °C and treated with 2.1 equiv of lithium diisopropylamide in THF. After stirring for 2 h at 0 °C, the mixture was transferred to a solution of 1,4-dibromobutane (30.0 g; 0.14 mol) in 25 mL of THF. After stirring for several hours, the reaction was quenched by the addition of 1N HCl and extracted into ether. The ether layer was dried, concentrated, and chromatographed (5% methanol/methylene chloride) to obtain 1.70 g (18%) of 2(*R,S*)-benzyl-6-bromoheptanoic acid.

A solution of fluorene (1.09 g; 6.56 mmol) in 35 mL of THF was cooled to 0 °C and treated with 3.42 mL of a 2.5 M solution of *n*-butyllithium in hexanes (1 equiv). This mixture was stirred for 2.5 h. In a separate flask, the above heptanoic acid was dissolved in THF (30 mL), cooled to 0 °C, treated with 1 equiv of *n*-BuLi, and stirred for 2.5 h. The lithiofluorene solution was transferred into the solution of the carboxylate salt, and the resulting mixture was stirred for 3 h at room temperature before quenching with 1 N HCl and extracting into methylene chloride. The crude material obtained upon removal of the solvent was passed through a flash column, eluting with 2.5% methanol in methylene chloride, to obtain 1.61 g (73%) of 7 as a white solid: mp 107.5–108.5 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.09–1.15 (m, 2H), 1.26–1.33 (m, 2H), 1.36–1.43 (m, 1H), 1.50–1.56 (m, 1H), 1.95–2.01 (m, 2H), 2.55–2.60 (m, 1H), 2.63–2.69 (m, 1H), 2.88–2.93 (m, 1H), 3.94–3.96 (t, 1H,  $J = 5.6$ ), 7.10–7.12 (m, 2H), 7.16–7.37 (m, 7H), 7.46 (d, 2H,  $J = 7.4$ ), 7.74 (d, 2H,  $J = 7.5$ ). Anal. ( $\text{C}_{26}\text{H}_{26}\text{O}_2$ ) C, H.

**Synthesis of (9H-Fluorenyl)-4-benzoic Acid (10).** 2-(4-Bromophenyl)-4,4-dimethyl-2-oxazoline (prepared from 4-bromobenzoic acid and 2-amino-2-methylpropanol according to the procedure of Meyers et al.;<sup>13</sup> 1.0 g; 3.90 mmol) was dissolved in 20 mL of THF and cooled to –78 °C under an argon atmosphere. After treating with 1.9 mL of 2.5 M *n*-BuLi (4.65 mmol), the mixture was stirred for 20 min and then treated with 0.70 g (3.9 mmol) of fluorene-9-one in 10 mL of THF. After coming to room temperature, the reaction was quenched with saturated ammonium chloride and extracted into ethyl acetate. The organic phase was washed with brine, dried, and concentrated, and the crude residue was recrystallized from ethyl acetate/hexane to obtain 1.0 g of the hydroxyoxazolyl compound. This material was suspended in 30 mL of 6 N HCl and refluxed for 4 h. After cooling, the white precipitate was filtered off, washed with water, and dried under vacuum to obtain 710 mg of product. The crude hydroxy acid was dissolved in 45 mL of a 1:1:1 mixture of EtOAc:AcOH:MeOH, treated with 700 mg of palladium hydroxide on carbon, and hydrogenated at 50 psi for 1 h. The mixture was filtered through Celite to remove the catalyst and concentrated; the crude product was recrystallized from methanol/water to obtain 10 as a white solid: mp >230 °C dec;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  5.11 (s, 1H), 7.18–7.50 (m, 8H), 7.81–7.83 (d, 2H,  $J = 7.7$ ), 7.99–8.10 (d, 2H,  $J = 8.3$ ). Anal. ( $\text{C}_{20}\text{H}_{14}\text{O}_2 \cdot 0.25\text{H}_2\text{O}$ ) C, H.

**Synthesis of (9H-Fluoren-9-ylmethylene)-4-benzoic Acid (11).** To a solution of fluorene (3.7 g; 20 mmol) in THF (50 mL) at 0 °C was added *n*-BuLi (8 mL of a 2.5 M solution in hexanes; 20 mmol). The anion solution was stirred for 1 h and then transferred via canula to a solution of methyl

4-(bromomethyl)benzoate (4.3 g; 20 mmol) in 30 mL of THF at –30 °C. After being allowed to warm to room temperature, the reaction was quenched with dilute HCl and the solvent was removed under reduced pressure. The residue was partitioned between water and ethyl acetate; the organic layer was washed with brine, dried, and concentrated. The product was recrystallized from ethyl acetate/hexane to obtain 7.50 g (90%) of the ester:  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  3.43 (d, 2H,  $J = 7.5$ ), 3.84 (s, 3H), 4.41 (t, 1H,  $J = 7.5$ ), 7.32 (m, 8H), 7.83 (m, 4H). This ester was hydrolyzed according to the general procedure given above to obtain 11 in 95% yield: mp 224–225 °C;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  3.25 (d, 2H,  $J = 7.0$ ), 4.38 (t, 1H,  $J = 7.0$ ), 7.21–7.41 (m, 8H), 7.81–7.92 (m, 4H), 12.82 (s, 1H); IR (KBr) 3013, 2892, 1681, 1419, 1290, 750  $\text{cm}^{-1}$ . Anal. ( $\text{C}_{21}\text{H}_{16}\text{O}_2$ ) C, H.

**Synthesis of 2,7-Di-*tert*-butyl-9H-fluorene.** Fluorene (5.0 g; 30 mmol) was dissolved in 50 mL of methylene chloride, and 2-chloro-2-methylpropane (12 mL) was added. Anhydrous ferric chloride (1.50 g) was added in portions, gas evolution began, and the reaction became somewhat vigorous. After all the  $\text{FeCl}_3$  had been added and the reaction had subsided, the mixture was stirred for 1 h. The solvent was removed *in vacuo*, and the residue was partitioned between ethyl acetate (200 mL) and 10% HCl (200 mL). The organic layer was washed with brine, dried, and concentrated; the residue was purified through a silica gel column, eluting with 20% ether/hexane, to obtain 6.34 g (76%) of the desired compound:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.37 (s, 18H), 3.83 (s, 2H), 7.38 (dd, 2H,  $J = 1.8$ , 8.0), 7.55 (d, 2H,  $J = 1.8$ ), 7.65 (d, 2H,  $J = 8.0$ ).

**Synthesis of 2,7-Dichloro-9H-fluorene.** This compound was synthesized according to the procedure of Perumattam et al.<sup>14</sup>

**Biological Assays. Adherence of PMN to Serum-Coated Wells. Isolation of PMN.** Blood was drawn into syringes containing heparin (1 unit/mL) from human volunteers (free of any medication for at least 48 h). Blood (30 mL) was layered over a gradient of 10 mL of 1.077 g/mL and 15 mL 1.119 g/mL Histopaque (Sigma, St. Louis, MO) and centrifuged at room temperature, 400g, for 20 min. All subsequent manipulations were carried out at room temperature, with endotoxin-free reagents, and using polypropylene pipettes and tubes, to prevent activation of the PMN. The pinkish PMN layer immediately above the RBC was collected and washed twice with Hank's buffered salt solution with calcium and magnesium, 0.2% glucose, and 10 mM HEPES, pH 7.2 (HBSS++) by centrifugation at 400g for 10 min. Pellets were suspended in 8 mL of 0.9% NaCl, and RBC was lysed by the addition of 24 mL of water for 40 s, followed by the addition of 8 mL of 3.6% NaCl. After centrifugation at 200g for 8 min, RBC ghosts were aspirated from the top of the PMN pellet and cells washed with HBSS++.

**PMN Adherence to Protein Substrates.** 96-well tissue culture plates were coated with 10% pooled human serum (North American Biologicals, Miami, FL) in phosphate-buffered saline (PBS) for 1 h at 37 °C, followed by washing four times with PBS. Test compounds were prepared in DMSO as 100× stocks. PMN ( $4 \times 10^6/\text{mL}$ ) were preincubated with test compounds for 10 min at 37 °C in serum-coated wells. TNF- $\alpha$  was added to 0.6 nM or PAF to 1  $\mu\text{M}$ , and adherence is carried out for 20 or 10 min, respectively. Nonadherent cells were aspirated, and wells were washed two times with warm PBS with calcium and magnesium, with blotting of the inverted plate following each aspiration. Adherent PMN were quantitated using a BCA protein assay (Pierce, Rockford, IL).

**INF- $\gamma$  HUVEC Assay.** Primary human umbilical vein endothelial cells (HUVECs) from Clonetics Corp. (San Diego, CA) were seeded at 20 000 cells per well in a 96-well tissue culture plate that had been coated with 5  $\mu\text{g}/\text{mL}$  fibronectin. The cells were allowed to adhere for 45 min, and the wells were then twice rinsed with phosphate-buffered saline. To the culture media (M199, 80%; FBS, 20%; 100  $\mu\text{g}/\text{mL}$  endothelial cell growth factor; 0.1 mg/mL heparin; 5%  $\text{CO}_2$ ; 37 °C) was added INF- $\gamma$  (R&D Systems) at 1 unit/mL for 20 h. Isolated PMNs were loaded with the fluorescent dye BCECF-AM (Molecular Probes) by incubating  $1 \times 10^7$  cells/mL in HBSS with 4  $\mu\text{M}$  dye for 30 min at 37 °C. The PMNs were incubated

with drug for 10 min at 37 °C, activated with 50 ng/mL TNF- $\alpha$ , and immediately added to the HUVECs which had been rinsed twice with HBSS + 1% human serum. The coculture plate was incubated at 37 °C for 20 min followed by gentle washing (twice) with HBSS + 1% human serum. The extent of adherence was assessed by measuring the amount of fluorescence (485–535 nm filter) in each well with a fluorescence plate reader (IDEXX).

**Measurement of Cell Viability.** PMN (50  $\mu$ L) or EC suspension (as above, but without phenol red) containing test compound was added to serum-coated wells and incubated at 37 °C for 30 min; 50  $\mu$ L of 2  $\mu$ M Calcein AM (Molecular Probes, Eugene, OR) in HBSS++ without phenol red was added and incubation continued for 30 min. Fluorescence was quantitated with an IDEXX plate reader using excitation/emission of 485/535 nm. Viable cells cleave the nonfluorescent AM ester to the fluorescent-free Calcein.

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