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Prodrugs of a CXC Chemokine-12 (CXCL12) Neutraligand Prevent Inflammatory Reactions in an Asthma Model in Vivo

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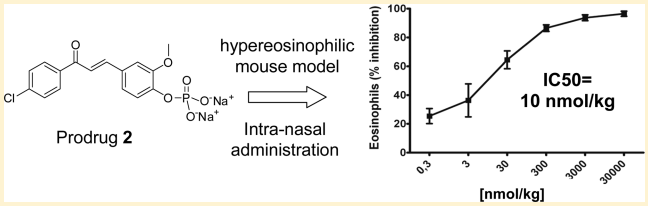
Supporting Information

ABSTRACT: Chalcone 4 (compound **1**) is a small molecule that neutralizes the CXC chemokine CXCL12 and prevents it from acting on the CXCR4 and CXCR7 receptors. To overcome its poor solubility in aqueous buffers, we designed highly soluble analogues of compound **1**, phosphate, L-seryl, and sulfate, all inactive by themselves on CXCL12 but when cleaved in vivo into **1**, highly active locally at a low dose in a mouse airway hypereosinophilia model.



KEYWORDS: *prodrug, solubility, CXCL12 chemokine, CXCR4 receptor, GPCR, asthma*

The chemokine CXCL12 plays a pivotal role in normal and pathological situations, including brain development, hematopoiesis, and chronic inflammation.[1] In screening a proprietary chemical library,[2] we recently identified compounds belonging to the chalcone family and preventing CXCL12 from binding to its CXCR4 or CXCR7 receptors.[3] These compounds have an original mechanism of action: they bind to the chemokine rather than to the receptors. The highest affinity molecule, chalcone 4[3] (compound **1** in Scheme 1, now commercialized by Sigma-Aldrich, C7870), inhibits both chemotaxis of human peripheral blood lymphocytes and eosinophil infiltration in a mouse model of airway hypereosinophilia.[3] The observation of these effects highlights the enormous potential of such a ligand neutralization strategy.[3−5] Because of its mechanism of action, compound **1** does not affect the resting/basal level of the receptors for either calcium or chemotactic responses.[3] By analogy to the activity of neutralizing antibodies, we named compound **1** a "neutraligand".[4] It is a tool that complements traditional antagonists of the CXCR4 receptor, such as AMD3100, AMD3465, ALX40-4C, and T22/T140,[6−8] but has the disadvantage of poor solubility in aqueous buffer (9 μM). The aim of the work reported here was to increase its solubility to enable in vivo access to its pharmacological targets. We report the design and synthesis of highly soluble derivatives of compound **1** that behave as prodrugs[9,10] converted into the active compound **1** and have in vivo anti-inflammatory activity at low doses upon intranasal administration in a mouse model of asthma.

The requirements for suitable prodrugs of compound **1** were (1) inclusion of a functional group enzymatically cleavable in vivo,[11] (2) improved aqueous solubility to facilitate in vivo intranasal administration, and (3) rapid and efficient synthesis to allow large-scale preparation. To fulfill these requirements, we decided to introduce three types of functional groups on the hydroxyl moiety of compound **1**: a phosphate, an L-seryl, and a sulfate group. Prodrugs **2**, **4**, and **5** were prepared according to Scheme 1 starting from compound **1**, itself obtained following a Claisen−Schmidt reaction with a 67% overall yield.[12]

The phosphate derivative **2** was obtained by phosphorylation of the phenol group with dibenzylphosphonate in CCl_4, followed by benzyl removal in the presence of trimethylsilylbromide (37% overall yield).[13] An alternative one-pot procedure involved phosphorylation of the 4-hydroxyphenyl in the presence of phosphorus oxichloride, followed by basic hydrolysis to obtain the disodium phosphate analogue with a 45% yield.[14] The L-serine conjugate of compound **1** was synthesized in two steps. First, an ester was prepared with Boc-L-Ser(*t*Bu)-OH in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDCI) and a catalytic amount of *N*,*N*-dimethyl-4-aminopyridine (4-DMAP). Subsequent deprotection in acidic media provided the expected prodrug **4** as the hydrochloride salt, with a 70% overall yield. The sulfate analogue **5** was obtained by direct sulfation of the 4′-hydroxyphenyl with a chlorosulfonic acid/pyridine mixture and then treatment with aqueous NaOH to obtain the sodium salt (34%).[15] The structure and purity of all compounds were assessed by ^{1}H, ^{13}C NMR, high-resolution mass spectrometry (HRMS), and RP-HPLC analyses.

Thermodynamic solubility was measured in an aqueous 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (pH 7.4, 22 °C). As expected, the solubility of the phosphate **2** was very high, so high that saturation was not achieved at a 30 mM

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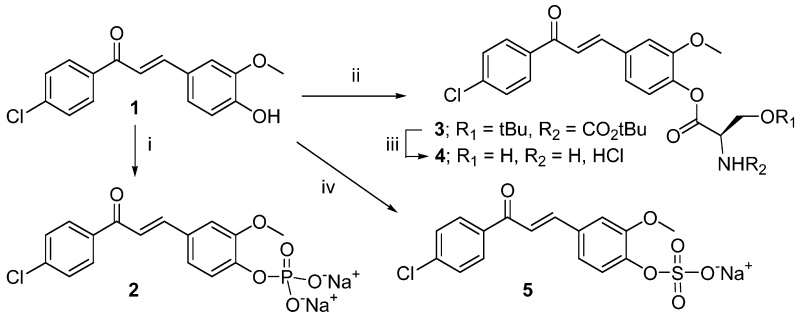
Scheme 1. Prodrugs 2, 4, and 5 Preparation[a] from Compound 1



[a]Reagents: (i) (a) $POCl_3$ (3 equiv), Et_3N (10 equiv), CH_2Cl_2, room temperature, overnight; (b) aqueous NaOH. (ii) Boc-Ser(*t*Bu)-OH (1.5 equiv), DMAP (0.1 equiv), EDCI (1.5 equiv), CH_2Cl_2, room temperature, 1 h. (iii) 4 N HCl/dioxane, 60 °C, 4 h. (iv) (a) Chlorosulfonic acid (10 equiv), pyridine, room temperature, overnight; (b) aqueous NaOH.

Table 1. Characterization of Compounds 1, 2, 4, and 5

					stability[d] half-life ($T_{1/2}$)			
compd	isolated yield (%)	CHI[a]	Log *D* calcd[b]	solubility (μM)[c]	PBS	murine serum	lung homogenate	binding inhibition (%)[e]
1		90	3.4	9 ± 1	>10 h	>10 h	>6 h	90 ± 10 (K_i = 53 ± 31nM)
2	40	43	1.0	>30000	>10 h	2 h	<15 min	5 ± 2
4	70	76	2.7	42 ± 3	12 min	<5 min	<5 min	15 ± 5
5	34	64	2.1	1434 ± 3	>10 h	>10 h	30 min	4 ± 3

[a]Determined by RP-HPLC[14] (C18 Luna column, 2 mL/min, 365 nm, solvent A = pH 7.4 ammonium acetate buffer, solvent B = acetonitrile). [b]Calculated from CHI. [c]Measured at pH 7.4, HEPES buffer, 24 h of incubation, 22 °C. [d]37 °C. [e]Inhibition of CXCL12-TR binding to EGFP-CXCR4 determined by FRET at 5 μM.

concentration. Its solubility is thus at least 3000 times higher than the 9 μM solubility of compound **1**. The solubility measured for the sulfate **5** under the same experimental conditions was 1430 μM, that is, greater than that required for satisfactory drug development. The yield of the serine **4** was better than that of the other two compounds (70%), but its solubility was only moderately better than that of compound **1** (4.6-fold; 42 μM as compared to 9 μM).

The partition coefficients of compounds **1**, **2**, **4**, and **5** at pH 7.4, expressed as Log *D*, are derived directly from measurements of their chromatographic hydrophobicity index (CHI), as compared with reference compounds.[16] Log *D* decreased from 3.4 for the original compound **1** to 2.7 for the serine **4**, 2.1 for the sulfate **5**, and 1.0 for the phosphate **2**.

The stability of the four compounds was evaluated by LC-MS in three media: phosphate buffer saline (PBS), mouse serum, and mouse lung homogenates (Table 1). All experiments were conducted with extemporaneously prepared solution. Compound **1** at 10 μM is stable after incubation in all three media ($T_{1/2}$ > 6−10 h). The phosphate **2** showed good stability in PBS, with 50% of it converted into **1** after 2 h of incubation in mouse serum (Table 1 and Figure 1) and within less than 15 min in lung homogenates (Table 1). The serine ester **4** was rapidly hydrolyzed into **1** with a $T_{1/2}$ = 12 min in PBS; it disappeared in less than 5 min in mouse serum and lung homogenate. This instability is the combined effect of the strong electron withdrawing effect of the protonated aminogroup activating the ester linkage toward hydrolysis and intramolecular assistance of this aminogroup, as already reported for α-aminoester prodrugs.[17] The sulfate **5** was stable in PBS and murine serum ($T_{1/2}$ > 10 h) but rapidly converted into **1** in lung homogenates ($T_{1/2}$ = 30 min). We therefore confirmed the high rate of conversion of each prodrug candidate into **1** in lung homogenates (Table 1).

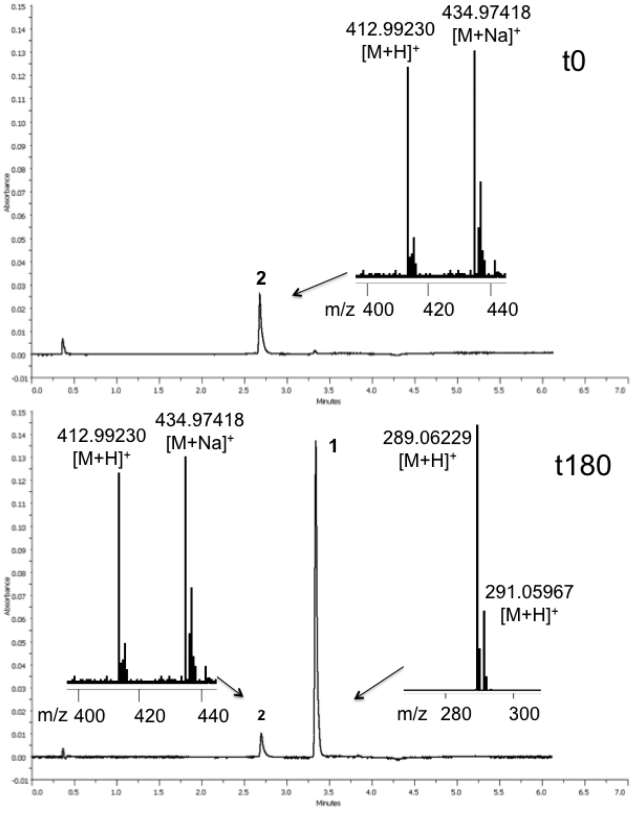


Figure 1. RP-HPLC chromatogram (C18 Luna column, 2 mL/min, 365 nm, solvent A = pH 7.4 ammonium acetate buffer, solvent B = acetonitrile). Gradient: 0−0.2 min, 0% B; 0.2−2.7 min, 0−100% B; 2.7−3.2 min, 100% B; 3.2−3.4 min, 100−0% B; and 3.4−6.2 min, 0% B. Positive ESI-MS spectrum of compound **2** at t_0 and t_{180} at 37 °C in mouse serum.

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In vitro inhibition of binding of Texas Red (TR)-labeled CXCL12 (CXCL12-TR, 100 nM) to enhanced green fluorescent protein (EGFP)-tagged CXCR4 by compounds **1**, **2**, **4**, and **5** at 5 μM was determined by fluorescence resonance energy transfer (FRET) (Table 1 and Figure 2) as previously described.[3]

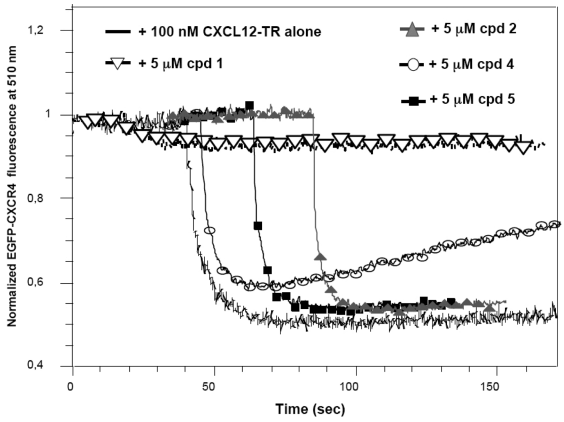

Figure 2. Real-time monitoring of fluorescent CXCL12-TR to EGFP-CXCR4 at 510 nm as a function of time. Upon addition of CXCL12-TR (100 nM), the fluorescence intensity at 510 nm declines as a result of interactions between CXCL12 and CXCR4. Compound **1** inhibits binding of CXCL12-TR to EGFP-CXCR4, whereas prodrugs **2**, **4**, and **5** (5 μM) do not.

Compound **1**, the reference compound, at 5 μM reduces this binding by 90% with a K_i of 53 nM. Compounds **2**, **4**, and **5** are, as expected, almost inactive in this in vitro binding assay (Figure 2). When serine was substituted in **4**, prolonged incubation times tended to inhibit CXCL12 binding to CXCR4. The inadequate stability of **4** in buffer or serum (Table 1), due to the progressive conversion of the inactive prodrug **4** into the active **1**, may explain this result.

The in vivo activity of the prodrugs was assessed in an 8 day mouse model of airway hypereosinophilia in ovalbumin (OVA)-sensitized mice challenged with OVA or control saline. Eosinophil number in the bronchoalveolar lavage fluid increased significantly after OVA challenge, as compared with saline, in OVA-sensitized animals, as well as macrophage, neutrophil, and lymphocyte recruitment, although those were more moderate (Figure 3). Because topical administration is a well-accepted and preferred route for treatment of inflamed airways, especially in asthma, we developed our model with intranasal administration of the compounds in mice to mimic the inhalation route used in humans.

We first investigated the activity of compound **1** and compared it with that of phosphate **2**, the most soluble compound, after intranasal administration to the airways (2 h before each challenge with OVA or saline on 3 consecutive days) (Figure 3A). We evaluated the activity of **1** and **2** administered at doses of 22 nmol/kg, that is, the dose corresponding to the maximal solubility of **1** in PBS (9 μM, 25 μL/mouse). The compounds per se had no effect in the control unsensitized groups. In the OVA-challenged mice, **1** had no effect on inflammatory cell recruitment (Figure 3A). By contrast, the phosphate **2** significantly inhibited OVA-induced eosinophil (56 ± 9%) and neutrophil recruitment, with a trend toward significance for lymphocytes (Figure 3A). The dose–response activity of phosphate **2** on eosinophil recruitment showed an IC_{50} of 10 nmol/kg (Figure 3B), thereby suggesting that the prodrug provides more efficient distribution of compound **1** in the target tissue. Contrastingly, no effect of **2** was noticed on macrophage recruitment, corroborating results on the activity of anti-CXCR4 or -CXCL12 neutralizing antibodies[18] or CXCR4 antagonist.[19] LC-MS analysis shows that **1** appears in lung tissue as early as 15 min after intranasal administration of **2**, thus revealing its hydrolysis into the active **1**, thereby delivered at high concentrations to the lung.

The activity of the other two prodrugs, the serine **4** and sulfate **5** derivatives, was compared to that of the phosphate **2** in the same airway hypereosinophilia mouse model. The intranasally administered dose was 30 nmol/kg. The activity levels of **4** and **5** shows similarity to that of **2** with significant reduction of the recruitment of eosinophils (67, 69, and 64% inhibition, respectively) and neutrophils by the three prodrugs and at a lower level for lymphocytes, with no effect on macrophage recruitment (Figure 4). This clearly indicates that all three prodrugs release the same amount of the active species **1** when administered to the airways. This finding is consistent with the rapid release of **1** from the three different prodrugs in lung homogenates in vitro (Table 1), its limited distribution in

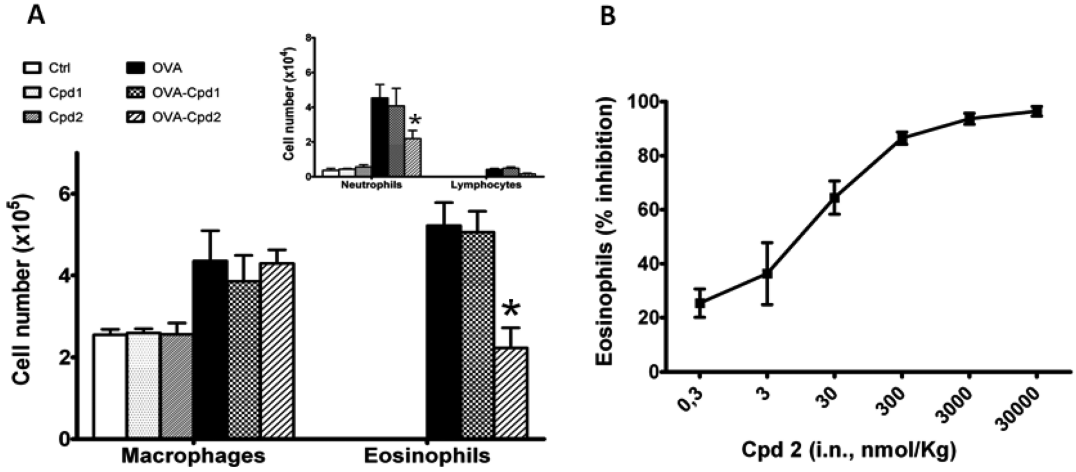

Figure 3. (A) Intranasal treatment with compounds **1** and **2** (22 nmol/kg in PBS) 2 h before each challenge in the 8 day mouse model of hypereosinophilia. Blocks (means) and bars (SEM) of $n = 6$ mice/group. *$p \leq 0.05$ as compared to saline-treated OVA group. (B) Dose–response inhibition of eosinophil number of compound **2** (percent). Means (filled squares) and bars (SEM) of $n = 6$ mice/group.

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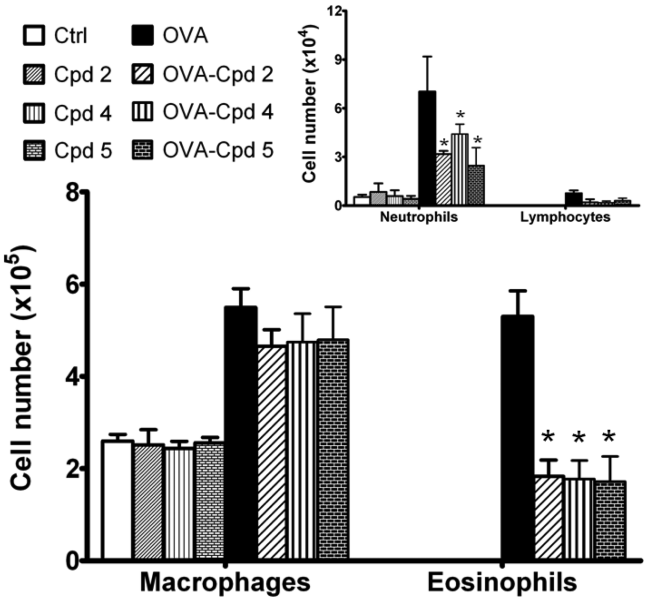

Figure 4. Intranasal administration of compounds **2**, **4**, and **5** (30 nmol/kg) 2 h before each challenge in a hypereosinophilic mouse model. Absolute numbers of cells in BAL are shown. Means (blocks) and SEM (bars) of $n = 6$ mice/group. *$p \leq 0.05$ as compared to OVA.

vivo, and efficient trapping of the pro-inflammatory chemokine, CXCL12, locally in the inflamed lung tissue.

Three analogues of compound **1**, a neutraligand of the CXC chemokine CXCL12, bearing a phosphate, an L-seryl, and a sulfate moiety, are highly soluble and bioavailable when administered to the airways. They behave as prodrugs, remaining inactive until compound **1** is released. Intranasal application of each of these three analogues inhibits eosinophil recruitment in the airways by ≥50% at a dose as low as 30 nmol/kg and without any signs of toxicity. This prodrug strategy, which results in a low-dose application for topical effect, therefore, appears to be a powerful strategy for obtaining highly soluble compounds that act directly in the airways and promote the desired local action while avoiding systemic adverse effects on other CXCL12-related functions.

EXPERIMENTAL PROCEDURES

For (*E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-enyl)-2-methoxyphenyl-phosphate, sodium salt **2**, under argon atmosphere, $POCl_3$ (186 μL, 2 mmol) was dissolved in anhydrous CH_2Cl_2 (8 mL); the mixture was then cooled to 0 °C, and anhydrous triethylamine (700 μL, 5 mmol) was added. After 5 min of stirring, compound **1** (114 mg, 0.39 mmol dissolved in 1 mL of anhydrous CH_2Cl_2) was added dropwise to the reaction mixture. The resulting mixture was stirred for 1 h at 0 °C and then allowed to warm to room temperature overnight. The crude mixture was then evaporated to dryness under reduced pressure to remove the excess NEt_3 and $POCl_3$. The residue was dissolved in CH_2Cl_2 and evaporated to dryness again. The procedure was repeated twice. Finally, the residue was dissolved in a tetrahydrofuran−water solution (2/1 v/v, 1.5 mL), and the mixture was vigorously stirred for 1 h. The solution was diluted with water (500 μL) and cooled down to 0 °C. A 0.1 N, NaOH aqueous solution was added dropwise to reach a pH of 12. After concentration under reduced pressure, compound **5** was isolated by flash chromatography on an RP18 column, with a linear gradient of acetonitrile in water. Following a freeze/drying step, compound **2** was recovered as a yellow solid (74 mg, 45% yield).

For (*R*,*E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-2-methoxyphenyl-3-(*tert*-butoxy)-2-(*tert*-butyloxycarbonylamino)-propanoate **3**, under argon atmosphere, **1** (100 mg, 0.35 mmol) was dissolved in anhydrous CH_2Cl_2 (1.5 mL), and *N*,*N*-dimethyl-4-aminopyridine (4-DMAP) (4 mg, 0.034 mmol) was added. To this solution was added a solution of Boc-L-Ser(O*t*Bu)-OH (136 mg, 0.52 mmol) and EDCI hydrochloride (100 mg, 0.52 mmol) in anhydrous CH_2Cl_2 (1.5 mL), after it was prepared, and stirred for 15 min at room temperature in the dark. The combined mixture was stirred at room temperature in the dark for 1 h, the solvent was removed, and the crude product was purified by chromatography on Si40 silica (3/7 ethyl acetate/heptane). Compound **3** was recovered as a pale yellow oil (170 mg, 92% yield).

For (*R*,*E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-2-methoxyphenyl 2-amino-3-hydroxypropanoate, hydrochloride salt **4**, compound **3** (51 mg, 0.096 mmol) was dissolved in a mixture of dioxane and 4 N HCl aqueous solution (1/1 v/v, 510 μL). The mixture was heated at 60 °C for 3 h. After completion, the precipitate was recovered by filtration and triturated twice with ether. After it was dried, **4** was obtained as a pale yellow solid (30 mg, 76% yield).

For (*E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-2-methoxyphenyl sulfate, sodium salt **5**, under argon atmosphere, **1** (100 mg, 0.35 mmol) was dissolved in anhydrous pyridine (10 mL), and the mixture was cooled to −10 °C. Chlorosulfonic acid (233 μL, 3.5 mmol) was added dropwise (warning: violent reaction!). The mixture was stirred at room temperature overnight. The solvent was then evaporated under reduced pressure, and the residue was dissolved in water and acidified to pH 2 by addition of 1 N HCl aqueous solution. The mixture was extracted three times with CH_2Cl_2. The combined organic layers were concentrated under reduced pressure. The residue was dissolved in EtOH (1 mL) and alkalinized with 50% NaOH aqueous solution (1 equiv). The solvent was evaporated under reduced pressure at room temperature, and the crude oily residue was purified by flash chromatography on Reverse-Phase 18 column (eluent water-acetonitrile). Compound **5** was recovered as a yellow solid (46 mg, 34% yield).

After mice were exposed for 15 min to intranasally administered compounds **2**, **4**, and **5**, they were killed, and their lungs were collected, homogenized, and solid−liquid extracted with acetonitrile. After 3000*g* centrifugation, the supernatant was collected, evaporated, and resuspended in 50% water−50% acetonitrile, 0.1% TFA before injection. Analyses were conducted with RP-HPLC, as shown in Figure 1, or LC-MS.

The activity of each compound was assessed in vivo in an 8 day model of hypereosinophilia. Briefly, 9 weeks old male Balb/c mice were sensitized by intraperitoneal injection of 50 μg of OVA (grade V, Sigma-Aldrich) adsorbed on 2 mg of aluminum hydroxide (Sigma-Aldrich) in 0.1 mL of saline on days 0, 1, and 2. Mice were challenged intranasally [10 μg of OVA in 25 μL of saline (12.5 μL/nostril)] on days 5, 6, and 7. Control mice received intraperitoneal and intranasal administrations of saline alone. Intranasal administrations were performed under anesthesia with intraperitoneal 50 mg/kg ketamine and xylazine at 3.33 mg/kg. Food and water were supplied ad libitum. Animal experimentation was conducted with the approval of the government body that regulates animal research in France. Two hours before each OVA or saline challenge, compound **1** or **2**, **4**, or **5** in PBS (25 μL) was administered intranasally.

Bronchoalveolar lavage (BAL) was performed 24 h after the last OVA challenge. Mice were deeply anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. A plastic canula was inserted into the trachea, and airways were lavaged by 10 instillations of 0.5 mL of ice-cold saline supplemented with 2.6 mM ethylenediaminetetraacetic acid (EDTA) (saline-EDTA). BAL fluids were centrifuged (300*g*, 5 min, 4 °C) to pellet cells, and erythrocytes were lysed by hypotonic shock. Cells were resuspended in 500 μL of ice-cold saline-EDTA, and total cell counts were determined with a hemocytometer (Neubauer's chamber). Differential cell counts were assessed on cytologic preparations (Cytospin, Shandon Ltd.) (250 000 cells/mL in ice-cold saline-EDTA) stained with Hemacolor (Merck) on counts of at least 400 cells and expressed as absolute numbers or percentage of total cell number.

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■ ASSOCIATED CONTENT

Supporting Information

Detailed synthesis and full characterization of compounds **1**, **2**, **4**, and **5**, the solubility and stability determinations, and the in vitro binding experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BAL, bronchoalveolar lavage; CHI, chromatography hydrophobicity index; 4-DMAP, *N*,*N*-dimethyl-4-aminopyridine; EDCI, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide; EDTA, ethylenediaminetetraacetic acid; EGFP, enhanced green fluorescent protein; FRET, fluorescence resonance energy transfer; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HRMS, high-resolution mass spectrometry; OVA, ovalbumin; SEM, standard error of the mean; TR, Texas Red; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol

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