Synthesis of Gallic Acid Analogs as Histamine and Pro-Inflammatory Cytokine Inhibitors for Treatment of Mast Cell-Mediated Allergic Inflammation

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Abstract: Gallic acid (3,4,5-trihydroxybenzoic acid), is a natural product found in various foods and herbs that are well known as powerful antioxidants. Our previous report demonstrated that it inhibits mast cell-derived inflammatory allergic reactions by blocking histamine release and pro-inflammatory cytokine expression. In this report, various amide analogs of gallic acid have been synthesized by introducing different amines through carbodiimide-mediated amide coupling and Pd/C-catalyzed hydrogenation. These compounds showed a modest to high inhibitory effect on histamine release and pro-inflammatory cytokine expression. Among them, the amide bearing (S)-phenylglycine methyl ester 3d was found to be more active than natural gallic acid. Further optimization yielded several (S)- and (R)-phenylglycine analogs that inhibited histamine release in vitro. Our findings suggest that some gallamides could be used as a treatment for allergic inflammatory diseases.

Keywords: gallic acid; allergic inflammation; histamine; pro-inflammatory cytokine

1. Introduction

Gallic acid (3,4,5-trihydroxybenzoic acid), a polyphenol natural product obtained from various herbs, is known to have diverse biological effects such as anti-oxidation, anti-inflammation, and anti-cancer. In a previous research, Shin and coworkers demonstrated that gallic acid inhibits mast cell-mediated inflammatory allergic reactions by blocking histamine release and pro-inflammatory cytokine expression [1].

The prevalence rate of allergic diseases has been rising globally for more than 50 years. Approximately 50% of children are sensitized to common allergens [2]. Allergic inflammation is classified into three phases: early-phase, late-phase, and chronic allergic inflammation. In early-phase reactions, histamine, a major factor in the allergic response, is released from mast cells and induces vasodilation, increases vascular permeability, and recruits leukocytes. Repetitive allergen exposure alters organ function by affecting structural cells and increases production of cytokines resulting in chronic allergic inflammation [3].

Mast cells play key roles in immunoglobulin E (IgE)-mediated allergic reactions through secretion of preformed or newly synthesized mediators such as histamine, lipid-derived mediators, chemokines, cytokines, and growth factors [4]. The signaling pathway of mast cells triggered by antigen cross-linking of IgE bound to FcεRI has been previously described. Stimulation of FcεRI increases degranulation, production of lipid-derived mediators, and expression of cytokines [5]. Therefore,
suppression of histamine and pro-inflammatory cytokine release might be an appropriate therapeutic
target to reduce allergic inflammation. Human mast cells (HMC-1) are known as an appropriate model
for studying allergic reactions characterized by release of histamine and expression of pro-inflammatory
cytokines [6,7].

2. Results and Discussion

To increase the efficacy of gallic acid, gallic acid analogs were designed and synthesized by the
amidation of gallic acid with various amines (Figure 1) [8]. In addition, 3,4,5-trimethoxybenzamides
and 3,4,5-trisbenzyloxybenzamides were synthesized in a similar fashion.

Our synthesis of 3,4,5-trihydroxybenzamides commenced with formation of 3,4,5-
tris(phenylglycine methyl ester)-phenylglycine methyl ester (EDCI) was chosen as a carboxyl-activating agent for the amide coupling with amines such as
p-toluidine, p-methoxybenzylamine, (R)- and (S)-phenylglycine methyl ester, propargyl amine, benzylamine,
and Boc-piperazine. Thus, we obtained the amides 3a–3g from the respective amines, followed by hydrogenolysis under H2, Pd/C to obtain the resulting 3,4,5-trihydroxybenzamides 5b–5h in good
yield. Similarly, 3,4,5-trimethoxybenzamides 4b–4f were prepared from 3,4,5-trimethoxybenzoic acid
(Scheme 1). Compounds 4a, 4e and 5h were previously reported in the literature [9–14].

The release of histamine via degranulation is a key characteristic of activated mast cells, and
HMC-1 cells stimulated with phorbol 12-myristate 13-acetate and the calcium ionophore A23187
(PMACI) also released a high level of histamine. Gallic acid, a known inhibitor of histamine release,
was used as a positive control. Pretreatment with variously modified compounds (10 µM) differently
inhibited the level of histamine released by PMACI-stimulated HMC-1 cells as shown in Tables 1
and 2. Since the p-toluidine analog 3a showed the weakest inhibition rate (8.9% at 10 µM), neither

![Figure 1. Gallic acid and its amide analogs.](image)

![Scheme 1. Synthesis of 3,4,5-trisbenzyl-, 3,4,5-trimethoxy- and 3,4,5-trihydroxybenzamides.](image)
the trimethoxybenzamide nor trihydroxybenzamide of p-toluidine 4a and 5a was evaluated further. The gallamides with (R)-phenylglycine methyl ester 3c and 4c were more potent than (S)-enantiomers 3d and 4d. Unfortunately, reduction of trisbenzyloxy to trihydroxybenzamides 5c and 5d decreased the inhibition rate (24% and 36% at 10 μM). In contrast, p-methoxybenzylamine derivatives 3b and 5b exhibited a lower inhibition rate than did gallic acid. Trimethoxybenzamide 4b enhanced the histamine release by HMC-1 cells rather than having an inhibitory effect; consequently, 4b may trigger an allergic reaction. The N-propargylamide analog 3e and N-propylamide 5h, which was obtained by reduction of 3e, exhibited a lower histamine inhibition rate (39.4% and 31.2% at 10 μM) than did gallic acid. However, the trimethoxybenzamide analog 4e showed a slightly increased inhibitory activity (67% at 10 μM) compared with that of gallic acid. Compared with gallic acid, 3,4,5-trihydroxybenzamide analogs did not have an improved effect on histamine inhibition in vitro; however, several compounds such as 3c, 3d, 3g, 4c, 4d, 4e, and 5b exhibited similar or better inhibitory effects on histamine release.

Table 1. Inhibitory activity of 3,4,5-trisbenzyloxy- and 3,4,5-trihydroxybenzamide analogs (10 μM) on histamine release in HMC-1 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>58.9</td>
</tr>
<tr>
<td>3a</td>
<td>8.9</td>
</tr>
<tr>
<td>3b</td>
<td>48.3</td>
</tr>
<tr>
<td>3c</td>
<td>74.3</td>
</tr>
<tr>
<td>3d</td>
<td>54.9</td>
</tr>
<tr>
<td>3e</td>
<td>39.4</td>
</tr>
<tr>
<td>3f</td>
<td>20.9</td>
</tr>
<tr>
<td>3g</td>
<td>59.9</td>
</tr>
<tr>
<td>5b</td>
<td>52.1</td>
</tr>
<tr>
<td>5c</td>
<td>24.3</td>
</tr>
<tr>
<td>5d</td>
<td>35.8</td>
</tr>
<tr>
<td>5g</td>
<td>41.9</td>
</tr>
<tr>
<td>5h</td>
<td>31.2</td>
</tr>
</tbody>
</table>

Cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 are released by mast cells and are more important in chronic-phase allergic reactions. TNF-α play a key role in immunity by activating NF-κB and regulating immune cells. IL-6 maintains CD4+ T cell survival and promotes Th2 modulation; moreover, local accumulation of IL-6 produced by mast cells is known to induce passive cutaneous anaphylaxis (PCA) reaction. Therefore, the inhibition of pro-inflammatory cytokines is also a key strategy to treat allergic inflammation. To determine whether gallic acid and its amide analogs can inhibit inflammation, the expression of pro-inflammatory cytokines such as TNF-α and IL-6 was evaluated by real-time PCR. Some compounds showed different inhibitory effects on the gene expression of TNF-α and IL-6. As shown in Table 3, 3d and 5d significantly reduced the gene expression of TNF-α, and five compounds—3c, 3d, 3e, 3g, and 5d—reduced expression of IL-6.
Table 2. Inhibitory activity of 3,4,5-trimethoxylamides (10 μM) on histamine release in HMC-1 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>63.0</td>
</tr>
<tr>
<td>4b</td>
<td>-12.9</td>
</tr>
<tr>
<td>4c</td>
<td>69.6</td>
</tr>
<tr>
<td>4d</td>
<td>57.2</td>
</tr>
<tr>
<td>4e</td>
<td>67.0</td>
</tr>
<tr>
<td>4f</td>
<td>44.5</td>
</tr>
</tbody>
</table>

Table 3. Gene expression of pro-inflammatory cytokines. (Values are related to β-actin) * significant inhibition of cytokines.

<table>
<thead>
<tr>
<th>PMACI</th>
<th>Compounds (10 μM/L)</th>
<th>Gallic acid (10 μM/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>3</td>
<td>3g</td>
</tr>
<tr>
<td>+</td>
<td>3a</td>
<td>3a</td>
</tr>
<tr>
<td>+</td>
<td>3b</td>
<td>3c</td>
</tr>
<tr>
<td>+</td>
<td>3c</td>
<td>3d</td>
</tr>
<tr>
<td>+</td>
<td>3e</td>
<td>3f</td>
</tr>
<tr>
<td>+</td>
<td>3f</td>
<td>3g</td>
</tr>
<tr>
<td>+</td>
<td>5b</td>
<td>5c</td>
</tr>
<tr>
<td>+</td>
<td>5c</td>
<td>5d</td>
</tr>
<tr>
<td>+</td>
<td>5d</td>
<td>5g</td>
</tr>
<tr>
<td>+</td>
<td>5g</td>
<td>5h</td>
</tr>
<tr>
<td></td>
<td>0.35 ± 0.03</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>0.71 ± 0.06</td>
<td>1.05 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>0.59 ± 0.02 *</td>
<td>0.67 ± 0.02 *</td>
</tr>
<tr>
<td></td>
<td>0.93 ± 0.22</td>
<td>0.84 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>1.10 ± 0.03</td>
<td>0.81 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>0.88 ± 0.07</td>
<td>0.67 ± 0.05 *</td>
</tr>
<tr>
<td></td>
<td>0.46 ± 0.04 *</td>
<td>0.71 ± 0.08 *</td>
</tr>
<tr>
<td></td>
<td>0.65 ± 0.12</td>
<td>0.59 ± 0.09 *</td>
</tr>
<tr>
<td></td>
<td>0.67 ± 0.12</td>
<td>0.85 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>0.85 ± 0.08</td>
<td>0.68 ± 0.04 *</td>
</tr>
<tr>
<td></td>
<td>0.96 ± 0.22</td>
<td>0.80 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>0.81 ± 0.20</td>
<td>0.81 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>0.50 ± 0.07 *</td>
<td>0.45 ± 0.09 *</td>
</tr>
<tr>
<td></td>
<td>0.92 ± 0.16</td>
<td>0.83 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>0.88 ± 0.11</td>
<td>0.81 ± 0.17</td>
</tr>
</tbody>
</table>

Combined with the result of the inhibitory effect on histamine release, 3c and 3d significantly inhibited both histamine release and expression of TNF-α and IL-6. Due to the inhibitory effect on both histamine and pro-inflammatory cytokine release, 3c and 3d were targeted for more modifications in order to improve their biological activity and drug-like properties. In addition, the analogs
derived from 3c and 3d could enable us to elucidate structure-activity relationships and modes of action (Scheme 2). Thus, the methyl ester 3d was hydrolyzed by LiOH in H$_2$O/THF to furnish the carboxylic acid 6a. In a second modification, 3d was methylated with two equivalents of MeI in the presence of NaH at 0 °C to furnish the tertiary amide 6b. Various gallamides 6c–6i were prepared by EDCI-mediated coupling with (S)-leucine methyl ester, (R)- and (S)-phenylalanine methyl ester, (S)-phenylalanine tert-butyl ester, (S)-2-amino-2-phenylacetamide, (S)-(+)2-chloro-phenylglycine methyl ester and (±)-4-fluorophenylglycine methyl ester.

As shown in Table 4, we evaluated the inhibitory activity of the second-generation gallamide analogs at 10 nM on histamine release. We found that 3d and some derivatives markedly inhibited histamine release even at quite low concentrations. The carboxylic acid 6a and the dimethylated amide 6b of 3d exhibited a similar inhibitory activity on histamine release as did 3d. Additionally, (R)- and (S)-phenylalanine methyl esters 6c and 6d had a lower inhibition rate than did 3d. Similar to 4b, 6f and 6h increased the histamine concentration released by HMC-1 cells instead of inhibiting it. The incorporation of an electron-withdrawing group, such as chloro (compound 6h) and fluoro (compound 6i), at the aromatic ring of 3d was not tolerated. Interestingly, leucine amide 6e showed even better inhibitory activity than the initial compound 3d.
Table 4. Inhibitory activity of 3d and its analogs (10 nM) on histamine release in RBL-2H3 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3d</td>
<td>53</td>
</tr>
<tr>
<td>6a</td>
<td>52</td>
</tr>
<tr>
<td>6b</td>
<td>48</td>
</tr>
<tr>
<td>6c</td>
<td>32</td>
</tr>
<tr>
<td>6d</td>
<td>18</td>
</tr>
<tr>
<td>6e</td>
<td>55</td>
</tr>
<tr>
<td>6f</td>
<td>−42</td>
</tr>
<tr>
<td>6g</td>
<td>4</td>
</tr>
<tr>
<td>6h</td>
<td>−26</td>
</tr>
<tr>
<td>6i</td>
<td>11</td>
</tr>
</tbody>
</table>

3. Conclusions

It was well established that gallic acid inhibits mast cell-derived inflammatory allergic reactions by blocking histamine release and pro-inflammatory cytokine expression. However, the structure of gallic acid is too small and its hydrophilic polyphenol groups affect metabolism; therefore, it needs to be converted to drug-like compounds. In order to solve these problems, new gallamide analogs were synthesized. Fifteen of 17 compounds could inhibit histamine release; compounds 3c and 3d showed greatest inhibitory activity on release of both histamine and pro-inflammatory cytokines such as TNF-α and IL-6. The results give insight into the mechanism by which 3d exerts anti-allergic and anti-inflammatory activity on mast cells, and suggests that 3d might be considered as a therapeutic candidate for mast cell-mediated allergic inflammatory diseases.

4. Materials and Methods

4.1. General Information

All starting materials and reagents were obtained from commercial suppliers and were used without further purification. Air and moisture sensitive reactions were performed under an argon atmosphere. Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck, Darmstadt, Germany) with the indicated solvents. Thin-layer chromatography was performed using 0.25 mm silica gel plates (Merck, Darmstadt, Germany). 1H- (600 MHz) and 13C-NMR (150 MHz) spectra were recorded on an AVANCE III System 600 MHz spectrometer (Bruker, Billerica, MA, USA) as solutions in CDCl3, DMSO-d6 or methanol-d4. High-resolution mass spectra (HRMS) were obtained on JMS-700 instrument (JEOL, Akishima, Tokyo, Japan) with electrospray ionization. Spectral data of 3a–6i are available in Supplementary Materials.
4.2. Synthesis of Gallamide Analogs

3,4,5-Tris(benzyloxy)-N-p-tolylbenzamide (3a). To a DMF solution (2 mL) of 3,4,5-tris(benzyloxy)benzoic acid (110 mg, 0.25 mmol), p-toluidine (32 mg, 0.3 mmol), EDCI (60 mg, 0.4 mmol), HOBt (4 mg, 30 µmol) and TEA (87 µL, 0.50 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:2) to generate pure 3a (111 mg, 80%). ¹H-NMR (CDCl₃) δ 7.46 (d, J = 8.3 Hz, 2H), 7.44–7.32 (m, 12H), 7.29–7.25 (m, 5H), 7.17 (d, J = 8.1 Hz, 3H), 7.12 (s, 2H), 5.15 (s, 4H), 5.12 (s, 2H), 2.34 (s, 3H), ¹³C-NMR (CDCl₃) δ 166.41, 152.95, 141.52, 137.50, 136.74, 135.48, 134.32, 130.59, 129.71, 128.72, 128.70, 128.35, 128.20, 128.12, 127.69, 120.29, 107.13, 75.32, 71.57, 21.05; HRMS (EI) m/z calcd. for 559.2359; found 559.2361.

(R)-Methyl 2-phenyl-2-(3,4,5-tris(benzyloxy)benzamido)acetate (3c). To a DMF solution (2 mL) of 3,4,5-tris(benzyloxy)benzoic acid (110 mg, 0.25 mmol), (R)-2-Phenylglycine methyl ester hydrochloride (61 mg, 0.3 mmol), EDCI (59 mg, 0.4 mmol), HOBt (3.8 mg, 30 µmol) and TEA (90 µL, 0.50 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to yield pure 3c (98 mg, 70%). ¹H-NMR (CDCl₃) δ 7.21–7.41 (m, 15H), 7.05 (s, 2H), 6.90 (m, 2H), 5.11 (s, 4H), 5.08 (s, 2H), 4.53 (d, 2H), 3.81 (s, 3H); ¹³C-NMR (CDCl₃) δ 171.59, 166.90, 152.87, 141.48, 137.46, 136.68, 135.48, 134.32, 130.59, 129.71, 128.72, 128.70, 128.35, 128.20, 128.12, 127.69, 126.69, 125.71, 123.67, 122.34, 121.84, 121.48, 119.12, 118.89, 118.54, 118.37, 118.31, 117.92, 117.50, 75.49, 71.79, 57.25, 53.28; HRMS (EI) m/z calcd. for 599.2359; found 599.2361.

(S)-Methyl 2-phenyl-2-(3,4,5-tris(benzyloxy)benzamido)acetate (3d). To a DMF solution (2 mL) of 3,4,5-tris(benzyloxy)benzoic acid (110 mg, 0.25 mmol), (S)-2-Phenylglycine methyl ester hydrochloride (56 mg, 0.28 mmol), EDCI (115 mg, 0.60 mmol), HOBt (4.0 mg, 30 µmol) and TEA (90 µL, 0.50 mmol) were added. After stirring for 3 h, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to yield pure 3d (127 mg, 86%). ¹H-NMR (CDCl₃) δ 7.41–7.32 (m, 17H), 7.27–7.22 (m, 3H), 7.12 (s, 2H), 7.06 (d, J = 6.8 Hz, 1H), 6.69 (d, J = 6.9 Hz, 1H), 5.05 (s, 2H), 4.53 (s, 3H), 3.75 (s, 3H); ¹³C-NMR (CDCl₃) δ 171.59, 166.21, 152.83, 141.64, 137.46, 136.73, 136.52, 129.10, 128.91, 128.71, 128.66, 128.63, 128.28, 128.11, 128.05, 127.67, 127.48, 107.23, 75.23, 71.50, 70.03, 53.01; HRMS (EI) m/z calcd. for 587.2308; found 587.2307.

3,4,5-Tris(benzyloxy)-N-(prop-2-yn-1-yl)benzamide (3e). To a DMF solution (4 mL) of 3,4,5-tris(benzyloxy)benzoic acid (220 mg, 0.50 mmol), propargylamine (33 mg, 0.60 mmol), EDCI (115 mg, 0.60 mmol), HOBt (7.0 mg, 50 µmol) and TEA (90 µL, 0.50 mmol) were added. After stirring for 24 h, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to produce the amide 3e (212 mg, 89%). ¹H-NMR (CDCl₃) δ 7.41–7.21 (m, 15H), 7.08 (s, 2H), 6.40 (s, 1H), 5.07 (s, 2H), 5.06 (s, 4H), 4.17 (dd, J = 5.2, 2.5 Hz, 2H), 2.24 (t, J = 2.5 Hz, 1H); ¹³C-NMR (CDCl₃) δ 166.90, 152.87, 141.48, 137.46, 136.68,
N,3,4,5-Tetrakis(benzyloxy)benzamide (3f). To a DMF solution (2 mL) of 3,4,5-tris(benzyloxy)benzoic acid (110 mg, 0.25 mmol), O-benzylhydroxylamine hydrochloride (48 mg, 0.3 mmol), EDCI (58 mg, 0.3 mmol), HOBT (4 mg, 30 µmol) and TEA (90 µL, 0.50 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:2) to generate pure 3f (102 mg, 74%). ¹H-NMR (CDCl₃) δ 7.42–7.20 (m, 2H), 6.95 (s, 2H), 5.06 (s, 2H), 5.03 (s, 4H), 4.97 (s, 2H); ¹³C-NMR (CDCl₃) δ 166.20, 152.80, 141.56, 137.35, 136.51, 135.30, 129.39, 128.84, 128.65, 128.29, 128.15, 128.07, 127.64, 139.79, 137.55, 136.70, 130.42, 128.60, 128.26, 128.23, 128.03, 127.98, 127.38, 107.24, 80.29, 75.17, 71.18, 29.94; HRMS (EI) m/z calcd. for 359.1369; found 359.1370.

tert-Butyl 4-(3,4,5-tris(benzyloxy)benzoyl)piperazine-1-carboxylate (3g). To a DMF solution (2 mL) of 3,4,5-tris(benzyloxy)benzoic acid (110 mg, 0.25 mmol), 1-Boc-piperazine (56 mg, 0.3 mmol), EDCI (115 mg, 0.60 mmol), HOBT (5.0 mg, 40 µmol) and TEA (90 µL, 0.50 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to generate pure 3g (103 mg, 68%). ¹H-NMR (CDCl₃) δ 7.44–7.25 (m, 15H), 6.65 (t, J = 5.1 Hz, 1H), 4.51 (d, J = 5.7 Hz, 2H), 3.84 (s, 3H), 3.82 (s, 6H), 3.78 (s, 3H); ¹³C-NMR (CDCl₃) δ 170.07, 154.52, 152.68, 139.79, 137.55, 136.70, 130.42, 128.60, 128.26, 128.23, 128.03, 127.98, 127.38, 107.24, 80.29, 75.23, 71.18, 28.43; HRMS (EI) m/z calcd. for 608.2886; found 608.2888.

3,4,5-Trihydroxy-N-(4-methoxybenzyl)benzamide (4b). To a CH₂Cl₂ solution (3 mL) of 3,4,5-trimethoxybenzoic acid (100 mg, 0.471 mmol), 4-Methoxybenzylamine (78 mg, 0.566 mmol), EDCI (115 mg, 0.61 mmol), HOBT (70 mg, 0.46 mmol) and TEA (0.31 mL, 1.78 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 2:1) to generate 85 mg of 4b (yield 54%). ¹H-NMR (CDCl₃) δ 7.23 (d, J = 8.6 Hz, 2H), 7.01 (s, 2H), 6.83 (d, J = 8.6 Hz, 2H), 6.67 (t, J = 5.1 Hz, 1H), 4.51 (d, J = 5.7 Hz, 2H), 3.84 (s, 3H), 3.82 (s, 6H), 3.77 (s, 3H); ¹³C-NMR (CDCl₃) δ 167.26, 159.31, 153.39, 141.09, 130.61, 130.11, 129.54, 114.34, 104.66, 61.16, 56.52, 55.57, 43.94.; HRMS (EI) m/z calcd. for 399.2095; found 399.2098.

(R)-Methyl 2-phenyl-2-(3,4,5-trimethoxybenzamido)acetate (4c). To a CH₂Cl₂ solution (3 mL) of 3,4,5-trimethoxybenzoic acid (100 mg, 0.471 mmol), (R)-(−)-2-phenylglycine methyl ester hydrochloride (114 mg, 0.56 mmol), EDCI (117 mg, 0.613 mmol), HOBT (70 mg, 0.46 mmol) and TEA (0.24 mL, 1.41 mmol) were added. After stirring overnight, the reaction mixture was diluted with CH₂Cl₂ and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:1) to generate 85 mg of 4c (yield 50%). ¹H-NMR (CDCl₃) δ 7.46–7.33 (m, 2H), 7.19 (d, J = 6.9 Hz, 1H), 7.05 (s, 2H), 5.74 (d, J = 6.8 Hz, 1H), 3.89 (s, 6H), 3.88 (s, 3H), 3.78 (s, 3H); ¹³C-NMR (CDCl₃) δ 171.69, 166.37, 153.30, 141.40, 136.57, 129.15, 129.02, 128.75, 127.51, 104.72, 61.02, 57.10, 56.47, 53.06; HRMS (EI) m/z calcd. for 359.1369; found 359.1370.

(S)-Methyl 2-phenyl-2-(3,4,5-trimethoxybenzamido)acetate (4d). To a CH₂Cl₂ solution (3 mL) of 3,4,5-trimethoxybenzoic acid (100 mg, 0.471 mmol), (S)-(−)-2-phenylglycine methyl ester hydrochloride (110 mg, 0.66 mmol), EDCI (210 mg, 1.09 mmol), HOBT (70 mg, 0.46 mmol) and TEA (0.24 mL, 1.41 mmol) were added. After stirring overnight, the reaction mixture was diluted with CH₂Cl₂ and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:1) to produce 90 mg of 4d (yield 53%). ¹H-NMR (CDCl₃) δ 7.43–7.40 (m, 2H), 7.37–7.30 (m, 3H), 7.19
(d, J = 6.9 Hz, 1H), 7.04 (s, 2H), 5.72 (d, J = 6.9 Hz, 1H), 3.86 (s, 9H), 3.76 (s, 3H); 13C-NMR (CDCl3) δ 171.65, 166.35, 153.24, 141.33, 136.51, 129.08, 128.95, 128.69, 127.50, 104.69, 60.96, 57.07, 56.40, 52.99; HRMS (EI) m/z calcd. for 359.1369; found 359.1370.

3,4,5-Trimethoxy-N-(prop-2-yn-1-yl)benzamide (4e). To a CH2Cl2 solution (3 mL) of 3,4,5-trimethoxybenzoic acid (100 mg, 0.471 mmol), propargylamine (31 mg, 0.56 mmol), EDCI (250 mg, 1.32 mmol), HOBT (29 mg, 0.20 mmol) and TEA (0.3 mL, 1.71 mmol) were added. After stirring overnight, the reaction mixture was diluted with CH2Cl2 and washed with water and brine, dried over MgSO4 and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:1) to generate 48 mg of 4e (yield 52%). 1H-NMR (CDCl3) δ 7.01 (s, 2H), 6.40 (s, 1H), 4.23 (dd, J = 5.3, 2.6 Hz, 2H), 3.88 (s, 6H), 3.87 (s, 3H), 2.28 (s, 1H); 13C-NMR (CDCl3) δ 166.81, 153.11, 141.02, 129.04, 104.35, 79.40, 71.77, 60.83, 56.23, 56.11, 29.78; HRMS (EI) m/z calcd. for 317.0899; found 317.0900.

Glasses

3,4,5-Trihydroxy-N-(4-methoxybenzyl)benzamide (5b). A 10 mL round-bottom flask was charged with 3b (30 mg, 0.05 mmol), MeOH (3 mL), and a Teflon-coated magnetic stirring bar. 10% Pd/C (10 mg) were added, producing a black slurry. The flask was equipped with a hydrogen balloon attached to a stainless steel needle. The slurry was left to stir under hydrogen atmosphere. After 24 h, the hydrogen atmosphere of the flask was purged with nitrogen. The mixture was then filtered through a pad of Celite to give a dark green solution. This solution was concentrated to give 5b (14 mg, 90%). 1H-NMR (CD2OD) δ 7.24 (d, J = 8.7 Hz, 2H), 6.86 (d, J = 8.8 Hz, 4H), 4.44 (s, 2H), 3.76 (s, 3H); 13C-NMR (CD2OD) δ 170.20, 161.04, 146.50, 137.93, 132.29, 129.58, 126.05, 114.69, 107.69, 55.51, 43.73; HRMS (EI) m/z calcd. for 289.0950; found 289.0948.

(R)-Methyl 2-phenyl-2-(3,4,5-trihydroxybenzamido)acetate (5c). A 10 mL round-bottom flask was charged with 3a (89 mg, 0.15 mmol), MeOH (3 mL), and a Teflon-coated magnetic stirring bar. 10% Pd/C (12 mg) was added to the resulting solution, producing a black slurry. The flask was equipped with a hydrogen balloon attached to a stainless steel needle. The slurry was left to stir under hydrogen atmosphere. After 24 h, the hydrogen atmosphere of the flask was purged with nitrogen. The mixture was then filtered through a pad of Celite to give a dark green solution. This solution was concentrated to give 5c (43 mg, 88%). 1H-NMR (CD2OD) δ 7.54–7.43 (m, 5H), 6.89 (s, 2H), 5.63 (s, 1H), 3.71 (s, 3H); 13C-NMR (CD2OD) δ 193.7149, 168.75, 145.21, 137.04, 136.23, 128.48, 128.13, 127.51, 123.96, 106.76, 57.30, 51.75; HRMS (EI) m/z calcd. for 317.0899; found 317.0900.

(S)-Methyl 2-phenyl-2-(3,4,5-trihydroxybenzamido)acetate (5d). A 10 mL round-bottom flask was charged with 4a (60 mg, 0.1 mmol), MeOH (3 mL), and a Teflon-coated magnetic stirring bar. 10% Pd/C (5 mg) was added to the resulting solution, producing a black slurry. The flask was equipped with a hydrogen balloon attached to a stainless steel needle. The slurry was left to stir under hydrogen atmosphere. After 24 h, the hydrogen atmosphere of the flask was purged with nitrogen. The mixture was then filtered through a pad of Celite to give a dark green solution. This solution was concentrated to give 5d (30 mg, 92%) 1H-NMR (CD2OD) δ 7.28–7.41 (m, 5H), 6.86 (s, 2H), 5.60 (s, 1H), 3.68 (s, 3H); 13C-NMR (CD2OD) δ 171.50, 168.75, 145.24, 137.07, 136.25, 128.47, 128.12, 127.51, 123.96, 106.76, 57.30, 51.70; HRMS (EI) m/z calcd. for 317.0899; found 317.0900.
**5g**. A 10 mL round-bottom flask was charged with 7a (40 mg, 0.06 mmol), MeOH (3 mL), and a Teflon-coated magnetic stirring bar. 10% Pd/C (10 mg) was added to the resulting solution, producing a black slurry. The flask was equipped with a hydrogen balloon attached to a stainless steel needle. The slurry was left to stir under hydrogen atmosphere. After 24 h, the hydrogen atmosphere of the flask was purged with nitrogen. The mixture was then filtered through a pad of Celite to give a dark green solution. This solution was concentrated and washed with water and brine, dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/hexane = 6:4) to give 5g (20 mg, 91%). ¹H-NMR (CD₂OD) δ 6.36 (s, 2H), 3.64–3.29 (m, 8H), 1.38 (s, 9H); ¹³C-NMR (CD₂OD) δ 173.19, 156.18, 146.93, 136.53, 126.39, 107.80, 107.60, 81.60, 28.58; HRMS (EI) m/z calcd. for 338.1478; found 338.1477.

3,4,5-Trihydroxy-N-(prop-2-ynyl)benzamide (5h). A 10 mL round-bottom flask was charged with 5a (20 mg, 0.04 mmol), MeOH (3 mL), and a Teflon-coated magnetic stirring bar. 10% Pd/C (5 mg) was added to the resulting solution, producing a black slurry. The slurry was equipped with a hydrogen balloon attached to a stainless steel needle. The slurry was left to stir under hydrogen atmosphere. After 24 h, the hydrogen atmosphere of the flask was purged with nitrogen. The mixture was then filtered through a pad of Celite to give a dark green solution. This solution was concentrated and purified by Biotage® SNAP Ultra C18 reversed phase cartridge (from 10 to 90% ACN with 0.1% TFA) to give 5h (6 mg, 67%). ¹H-NMR (CD₂OD) δ 6.83 (s, 2H), 3.26 (t, J = 5.7 Hz, 2H), 1.59 (dd, J = 14.5, 7.3 Hz, 2H), 0.95 (t, J = 7.4 Hz, 3H); ¹³C-NMR (CD₂OD) δ 170.59, 146.62, 137.95, 126.41, 107.73, 58.32, 42.67, 23.79, 18.36, 11.75; HRMS (EI) m/z calcd. for 211.0845; found 211.0842.

(S)-2-Phenyl-2-(3,4,5-tris(benzyloxy)benzamido)acetic acid (6a). The 3c (150 mg, 0.26 mmol) was suspended in THF (3 mL) in a 25 mL round-bottomed flask. After 30 min, iodomethane (0.042 mL, 0.48 mmol) was added slowly. The solution was stirred for 10 min at room temperature, after which the reaction was quenched by adding an excess amount of saturated NH₄Cl aqueous solution, followed by extraction with ethyl acetate. The organic phase was washed with water and then dried over anhydrous MgSO₄. After the solution was filtered and the solvent was evaporated, the residue was subjected to silica gel column chromatography using 25% EtOAc in hexane to give 6a (80 mg, 55%). ¹H-NMR (CDCl₃) δ 7.90 (s, 1H), 7.51 (dd, J = 8.1, 0.9 Hz, 2H), 7.46–7.44 (m, 3H), 7.40–7.29 (m, 13H), 7.25–7.14 (m, 5H), 5.46 (s, 1H), 5.13 (s, 4H), 5.01 (s, 2H); ¹³C-NMR (CDCl₃) δ 174.62, 168.22, 154.02, 141.95, 141.24, 138.78, 138.32, 131.00, 129.74, 129.54, 129.33, 129.16, 129.06, 129.02, 128.92, 128.60, 128.36, 107.94, 76.19, 72.28, 61.10; HRMS (EI) m/z calcd. for 573.2151; found 573.2150.

Methyl 2-phenyl-2-(3,4,5-tris(benzyloxy)-N-methylbenzamido)propanoate (6b). Under a N₂ atmosphere, to a solution of (S)-methyl 2-phenyl-2-(3,4,5-tris(benzyloxy)benzamido)acetate (200 mg, 0.34 mmol) in DMF (2.5 mL), NaH (20 mg, 60% purity, 0.51 mmol) was added slowly at 0 °C. After 30 min, iodomethane (0.042 mL, 0.48 mmol) was added slowly. The solution was stirred for 10 min at room temperature, after which the reaction was quenched by adding an excess amount of saturated NH₄Cl aqueous solution, followed by extraction with ethyl acetate. The organic phase was washed with water and then dried over anhydrous MgSO₄. After the solution was filtered and the solvent was evaporated under vacuum, the residue was subjected to silica gel column chromatography using 25% EtOAc in hexane to give 6b (30 mg, 15%). H-NMR (CDCl₃) δ 7.62–7.51 (m, 2H), 7.46–7.18 (m, 19H), 6.87–6.79 (m, 2H), 5.18–5.07 (m, 6H), 3.66 (s, 3H), 2.38 (s, 3H), 1.96 (s, 3H); ¹³C-NMR (CDCl₃) δ 172.9, 172.1, 152.6, 140.3, 137.8, 137.5, 136.8, 131.0, 128.6, 128.5, 128.4, 128.3, 128.2, 127.9 (2), 127.4, 108.0, 75.2, 66.9, 52.6, 35.9, 18.6. HRMS (EI) m/z calcd. for 615.2621; found 615.2619.

Methyl (3,4,5-tris(benzyloxy)benzoyl)-d-phenylalaninate (6c). To a DMF solution (10 mL) of 3,4,5-tris(benzyloxy)benzoic acid (408 mg, 0.92 mmol), d-phenylalanine methyl ester hydrochloride (200 mg, 0.92 mmol), EDCI (196 mg, 1.02 mmol), HOBT (71 mg, 0.46 mmol) and TEA (0.30 mL, 2.04 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane =
Methyl (3,4,5-tris(benzyloxy)benzoyl)-l-phenylalaninate (6d). To a DMF solution (10 mL) of 3,4,5-tris(benzyloxy)benzoic acid (240 mg, 0.54 mmol), l-phenylalanine methyl ester hydrochloride (180 mg, 1.06 mmol), EDCI (230 mg, 1.20 mmol), HOBt (80 mg, 0.52 mmol) and TEA (0.37 mL, 2.09 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to generate pure 6d (420 mg, 66%). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 7.25–7.41 (m, 18H), 7.11 (dd, 2H, \(J = 1.8, 7.8\) Hz), 7.00 (s, 2H), 6.37 (d, 1H), 5.09 (s, 4H), 5.08 (s, 2H), 5.02–5.05 (m, 1H), 3.78 (s, 3H), 3.23 (qd, 2H, \(J = 7.8, 24.6\) Hz); \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) 172.0, 166.4, 152.7, 141.4, 137.4, 136.6, 135.8, 129.3, 129.2, 128.6, 128.5, 128.0, 127.9, 127.5, 127.2, 106.8, 75.1, 71.3, 53.5, 52.4, 37.8; HRMS (EI) m/z calcld. for 601.2464; found 601.2466.

Methyl (3,4,5-tris(benzyloxy)benzoyl)-l-leucinate (6e). To a CH\(_2\)Cl\(_2\) solution (3 mL) of 3,4,5-tris(benzyloxy)benzoic acid (190 mg, 1.00 mmol), HOBT (30 mg, 0.19 mmol) and TEA (0.14 mL, 0.85 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to generate pure 6e (yield 4%) . \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 7.44–7.17 (m, 15H), 7.10 (s, 2H), 6.93 (d, \(J = 8.1\) Hz, 1H), 4.85–4.79 (m, 1H), 3.74 (s, 3H), 1.77–1.56 (m, 3H), 0.96 (dd, \(J = 6.3, 2.7\) Hz, 6H); \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) 174.61, 166.67, 152.75, 141.35, 137.56, 136.85, 128.91, 128.62, 128.52, 128.21, 127.98, 127.92, 127.77, 106.89, 75.18, 71.32, 52.48, 51.36, 41.37, 25.06, 23.03, 21.85; HRMS (EI) m/z calcld. for 567.2421; found 567.2466.

tert-Butyl (S)-2-phenyl-2-(3,4,5-tris(benzyloxy)benzamido)acetate (6f). To a CH\(_2\)Cl\(_2\) solution (3 mL) of 3,4,5-tris(benzyloxy)benzoic acid (180 mg, 0.40 mmol), L-Phenylalanine tert-butyl ester hydrochloride (100 mg, 0.41 mmol), EDCI (90 mg, 0.47 mmol), HOBT (30 mg, 0.19 mmol) and TEA (0.14 mL, 0.85 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to generate pure 6f (yield 47%). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 7.46–7.27 (m, 19H), 7.21 (t, \(J = 6.1\) Hz, 1H), 7.19 (s, 2H), 5.65 (d, \(J = 7.0\) Hz, 1H), 5.12 (s, 6H), 1.46 (s, 9H); \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) 170.22, 165.96, 152.79, 141.48, 137.49, 137.32, 136.74, 129.18, 128.82, 128.63, 128.58, 128.27, 128.24, 128.05, 127.99, 127.66, 127.26, 107.13, 82.84, 75.18, 71.42, 57.40, 27.90; HRMS (EI) m/z calcld. for 629.2777; found 629.2774.

(S)-(S)-N-(2-Amino-2-oxo-1-phenylethyl)-3,4,5-tris(benzyloxy)benzamide (6g). To a CH\(_2\)Cl\(_2\) solution (3 mL) of 3,4,5-tris(benzyloxy)benzoic acid (390 mg, 0.88 mmol), (S)-2-amino-2-phenylacetamide (180 mg, 1.06 mmol), EDCI (190 mg, 1.00 mmol), HOBT (70 mg, 0.46 mmol) and TEA (0.31 mL, 1.78 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:1) to generate pure 6g (yield 57%). \(^1\)H-NMR (DMSO-d\(_6\)) \(\delta\) 8.75 (d, 1H, \(J = 7.8\) Hz), 7.72 (s, 1H), 7.51 (d, 2H, \(J = 7.2\) Hz), 7.46 (d, 4H, \(J = 7.2\) Hz), 7.23–7.40 (m, 17H), 5.66 (d, 1H, \(J = 7.8\) Hz), 5.17 (s, 4H), 4.99 (s, 2H); \(^{13}\)C-NMR (DMSO-d\(_6\)) \(\delta\) 172.26, 165.79, 152.37, 139.21, 137.36, 129.60, 128.84, 128.69, 128.63, 128.47, 128.33, 128.26, 128.12, 127.99, 127.95, 107.35, 74.65, 70.83, 60.18; HRMS (EI) m/z calcld. for 572.2311; found 572.2312.

Methyl 2-(2-chlorophenyl)-2-(3,4,5-tris(benzyloxy)benzamido)acetate (6h). To a CH\(_2\)Cl\(_2\) solution (3 mL) of 3,4,5-tris(benzyloxy)benzoic acid (286 mg, 0.65 mmol), (S)-(+-)2-chlorophenylglycine methyl ester...
hydrochloride (130 mg, 0.65 mmol), EDCI (137 mg, 0.71 mmol), HOBT (50 mg, 0.33 mmol) and TEA (0.34 mL, 1.9 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO\textsubscript{4} and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to generate 30 mg of 6h (yield 8%). \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta 7.46–7.26 (m, 18H), 7.13 (d, J = 6.9 Hz, 1H), 7.10 (s, 2H), 6.01 (d, J = 7.0 Hz, 1H), 5.10 (d, J = 6.6 Hz, 4H), 5.09 (s, 2H), 3.77 (d, J = 8.4 Hz, 3H); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}) \(\delta 142.80, 138.02, 124.72, 113.56, 109.35, 108.60, 106.68, 105.53, 102.65, 102.19, 101.79, 100.75, 100.56, 100.54, 100.18, 100.00, 99.94, 99.53, 99.30, 79.13, 47.12, 43.37, 27.29, 25.14; HRMS (EI) m/z calcd. for 621.1918; found 621.1917.

Methyl 2-(4-fluorophenyl)-2-(3,4,5-tris(benzyloxy)benzamido)acetate (6i). To a CH\textsubscript{2}Cl\textsubscript{2} solution (3 mL) of 3,4,5-tris(benzyloxy)benzoic acid (390 mg, 0.88 mmol), methyl amino(4-fluorophenyl)acetate hydrochloride (240 mg, 1.09 mmol), EDCI (310 mg, 1.62 mmol), HOBT (70 mg, 0.52 mmol) and TEA (0.57 mL, 3.2 mmol) were added. After stirring overnight, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over MgSO\textsubscript{4} and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/n-hexane = 1:3) to generate 320 mg of 6i (yield 48%). \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta 7.46–7.29 (m, 15H), 7.29–7.27 (m, 2H), 7.13–7.08 (m, 2H), 7.08–6.99 (m, 2H), 6.99–6.91 (m, 1H), 5.69 (d, J = 6.7 Hz, 1H), 5.16–5.12 (m, 4H), 5.10 (s, 2H), 3.79 (s, 3H); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}) \(\delta 171.37, 166.04, 163.59, 161.95, 152.82, 141.73, 137.35, 136.62, 132.46, 129.12, 129.06, 128.73, 128.59, 128.23, 128.08, 128.01, 127.60, 116.06, 115.92, 107.20, 75.18, 71.52, 56.20, 53.07; HRMS (EI) m/z calcd. for 605.2214; found 605.2217.

4.3. Determination of the Mast Cell Degranulation

4.3.1. Histamine Assay Use PMACl in HMC-1 Cells

To determine the mast cell degranulation, level of histamine in culture media was measured. HMC-1 cells were grown in Iscove’s modified Dulbecco’s medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin G, 100 mg/mL streptomycin, and 250 ng/mL amphotericin B at 37 °C in 5% CO\textsubscript{2} incubator. The passage ranging 4–8 of HMC-1 cells (5 \(\times\) 10\textsuperscript{5}/well in 24-well plates) were pretreated with or without drugs for 1 h and then stimulated with 4 nM of phorbol 12-mystate 13-acetate (PMA, Sigma-Aldrich, St Louis, MO, USA) and 1 \(\mu\)M of calcium ionophore A23187 (CI, Sigma-Aldrich) for 8 h. The cells were separated from the media by centrifugation at 150 g for 5 min at 4 °C. To separate histamine from serum and media, 0.1 N HCl and 60% perchloric acid were added. After centrifugation, the supernatant fluid transferred to eppendorf tube containing 5 N NaOH, 5 M NaCl, and n-butanol and then vortexed. The organic phase was gathered, shaken with 0.1 N HCl and n-haptane, and then centrifuged. The histamine in the aqueous phase is assayed using the o-phthaldialdehyde spectrofluorometric procedure as previously described [6].

4.3.2. Histamine Assay Use DNP-Human Serum Albumin in RBL-2H3 Cells

To evaluate mast cell degranulation, the level of histamine in culture media was measured. RBL-2H3 cells (5 \(\times\) 10\textsuperscript{5}/well in 24-well plates) were sensitized with anti-DNP IgE (50 ng/mL). After incubation overnight, cells were pretreated with or without compound for 1 h and then stimulated with DNP-HAS (100 ng/mL) for 4 h. The cells were separated from the media by centrifugation at 150 g for 5 min at 4 °C. To separate histamine from serum and media, 0.1 N HCl and 60% perchloric acid were added. After centrifugation, the supernatant fluid was transferred to an Eppendorf tube containing 5 N NaOH, 5 M NaCl, and n-butanol, then vortexed. The organic phase was gathered, shaken with 0.1 N HCl and n-haptane, then centrifuged. The histamine in the aqueous phase was assayed using the o-phthaldialdehyde spectrofluorometric procedure as previously described [6].
4.4. RNA Extraction and Real-Time PCR

Prior to isolation of total cellular RNA, HMC-1 cells (5 × 10^5/well in 24-well plates) were pretreated with or without compound for 1 h and then stimulated with PMA (40 nM) and CI (1 µM) for 1 h. RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan) was used to extract total RNA, in accordance with the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized from 2 µg of total RNA using the Maxime RT-Pre Mix Kit (iNtRON Biotechnology, Daejeon, Korea). Quantitative real-time PCR was carried out using the Thermal Cycler Dice TP850 (Takara Bio Inc.) according to the manufacturer’s protocol. The 25 µL reaction mixture was composed as follows: 1.5 µL of cDNA (150 ng), 1 µL of each of the forward and reverse primers (0.4 µM), 12.5 µL of SYBR Premix Ex Taq (Takara Bio Inc.), and 9 µL of D_2O [15].

4.5. Statistical Analysis

Each data represents the mean ± SE of 3 independent experiments. Statistical analyses were performed using Prism5 (GraphPad Software, San Diego, CA, USA), and treatment effects were analyzed using a one-way ANOVA followed by Dunnett’s test. A value of p < 0.05 was used to indicate a significant difference.

Supplementary Materials: The following are available online, Figures S1–S52: Spectral data.

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References


**Sample Availability:** Samples of the compounds are available from the authors.

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