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Morroniside cinnamic acid conjugate as an anti-inflammatory agent

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ABSTRACT

A morroniside cinnamic acid conjugate was prepared and evaluated on E-selectin mediated cell–cell adhesion as an important role in inflammatory processes. 7-*O*-Cinnamoylmorroniside exhibited excellent anti-inflammatory activity (IC_{50} = 49.3 μM) by inhibiting the expression of E-selectin; further, it was more active than another cinnamic-acid-conjugated iridoid glycoside (harpagoside; IC_{50} = 88.2 μM), 7-*O*-methylmorroniside, and morroniside itself. As a result, 7-*O*-cinnamoylmorroniside was observed to be a potent inhibitor of TNF-α-induced E-selectin expression.

Inflammations are associated with the extravasation of leukocytes from blood into tissue. Selectins are carbohydrate-binding type-1 membrane glycoproteins that act as adhesion molecules involved in the development of different inflammatory reactions. E-selectin is particularly noteworthy in the case of inflammatory diseases owing to its expression in activated endothelium and bone–skin microvascular linings, and to its role in cell rolling, cell signaling, and chemotaxis.[1] E-selectin mediates cell tethering and rolling interactions through the recognition of sialofucosylated Lewis carbohydrates expressed on structurally diverse protein–lipid ligands on circulating leukocytes; this phenomenon serves as an important trigger in inflammatory response.[2] Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), secreted by macrophages, induce expression of E-selectin in human endothelial cells and enhance vascular inflammation.[3] Therefore, compounds that exhibit the ability to regulate expression of E-selectin in endothelial cells may be useful as therapeutic agents for the treatment of inflammatory diseases.

Harpagoside, which is a phenylpropanoid-conjugated iridoid glycoside, is known to be a natural anti-inflammatory agent.[4] This compound is a major constituent of *Harpagophytum procumbens* (Devil's Claw), and was found to be one of the active agents in the extract, inhibiting the production of COX-2,[5–7] IL-1β, IL-6, and TNF-α by RAW 264.7 cells.[8] The structural characteristic of this compound is that cinnamic acid attached to iridoid moiety (Fig. 1). This

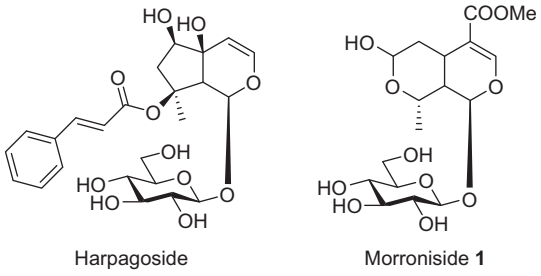

Figure 1. Chemical structures of harpagoside and morroniside.

structure is considered to be not only chemically interesting, but also medicinally important.

Morroniside (**1**) (Fig. 1), which is one of the iridoid glycoside, has been isolated from several plant families (for e.g., Cornaceae,[9,10] Caprifoliaceae,[11,12] Sarraceniaceae,[13] and Hydrangeaceae[14]); this compound is reported to exhibit antioxidative, α-glucosidase-inhibitory, and anti-inflammatory activities.[15–17] The use of such natural products as starting materials for the synthesis of biologically active compounds has received considerable attention. Recently, we reported a highly chemoselective etherification of unprotected **1**, catalyzed by molecular iodine. A series of alkyl ether derivatives were obtained and tested for their cytotoxic activity in a colon 26-L5 cell line. Among the tested compounds, 7-*O*-dodecylmorroniside showed moderated cytotoxic activity, having IC_{50} values of 20.9 μM; this value was higher than that of morroniside **1**.[18] These results motivated us to investigate synthetic approaches for deriving

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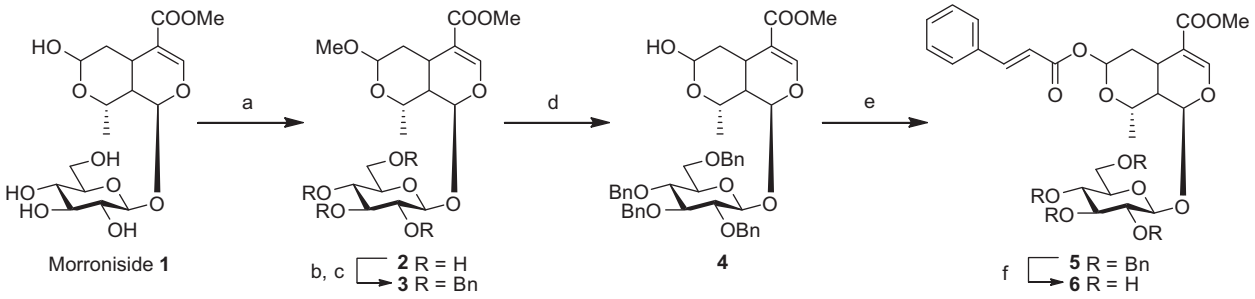

Scheme 1. Synthesis of morroniside cinnamic acid conjugate **6**. Reagents and conditions: (a) iodine, MeOH, acetone, rt, 15 min, 84%; (b) BnBr, NaH, DMF, 0 °C → rt, 1 h; (c) MeI, K_2CO_3, DMF, 1 h, 99% in two steps; (d) concd HCl, AcOH, H_2O, −10 °C → rt, 6 h, 44%; (e) cinnamoyl chloride, Et_3N, DMAP, THF, 60 °C, 5 h, 79%; (f) DDQ, 1,2-dichloroethane, H_2O, rt, 4 days, 64%.

bio-active compounds from **1**. We now report both the preparation and biological activity of such derivatives.

Morroniside (**1**) was isolated from the methanolic root extract of *Strychnos cocculoides* (Loganiaceae) by repeated silica gel vacuum liquid chromatography eluting with dichloromethane/methanol (80:20).[19] The ^{1}H NMR spectrum of this compound revealed it to be a mixture of anomeric forms.[12,20]

We developed a five-step synthesis route for obtaining a morroniside cinnamic acid conjugate **6**. As a key step, we adapted chemoselective etherification of unprotected **1** using molecular iodine.[18] In other words, the treatment of **1** in acetone with methanol in the presence of molecular iodine at room temperature yielded 7-*O*-methylmorroniside (**2**)[18]; this compound was used as a key intermediate for the subsequent reactions. The hydroxy group of the glucoside of **2** was benzyl-protected. On the process of benzylation, methyl ester of 7-*O*-methylmorroniside (**2**) was deprotected. Therefore, methyl esterification by iodomethane was carried out to obtain 2′,3′,4′,6′-tetra-*O*-benzyl-7-*O*-methylmorroniside (**3**) in two steps.[21] The hydroxy group at C-7 in 2′,3′,4′,6′-tetra-*O*-benzylmorroniside (**4**) was obtained by the reaction of **3** with concentrated hydrochloric acid in acetic acid/water.[22] Conjugation of cinnamic acid to **4** was achieved by reaction of **4** in THF with cinnamoyl chloride in the presence of DMAP.[23] For deprotection, we first attempted to perform debenzylation by employing Hanessian's method,[24] Lewis acid,[25] and catalytic hydrogenation (using Pt–C[26] and Lindlar's catalyst[27]). However, the desired compound **6** was not obtained as there was no reaction or decomposition. Finally, 2′,3′,4′,6′-tetra-*O*-benzyl-7-*O*-cinnamoylmorroniside (**5**) was debenzylated using DDQ[28] in 1,2-dichloroethane/water mixture to yield the corresponding 7-*O*-cinnamoylmorroniside (**6**) as a morroniside cinnamic acid conjugate (Scheme 1).[29]

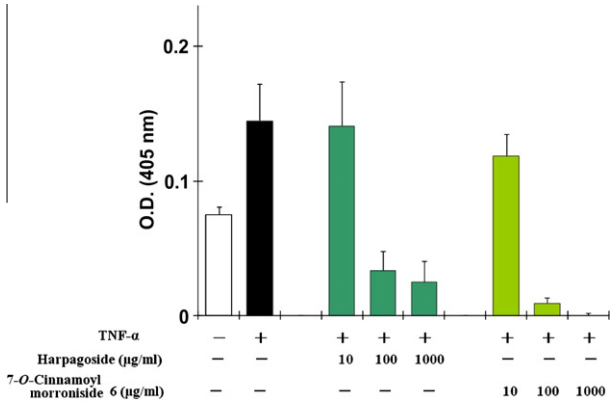

Figure 2. Inhibitory effects of tested compounds on TNF-α-induced E-selectin expression in HUVECs. HUVECs were incubated with 10 ng/ml of TNF-α in the presence and in the absence of the test specimens for 2 h. The expression level of E-selectin in HUVECs was measured by an ELISA. (Mean ± SEM, $n = 3$.)

In order to examine the anti-inflammatory effects of the tested compounds (harpagoside, **1**, **2**, and **6**), human umbilical vein endothelial cells (HUVECs) were stimulated with TNF-α in the presence and absence of these compounds, and the expression level of E-selectin was measured by an enzyme-linked immunosorbent assay (ELISA).[30] E-selectin was induced by stimulation with TNF-α in HUVECs. The induction was inhibited by addition of tested compounds (harpagoside and **6**). However, **1** and **2** did not inhibit the expression of E-selectin by the stimulation with TNF-α. Harpagoside selected as a reference anti-inflammatory agent showed IC_{50} values of 88.2 μM; in contrast, **6**, found to be a potent inhibitor of TNF-α-induced E-selectin expression, showed IC_{50} values of 49.3 μM. To determine if the treatment of HUVECs with these compounds induced cell death and influenced TNF-α-induced E-selectin expression, we investigated the effects of harpagoside, **1**, **2**, and **6** on the viability of HUVECs using a lactose dehydrogenase (LDH) assay. The viability of the cells was not affected by treatment with these compounds (data not shown).

These results indicated that harpagoside and **6** exhibited anti-inflammatory activity by inhibiting the expression of E-selectin. In addition, **6** is the best anti-inflammatory agent in comparison with the others (Fig. 2).

It is well known that iridoid glycosides exhibit anti-inflammatory activity, but the mechanism by which they exhibit this activity remains unclear.[31] In this study, our data shows that unconjugated iridoid glycosides (morroniside (**1**) and 7-*O*-methylmorroniside (**2**)) do not inhibit the expression of E-selectin in endothelial cells. However, cinnamic-acid-conjugated iridoid glycosides (harpagoside and 7-*O*-cinnamoylmorroniside (**6**)) exhibit anti-inflammatory activity by inhibiting the expression of E-selectin by stimulation with TNF-α. This fact suggests that the cinnamoyl moiety in iridoid glycoside derivatives contributes to the anti-inflammatory activity in such compounds. As **6** is the most potent among the tested compounds, it may be useful as a therapeutic agent for the regulation of vascular inflammation.

In conclusion, we have shown that **6** indicates its impressive activity. It is anticipated that further structural modifications would result in products that will enhance anti-inflammatory activity. These investigations on chemical modification using morroniside **1** are ongoing. The results of these investigations will be reported in the near future.

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21. *Procedure for synthesis of 2′,3′,4′,6′-tetra-O-benzyl-7-O-methylmorroniside* (**3**)*:* A solution of **2** (195.6 mg, 0.465 mmol) in anhydrous DMF (5.0 ml) containing benzylbromide (440.9 μl, 3.722 mmol) and sodium hydride (60% oil suspension, 123.0 mg, 2.05 mmol) was stirred under N_2 on ice for few minutes, and then at room temperature for 1 h. Methanol (10 ml) was added to this mixture and the reaction mixture was stirred for 10 min. The resultant solution was partitioned with EtOAc, washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated in vacuo to obtain a residue; this residue was then dissolved in anhydrous DMF (5.0 ml) containing methyl iodide (86.9 μl, 1.39 mmol) and potassium carbonate (191.9 mg, 1.39 mmol), and was stirred for 1 h. This resultant reaction mixture was thereafter partitioned with EtOAc, washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated in vacuo to obtain another residue; this residue was purified by silica gel column chromatography (EtOAc/*n*-hexane = 1:4–1:2) to afford **3** (360.0 mg, 99%) as a white amorphous solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.36 (3H, d, J = 6.9 Hz, H-10β), 1.43 (3H, d, J = 6.9 Hz, H-10α), 1.70–1.74 (1H, m, H-9β), 1.76–1.79 (1H, m, H-9α), 1.92–1.96 (2H, m, H-6β), 2.04–2.07 (2H, m, H-6α), 2.82 (1H, dt, J = 4.5, 13.0 Hz, H-5α), 3.07–3.12 1lH, m, H-5β), 3.47–3.77 (4H, m, H-2′, H-3′, H-4′, and H-5′), 3.36 (3H, s, 7-OMe), 3.72 (3H, s, $COOCH_3$), 3.74–3.75 (1H, m, H-8α), 3.78–3.80 (1H, m, H-8β), 4.26 (1H, dd, J = 3.0, 7.5 Hz, H-6′α), 4.42–4.45 (1H, m, H-6′β), 4.61–4.93 (8H, m, Ph-CH_2), 4.85 (1H, d, J = 8.2 Hz, H-1′), 4.95 (1H, d, J = 4.0 Hz, H-7), 5.84 (1H, d, J = 8.6 Hz, H-1), 7.20–7.36 (20H, m, Ar of Bn), 7.48 (3H, s, H-3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 19.3 (C-10), 26.6 (C-5β), 29.8 (C-6β), 30.4 (C-5α), 32.7 (C-6α), 39.8 (C-9), 51.4 ($COOCH_3$), 54.7 (7-OMe), 68.9 (C-6′), 72.6 (C-8), 75.7 (C-2′), 73.6, 75.1, 75.2, and 75.8, (4C, Ph-CH_2), 77.9 (C-4′), 82.2 (C-5′), 84.7 (C-3′), 94.8 (C-7), 98.0 (C-1), 99.3 (C-1′), 111.2 (C-4), 127.6–129.0 (20C, Ar of Bn), 138.2–138.7 (4C, Ar of Bn), 153.1 (C-3), 167.1 (C═O); $[\alpha]_D^{26}$ −40.1 (*c* 1.0, $CHCl_3$); FABMS *m*/*z* 804 $[M+H]^+$.
22. *Procedure for synthesis of 2′,3′,4′,6′-tetra-O-benzylmorroniside* (**4**)*:* A solution of **3** (116.5 mg, 0.149 mmol) in AcOH (2.5 ml) and H_2O (0.15 ml) was treated with concd HCl (20 μl) at −10 °C, and stirred for 6 h at room temperature. The resultant solution was partitioned with EtOAc, washed with saturated $NaHCO_3$ aq, dried over anhydrous Na_2SO_4, and concentrated in vacuo to obtain a residue; this residue was purified by silica gel column chromatography (EtOAc/*n*-hexane = 1:3–1:1) to afford **4** (50.0 mg, 44%) as a white amorphous solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.34 (3H, d, J = 6.9 Hz, H-10β), 1.42 (3H, d, J = 6.3 Hz, H-10α), 1.69–1.72 (1H, m, H-9β), 1.77–1.81 (1H, m, H-9α), 1.93–1.97 (2H, m, H-6β), 2.11–2.14 (2H, m, H-6α), 2.81 (1H, dt, J = 4.6, 12.6 Hz, H-5α), 3.16 (1H, dt, J = 4.9, 13.6 Hz, H-5β), 3.45–3.71 (4H, m, H-2′, H-3′, H-4′, and H-5′), 3.73 (3H, s, $COOCH_3$), 3.76–3.78 (1H, m, H-8α), 3.79–3.80 (1H, m, H-8β), 3.87–3.89 (1H, m, H-6′α), 4.52–4.54 (1H, m, H-6′β), 4.56–4.94 (8H, m, Ph-CH_2), 4.84 (1H, d, J = 8.6 Hz, H-1′), 4.95 (1H, d, J = 2.9 Hz, H-7), 5.82 (1H, d, J = 8.6 Hz, H-1), 7.21–7.35 (20H, m, Ar of Bn), 7.48 (3H, s, H-3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 19.3 (C-10), 26.6 (C-5β), 29.8 (C-6β), 30.4 (C-5α), 32.7 (C-6α), 39.8 (C-9), 51.5 ($COOCH_3$), 68.8 (C-6′), 72.9 (C-8), 75.7 (C-2′), 73.7, 75.1, 75.2, and 75.8, (4C, Ph-CH_2), 77.8 (C-4′), 82.1 (C-5′), 84.7 (C-3′), 95.1 (C-7), 96.1 (C-1), 99.3 (C-1′), 111.1 (C-4), 127.6–128.5 (20C, Ar of Bn), 138.2–138.7 (4C, Ar of Bn), 153.2 (C-3), 167.0 (C═O); $[\alpha]_D^{26}$ −36.0 (*c* 1.0, $CHCl_3$); FABMS *m*/*z* 767 $[M+H]^+$.
23. *Procedure for synthesis of 2′,3′,4′,6′-tetra-O-benzyl-7-O-cinnamoylmorroniside* (**5**)*:* A solution of **4** (50.0 mg, 0.0652 mmol) in anhydrous THF (2.0 ml) containing cinnamoylchloride (26.2 mg, 0.157 mmol), triethylamine (27.3 μl, 0.196 mmol), and *N*,*N*-dimethylaminopyridine (8.0 mg, 0.0655 mmol) stirred at 60 °C for 5 h. The resultant solution was concentrated in vacuo to obtain a residue; this residue was purified by silica gel column chromatography (EtOAc/*n*-hexane = 1:4–1:3) to afford **5** (58.5 mg, 79%) as a white amorphous solid. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 1.37 (3H, d, J = 6.8 Hz, H-10β), 1.46 (3H, d, J = 6.9 Hz, H-10α), 1.78–1.81 (1H, m, H-9β), 1.85–1.88 (1H, m, H-9α), 2.04–2.08 (2H, m, H-6β), 2.17–2.20 (2H, m, H-6α), 2.94 (1H, dt, J = 4.8, 12.8 Hz, H-5α), 3.20 (1H, dt, J = 4.8, 13.2 Hz, H-5β), 3.46–3.69 (4H, m, H-2′, H-3′, H-4′, and H-5′), 3.71–3.72 (1H, m, H-8α), 3.74 (3H, s, $COOCH_3$), 3.77–3.81 (1H, m, H-8β), 4.05 (1H, dd, J = 2.1, 9.0 Hz, H-6′α), 4.41 (1H, dd, J = 2.7, 7.5 Hz, H-6′β), 4.58–4.97 (8H, m, Ph-CH_2), 4.85 (1H, d, J = 8.1 Hz, H-1′), 5.85 (1H, d, J = 8.9 Hz, H-1α), 5.90 (1H, d, J = 8.9 Hz, H-1β), 5.93 (1H, d, J = 7.6 Hz, H-7), 6.31 (1H, d, J = 15.5 Hz, H-8″), 7.16–7.43 (25H, m, Ar), 7.52 (3H, s, H-3), 7.68 (1H, t, J = 15.5 Hz, H-7″); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 19.3 (C-10), 26.6 (C-5β), 29.8 (C-6β), 30.4 (C-5α), 32.7 (C-6α), 39.8 (C-9), 51.5 ($COOCH_3$), 68.8 (C-6′), 72.9 (C-8), 75.7 (C-2′), 73.7, 75.1, 75.2, and 75.8, (4C, Ph-CH_2), 77.8 (C-4′), 82.1 (C-5′), 84.7 (C-3′), 95.1 (C-7), 96.1 (C-1), 99.3 (C-1′), 111.1 (C-4), 112.5 (C-8″), 127.6–128.5 (24C, Ar of Bn, C-2″, C-3″, C-5″, and C-6″), 131.6 (C-4″), 136.0 (C-1″), 138.2–138.7 (4C, Ar of Bn), 140.4 (C-7″), 153.2 (C-3), 165.3 (C-9″), 167.0 (C═O); FABMS *m*/*z* 880 $[M+H-H_2O]^+$.
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29. *Procedure for synthesis of 7-O-cinnamoylmorroniside* (**6**)*:* A solution of **5** (5.0 mg, 0.00574 mmol) in 1.65 ml of 1,2-dichloroethane and H_2O = (10:1) containing 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (25.2 mg, 0.111 mmol) was stirred at room temperature for 4 days. The resultant solution was partitioned with EtOAc, washed with saturated $NaHCO_3$ aq, $NaHSO_3$ aq, dried over anhydrous Na_2SO_4, and concentrated in vacuo to obtain a residue; this residue was purified by silica gel column chromatography (EtOAc/*n*-hexane = 1:3–1:1 → CH_2Cl_2/MeOH = 10:1–10:2) to afford **6** (1.9 mg, 64%) as a white amorphous solid. ^{1}H NMR (CD_3OD, 600 MHz): δ 1.30 (3H, d, J = 6.9 Hz, H-10β), 1.36 (3H, d, J = 6.9 Hz, H-10α), 1.68–1.72 (1H, m, H-9β), 1.74–1.78 (1H, m, H-9α), 1.87–1.91 (2H, m, H-6β), 2.01–2.04 (2H, m, H-6α), 2.82 (1H, dt, J = 4.1, 12.4 Hz, H-5α), 3.18 (1H, t, J = 8.6 Hz, H-5β), 3.26–3.49 (4H, m, H-2′, H-3′, H-4′, and H-5′), 3.72 (3H, s, $COOCH_3$), 3.86 (1H, dd, J = 2.0, 6.3 Hz, H-8α), 3.94 (1H, dd, J = 2.0, 6.3 Hz, H-8β), 4.09 (1H, dd, J = 6.9, 14.3 Hz, H-6′α), 4.52–4.56 (1H, m, H-6′β), 4.82 (1H, d, J = 8.2 Hz, H-1′), 5.82 (1H, d, J = 9.2 Hz, H-1α), 5.87 (1H, d, J = 8.9 Hz, H-1β), 5.96 (1H, d, J = 12.4 Hz, H-7), 6.36 (1H, d, J = 14.9 Hz, H-8″), 7.24–7.31 (3H, m, H-3″, H-4″, H-5″), 7.37 (d, J = 6.9 Hz, H-2″, H-6″), 7.51 (3H, s, H-3), 7.60 (1H, t, J = 14.9 Hz, H-7″); ^{13}C NMR (CD_3OD, 150 MHz): δ 19.9 (C-10), 27.5 (C-5β), 32.0 (C-5α), 34.5 (C-6β), 37.2 (C-6α), 40.2 (C-9α), 41.0 (C-9β), 51.8 ($COOCH_3$), 62.8 (C-6′), 66.0 (C-8β), 71.7 (C-2′), 74.0 (C-4′), 75.6 (C-8α), 77.6 (C-5′), 78.4 (C-3′), 96.0 (C-7), 97.1 (C-1), 100.1 (C-1′), 111.4 (C-4), 112.3 (C-8″), 128.5 (C-2″, C-6″), 129.2 (C-3″, C-5″), 131.4 (C-4″), 135.7 (C-1″), 140.1 (C-7″), 154.4 (C-3), 165.4 (C-9″), 168.6 (C═O); $[\alpha]_D^{26}$ −11.2 (*c* 1.0, MeOH); FABMS *m*/*z* 519 $[M+H-H_2O]^+$.
30. *ELISA analysis:* For quantification of E-selectin protein expression, an ELISA-based method was used. 4×10^4 cells of HUVEC were seeded into a 96-well microtiter plate-and grown with EBM-2 overnight. Medium was replaced to DMEM before the cells were pretreated with 10 ng/ml of TNF-α. Two hours after the addition of TNF-α, the medium was removed and the cell layer was washed three times with ice-cold PBS/1% BSA and fixed with 4% paraformaldehyde phosphate buffer. After being washed with PBS five times, the cells were incubated with PBS/1% BSA for 60 min. After being washed with PBS three times, the cells were incubated with an anti-E-selectin antibody at 4 °C overnight (1:1000 in PBS/1% BSA). After being washed with PBS three times, the AP-conjugated secondary antibody (1:500 in PBS/1% BSA) was added for 120 min. After the addition of *p*-nitrophenyl phosphate (*p*NPP) solution, the enzymatic reaction was allowed to proceed for 60 min at room temperature; the optical density (OD: 405 nm) was measured using the ELISA plate reader. At least three independent experiments (each performed in triplicate) were used for the statistical interpretation of the data (means ± SEM).
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