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Design, Synthesis, and Immunosuppressive Activity of New Deoxybenzoin Derivatives

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In the search for potential immunosuppressive agents with high efficacy and low toxicity, a series of new deoxybenzoins were synthesized and evaluated for their cytotoxicity and immunosuppressive activity. Among the synthesized compounds, four deoxybenzoin oximes (compounds **31**, **32**, **37**, and **38**) exhibited lower cytotoxicity and higher inhibitory activity toward anti-CD3/anti-CD28 co-stimulated T-cell proliferation than other compounds. More significantly, compound **31** is >100-fold less cytotoxic than cyclosporine A (CsA) and is also more potent (**31**: SI > 684.64, CsA: SI = 235.44). The preliminary inhibition mechanism of compound **31** was also identified by flow cytometry, and this compound exerts immunosuppressive activity by inducing apoptosis in activated lymph node cells in a dose-dependent manner. In addition, the mechanism of apoptosis was detected by western blot analysis.

Introduction

Immunosuppressants are an important class of clinical drugs for an array of medical processes, including transplant rejection and treatment of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and psoriasis.[1,2] The knowledge that T-lymphocytes play an integral role in transplant rejection brought cyclosporine A (CsA), tacrolimus (FK506), and sirolimus (rapamycin) to the fore as therapeutic immunosuppressants.[3] Although immunosuppressive drugs have been successfully used for organ transplantation and treatment of autoimmune diseases in the clinic, their side effects cannot be neglected.[4–9] Therefore, there is a clinical need for new therapeutic agents capable of modulating immune responses with high efficacy and low toxicity.

As the most abundant natural products, isoflavones have demonstrated potent biological effects such as antitumor,[10] anti-inflammatory,[11] estrogenic,[12] and immunosuppressive activities[13] with low toxicity. Deoxybenzoins, intermediates in the synthesis of isoflavones, are also found in several plants and marine plants,[14,15] and their structural similarities with isoflavone led us to be interested in studying their biological activity, similar to that of isoflavone. The hypothesis is supported by the fact that deoxybenzoin derivatives exhibit similar biological effects including antimicrobial,[16] estrogenic,[17] and FabH inhibitory activity.[18]

Among the deoxybenzoins in previous reports, many compounds with the oxime group displayed potent biological activities and low toxicity,[19] some of them have been used as clinical medical agents and some kinds of oximes were reported to show immunosuppressive properties.[20] Schiff bases also exhibited antibacterial,[21] antifungal,[22] and antitumor activity.[23] Based on the previous reports, we focused our interest on new synthetic deoxybenzoins derivatives with an oxime group and Schiff base to explore new immunosuppressive agents with high efficacy and low toxicity.

In this study, 30 novel deoxybenzoin derivatives were synthesized and screened for their immunosuppressive activity. The immunosuppressive activity of the synthesized compounds was evaluated with a T-cell functional assay and their cytotoxicity was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) method. Some of the synthetic compounds exhibited potent immunosuppressive activity. The preliminary mechanism of suppression by the most active compound was further examined by flow cytometry (FCM) and western blot assays.

Results and Discussion

Chemistry

A series of novel deoxybenzoins and their Schiff bases were synthesized by the routes outlined in Scheme 1. Using a method previously described by Wähälä et al.,[15] we synthesized deoxybenzoins **1**–**10** by the method detailed in our previous paper.[24] The synthesis started from phenols and phenylacetic acids. The dihydroxyphenols resorcinol and pyrogallol reacted with variously substituted phenylacetic acids giving excellent yields of deoxybenzoins by the Friedel–Crafts reaction catalyzed by boron trifluoride diethyl etherate. Schiff bases of deoxybenzoins **11**–**20** were then obtained in high yields using *para*-chloroaniline as the amine and titanium tetrachloride as the carbonyl activator. Compounds **11**–**20** were then submit-

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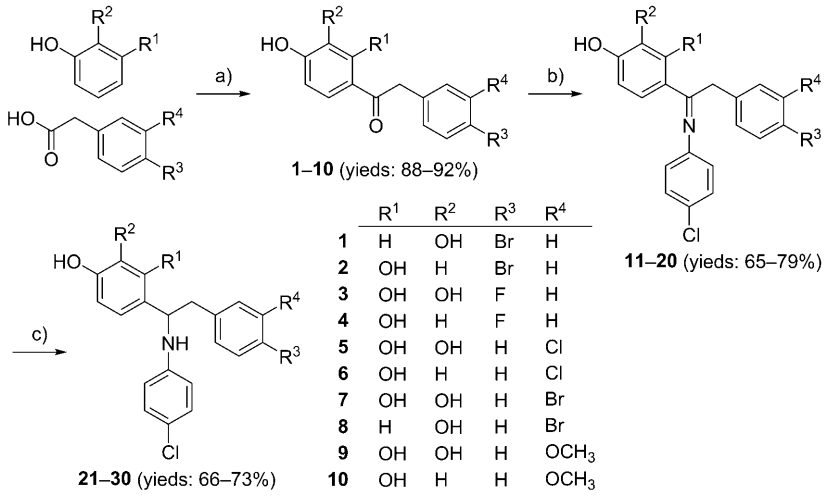


	R^1	R^2	R^3	R^4
1	H	OH	Br	H
2	OH	H	Br	H
3	OH	OH	F	H
4	OH	H	F	H
5	OH	OH	H	Cl
6	OH	H	H	Cl
7	OH	OH	H	Br
8	H	OH	H	Br
9	OH	OH	H	OCH_3
10	OH	H	H	OCH_3

Scheme 1. Synthesis of deoxybenzoin derivatives **1**–**30**: a) $BF_3{\cdot}OEt_2$, 80 °C, 2 h; b) $TiCl_4$, Et_3N, *p*-chloroaniline, THF, 60 °C, 6 h; c) Zn, HCOOH, THF, room temperature, 17 h.

ted to zinc/formic acid (aqueous) reducing conditions,[25] leading to the corresponding amines **21**–**30** with moderate to high overall yields. All new Schiff bases and amines (Table 1) were fully characterized by spectroscopic methods and elemental analysis.

Table 1. Structures of deoxybenzoin derivatives **11**–**40**.[a]

Compd	R^1	R^2	R^3	R^4	Compd	R^1	R^2	R^3	R^4
11	H	OH	Br	H	**26**	OH	H	H	Cl
12	OH	H	Br	H	**27**	OH	OH	H	Br
13	OH	OH	F	H	**28**	H	OH	H	Br
14	OH	H	F	H	**29**	OH	OH	H	OCH_3
15	OH	OH	H	Cl	**30**	OH	H	H	OCH_3
16	OH	H	H	Cl	**31**	H	OH	Br	H
17	OH	OH	H	Br	**32**	OH	H	Br	H
18	H	OH	H	Br	**33**	OH	OH	F	H
19	OH	OH	H	OCH_3	**34**	OH	H	F	H
20	OH	H	H	OCH_3	**35**	OH	OH	H	Cl
21	H	OH	Br	H	**36**	OH	H	H	Cl
22	OH	H	Br	H	**37**	OH	OH	H	Br
23	OH	OH	F	H	**38**	H	OH	H	Br
24	OH	H	F	H	**39**	OH	OH	H	OCH_3
25	OH	OH	H	Cl	**40**	OH	H	H	OCH_3

[a] See Scheme 1 for scaffold structures.

A series of novel oximes were synthesized by the routes outlined in Scheme 2. Our interest in this area was to design and synthesize oximes for SAR studies of immunosuppressive activity. Thus, the treatment of selected deoxybenzoins **1**–**10** with hydroxylamine hydrochloride in ethanol afforded the oximes **31**–**40** with sodium acetate as catalyst and alkaline environment provider. Based on the results of single X-ray determination and ^{1}H NMR spectroscopy, the *E* configuration was determined for these oximes. The crystal structure of compound **31** is shown in Figure 1.

The new synthetic Schiff bases and amines were tested in vitro for their cytotoxicity on lymph node cells and inhibition activity on anti-CD3/anti-CD28 co-stimulated lymph node cells with CsA as the control. The pharmacological results of these compounds are summarized in Table 2. The cytotoxicity of each compound was expressed as the concentration of compound that decreases cell viability to 50% (CC_{50}). The immunosuppressive activity of each compound was expressed as the concentration of compound that inhibits anti-CD3/anti-CD28 co-stimulated T-cell proliferation to 50% (IC_{50}) of the control value. The selectivity index (SI) value was used to evaluate the bioactivity of compounds. In the experiments testing the bioactivity of each compound, results were expressed as the mean ± standard error. Student's *t*-test was used to determine variances between groups where appropriate, although these data are not shown in Table 2.

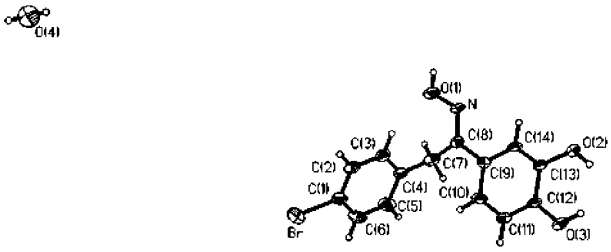
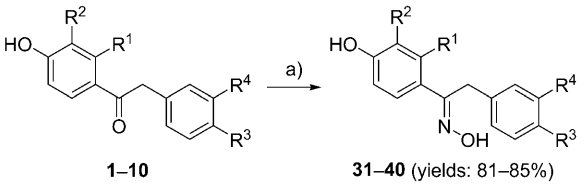


Figure 1. Single crystal structure of compound **31**.

As shown in Table 2, the SI values of the compounds differed greatly, ranging from 2.07 to 90.83. Some of the synthesized compounds (**11**, **18**, **21**, and **28**), especially **11** (SI = 90.83), showed good immunosuppressive activity but much lower than that elicited by CsA (SI = 235.44). The most active compound, **11**, (SI = 90.83) had a pyrocatechol and bromine group, and could be a promising lead for the further development of novel therapeutic agents. To compare the Schiff bases, we also synthesized the corresponding amines **21**–**30**. The activity results shown in Table 2 indicated that they were less effective than the Schiff bases.



Scheme 2. Synthesis of deoxybenzoin oximes **31**–**40**: a) $NH_2OH{\cdot}HCl$, NaOAc, EtOH, 60 °C, 4–4.5 h.

Table 2. In vitro cytotoxicity[a] and inhibitory effects[b] of the synthetic Schiff bases and amines.

Compd[c]	CC_{50} [μM]	IC_{50} [μM]	SI[d]
11	201.6 ± 22.5	2.22 ± 0.17	90.83
12	160.4 ± 12.4	4.91 ± 0.38	32.68
13	122.4 ± 10.3	24.5 ± 2.3	4.98
14	113.7 ± 8.8	38.2 ± 3.7	2.97
15	244.6 ± 21.5	50.1 ± 3.5	4.88
16	127.6 ± 8.6	20.8 ± 1.9	6.13
17	247.8 ± 28.5	5.97 ± 0.44	41.51
18	146.8 ± 13.7	2.03 ± 0.18	72.36
19	133.5 ± 10.3	53.0 ± 4.6	2.52
20	130.7 ± 11.7	59.3 ± 4.7	2.20
21	229.1 ± 20.5	3.01 ± 0.21	76.09
22	157.1 ± 13.8	5.08 ± 0.37	30.92
23	123.6 ± 7.7	30.3 ± 2.8	4.07
24	109.6 ± 6.5	39.1 ± 2.8	2.80
25	253.8 ± 22.4	58.0 ± 4.3	4.38
26	126.5 ± 8.1	35.6 ± 3.5	3.62
27	237.1 ± 19.5	7.97 ± 0.68	29.75
28	160.8 ± 12.5	2.95 ± 0.27	54.52
29	130.1 ± 10.3	58.4 ± 5.3	2.23
30	125.1 ± 7.6	60.3 ± 6.9	2.07
CsA	11.30 ± 1.53	0.05 ± 0.01	235.44

[a] On lymph node cells. [b] On lymph node cells co-stimulated by anti-CD3/anti-CD28. [c] The compounds tested for immunosuppressive activity are consistent with the description in the Experimental Section. [d] Selectivity index: ratio of the compound concentration that decreases cell viability by 50% (CC_{50}) to the compound concentration that inhibits proliferation by 50% (IC_{50}), relative to control values.

Table 3. In vitro cytotoxicity[a] and inhibitory effects[b] of the synthetic oximes.

Compd[c]	CC_{50} [μM]	IC_{50} [μM]	SI[d]
31	> 800	1.16 ± 0.13	> 684.64
32	164.6 ± 18.1	2.21 ± 0.37	74.5
33	147.4 ± 14.7	22.8 ± 3.5	6.47
34	127.5 ± 13.5	35.5 ± 4.3	3.59
35	> 320	41.7 ± 5.7	> 69.41
36	157.4 ± 16.2	21.3 ± 3.1	7.36
37	290.3 ± 26.5	4.16 ± 0.45	69.79
38	155.5 ± 14.7	1.12 ± 0.09	139.12
39	121.1 ± 10.1	40.6 ± 4.8	2.98
40	128.3 ± 11.6	43.7 ± 5.2	2.93
CsA	11.30 ± 1.42	0.05 ± 0.01	235.44

[a] On lymph node cells. [b] On lymph node cells co-stimulated by anti-CD3/anti-CD28. [c] The compounds tested for immunosuppressive activity are consistent with the description in the Experimental Section. [d] Selectivity index: ratio of the compound concentration that decreases cell viability by 50% (CC_{50}) to the compound concentration that inhibits proliferation by 50% (IC_{50}), relative to control values.

As we knew that among the deoxybenzoins, many compounds with oxime group indicated potent biological activities and low toxicity and to find more active deoxybenzoins showing immunosuppressive activity, the oximes **31**–**40** were synthesized. The new synthetic oximes were also tested in vitro for their cytotoxicity on lymph node cells and inhibition activity on anti-CD3/anti-CD28 co-stimulated lymph node cells with CsA as the control. The pharmacological results of these compounds were summarized in Table 3.

Structure–activity relationships (SAR) studies of these compounds were performed by modification of the parent compound to determine how the substituents of the subunits affect the antibacterial activities. In oximes with the same substituents at the R^3 and R^4 positions, the compounds (**31**, **38**) containing pyrocatechol as the subunits exhibited higher inhibition activity than those with resorcinol (**33**, **35**, **37**, **39**) or pyrogallol (**32**, **34**, **36**, **40**) as the substituents. In compounds **32** and **34**, replacement of a bromine atom (compound **32**, SI = 74.5) by a fluoride at the R^3 position on deoxybenzoins resulted in little loss of its inhibition activity (compound **34**, SI = 3.59). Similarly, **36** (SI = 7.36) is not as active as **38** (SI = 139.12). It is also clear that the compounds with a methoxy group (**39**, **40**) showed least activity. Compound **31** (SI > 684.64) showed excellent immunosuppressive activity compared with the other oximes, even compared with CsA (SI = 235.44), and therefore inspired us to continue the following work.

Our work is focused on finding new potential immunosuppressive agents with high efficacy and low toxicity; therefore, the acute oral toxicity of the most potent compound, **31**, was examined. The tested animals appeared normal immediately following administration and did not exhibit any indication of acute toxicity for 14 days afterward. The body weights in treated mice were similar to that of the control mice dosed with an equal volume of vehicle. The result indicated that compound **31** was nontoxic.

Cell apoptosis (apoptosis) is an important biological phenomenon in vivo in multicellular organisms. It can be divided into two continuous processes: commitment and execution. Caspase is considered to be the central executor, whose implementation depends on the apoptotic function of the enzyme activity of substrate proteins. Stimulated by a variety of physiological and pathological factors, caspase activates and degrades its substrate to cause terminal effect incidents by complex multifactor and multichannel activities. This activation can cause biochemical changes of characteristic morphology to complete the process of apoptosis. As a genomic testing and DNA repair enzyme, poly(ADP-ribose) polymerase (PARP) is the first enzyme which was identified as being degraded by caspase-3 and other cysteine proteases and is the most characteristic protease substrate solution.[26] As a representative of these derivatives, compound **31** was subjected to systematic in vitro investigation. We detected the mechanism of compound **31** inhibition effects by FCM (Figure 2), and we found that this compound could induce the apoptosis of activated lymph node cells in a dose-dependent manner.

As shown in Figure 2, lymph node cells stimulated with anti-CD3/anti-CD28 were treated with compound **31** at 3, 6, 12, and 24 μM for 48 h. The compound increased the percentage of apoptosis by Annexin V–FITC/PI staining in a dose-dependent manner. The result indicated that compound **31** induced apoptosis of anti-CD3/anti-CD28 stimulated lymph node cells.

To examine the status of the caspase-3 protein, we performed western blot analysis by using an anti-(cleaved caspase-3) antibody, which recognizes p17 cleaved caspase-3. The

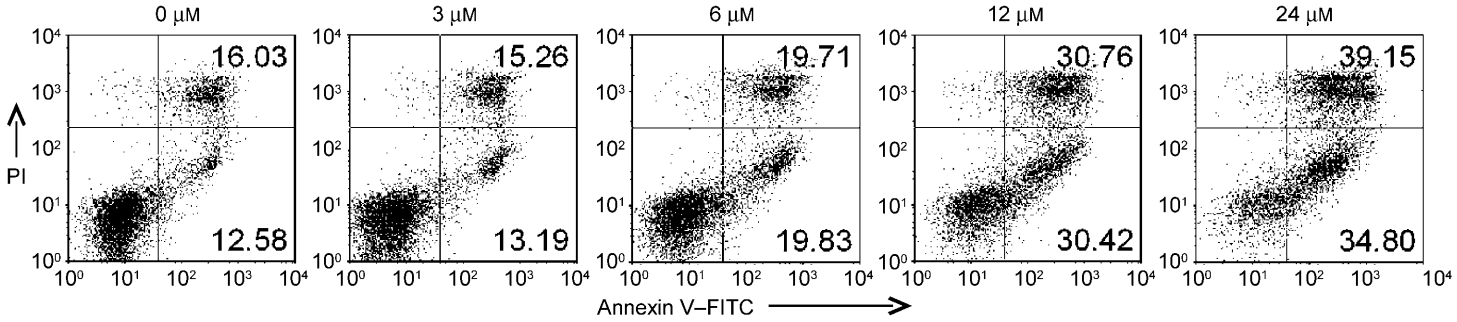


Figure 2. Lymph node cells isolated from naive mice were cultured with anti-CD3/anti-CD28 and various concentrations (0–24 μM, as indicated) of compound **31** for 48 h. Cells were stained by Annexin V–FITC/PI, and apoptosis was analyzed by flow cytometry.

p17 cleavage product appeared in the lysates of lymph node cells treated with compound **31** at 6, 12, and 24 μM. In addition, compound **31** at 12 and 24 μM also cleaved PARP to a band of 89 kDa. Compound **31**, at 6 μM, induced a slight cleavage of PARP (Figure 3). This clearly indicated that compound **31** activates caspase-3 and PARP to promote apoptosis. Based on the above results, we can conclude that the compound can stimulate cells to activate caspase-3. Activated caspase-3 can cleave the corresponding substrate PARP within the nuclei, cause PARP to lose its capacity for effecting DNA repair, and lead to the apoptosis of cells.

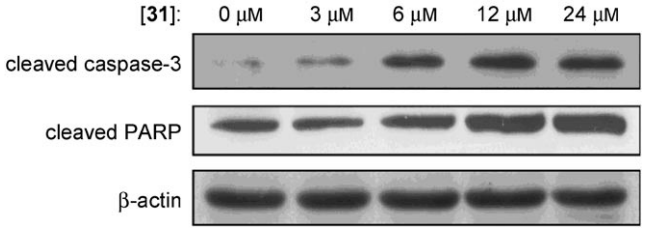


Figure 3. Protein levels of cleaved caspase-3 and cleaved PARP as a function of concentration of compound **31** (indicated) were examined by western blotting. Data are representative of three independent experiments.

Conclusions

A series of new deoxybenzoins were synthesized and their immunosuppressive activity and cytotoxicity were evaluated. Many deoxybenzoin oximes derivatives displayed good immunosuppressive activity. Compound **31** exhibited significant immunosuppressive activity with SI > 684.64, better than the reference compound CsA (SI = 235.44). In general, electron-donating substituents at R^2 and R^3 were favorable for the immunosuppressive activity of the synthesized deoxybenzoins. The cytotoxicity assay, using compound **31**, revealed potent immunosuppressive activity and indicated that this molecule did not exhibit significant cytotoxicity in inactivated mouse lymph node cells. Furthermore, the acute oral toxicity test demonstrated that compound **31** was nontoxic. The immunosuppression process is mediated by the promotion of apoptosis of the lymph node cells, where caspase-3 and PARP are involved. All the results outlined the great potential of these compounds for further exploitation as immunosuppressants.

Experimental Section

Chemistry

All chemicals (reagent grade) used were purchased from Sigma Aldrich (USA) and Sinopharm Chemical Reagent Co. Ltd. (China). TLC was run on silica gel coated aluminum sheets (silica gel 60 GF_{254}, E. Merck, Germany) and visualized by UV light (λ 254 nm). Melting points (uncorrected) were determined on a XT4 MP apparatus (Taike Corp., Beijing, China). ESIMS spectra were recorded on a Mariner System 5304 mass spectrometer, and ^{1}H NMR spectra were recorded on Bruker DPX-300, AV-300, or AV-500 spectrometers at 25 °C with $(CH_3)_4Si$ and solvent signals allotted as internal standards. Chemical shifts (δ) are reported in ppm. Elemental analyses were performed on a CHN-O-Rapid instrument. The purity of all tested compounds was determined by HPLC-DAD obtained on an LC–MS instrument (Agilent 6210 LC-TOF/MS, HPLC Agilent 1200) using the following procedure: compounds were dissolved at a concentration of 0.5 mg mL^{-1} in CH_3OH. Then, 1 μL of the sample was injected into an Agilent Zorbax SB-C_{18} HPLC column (50× 4.6 mm, particle size 1.8 μm) and were subjected to chromatography using a gradient of H_2O/CH_3OH from 80:10 to 0:100 for 15 min at a flow rate of 400 μL min^{-1}, starting the gradient after 10 min. General preparation procedures and the characterization of new compounds **11**–**40** can be found in the Supporting Information.

4-(2-(4-bromophenyl)-1-(4-chlorophenylimino)ethyl)benzene-1,2-diol (11): Deoxybenzoin **1** (1.84 g, 6 mmol) and *p*-chloroaniline (0.76 g, 6 mmol) were dissolved in THF (10 mL), then $TiCl_4$ (1.13 g, 0.006 mol) and Et_3N (1.39 mL, 0.01 mol) were added to the mixture at room temperature over the course of 10 min. The mixture was stirred at 60 °C for 6 h and then poured into an ice bath. The resulting mixture was extracted with EtOAc (3×30 mL), and the organic layer was washed with aqueous $NaHCO_3$ (4 g, 100 mL), dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography on silica gel, using a mixture of EtOAc and petroleum ether (PE). Compound **11** (1.85 g, 4.44 mmol) was obtained as a yellow solid. Yield: 74%; R_f = 0.17 (EtOAc/PE 1:2); mp: 154–155 °C; ^{1}H NMR (300 MHz, [D_6]DMSO), 4.42 (s, 2H), 6.67 (d, J = 8.2 Hz, 1H), 6.92 (dd, J = 8.2, 2.2 Hz, 2H), 7.21–7.36 (m, 4H), 7.89–7.96 (m, 4H), 9.13 (s, 1H), 11.07 (s, 1H); ^{13}C NMR (500 MHz, [D_6]DMSO): δ = 28.8, 113.5, 115.7, 118.1, 119.4, 123.1, 127.4, 130.4, 131.1, 131.6, 133.4, 137.3, 145.2, 146.5, 146.9, 154.6 ppm; ESIMS:

415.00 ([*M*+H]$^+$); Anal. calcd for $C_{20}H_{15}BrClNO_2$: C 57.65, H 3.63, Br 19.18, Cl 8.51, N 3.36%, found: C 57.63, H 3.60, Br 19.15, Cl 8.55, N 3.40%.

4-(2-(4-bromophenyl)-1-(4-chlorophenylamino)ethyl)benzene-1,2-diol (21): The resulting Schiff base **11** (3.75 g, 9 mmol) was dissolved in THF (20 mL), 70% $HCOOH_{(aq)}$ (17 mL) was added, and Zn powder (1.74 g, 26.7 mmol) was added portion-wise over 30 min after cooling the solution with an ice bath. The reaction mixture was stirred for 17–19 h at room temperature, filtered on sand, and washed with EtOAc (3×30 mL). The filtrate was neutralized with a concentrated ammonia solution to pH 8 and then extracted with EtOAc (3×30 mL). The organic phase was washed with H_2O (50 mL), dried over anhydrous Na_2SO_4, and evaporated in vacuo to give a residue that was purified by column chromatography on silica gel using a mixture of EtOAc and PE as the eluent. Compound **21** (2.52 g, 6.03 mmol) was obtained as a yellow solid. Yield: 67%; R_f=0.15 (EtOAc/PE 1:3); mp: 112–113 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO), 1.52 (s, 1H), 4.29 (s, 2H), 6.62 (d, J=8.1 Hz, 1H), 6.82 (dd, J=8.2, 2.1 Hz, 2H), 7.17–7.34 (m, 4H), 7.86–7.94 (m, 4H), 9.08 (s, 1H), 11.13 (s, 1H); ^{13}C NMR (500 MHz, [D$_6$]DMSO): δ= 36.7, 65.5, 112.4, 117.4, 119.2, 121.4, 114.1, 126.6, 127.4, 130.4, 131.1, 132.6, 135.3, 142.1, 145.5, 149.2 ppm; ESIMS: 417.01 ([*M*+H]$^+$); Anal. calcd for $C_{20}H_{17}BrClNO_2$: C 57.37, H 4.09, Br 19.08, Cl 8.47, N 3.35%, found: C 57.33, H 4.05, Br 19.10, Cl 8.49, N 3.38%.

(*E*)-2-(4-bromophenyl)-1-(3,4-dihydroxyphenyl)ethanone oxime (31): A solution of deoxybenzoin **1** (0.28 g, 0.90 mmol) and $NH_2OH{\cdot}HCl$ (0.38 g, 5.4 mmol) in EtOH (18 mL) was held at reflux for 4–4.5 h at 60 °C, and then NaOAc (0.074 g, 0.90 mol) was added. The reaction mixture was allowed to cool to room temperature and evaporated to dryness in vacuo, and the residue was partitioned between H_2O (10 mL) and CH_2Cl_2 (10 mL). The organic extract was washed with H_2O (10 mL), and the aqueous layer was extracted with CH_2Cl_2 (10 mL). The combined organic extracts dried over anhydrous Na_2SO_4 and evaporated in vacuo to give a residue that was purified by column chromatography on silica gel using a mixture of EtOAc and PE as eluent. Compound **31** (0.24 g, 0.73 mmol) was obtained as a yellow crystalline solid. Yield: 81%; R_f=0.13 (EtOAc/PE 1:3); mp: 144–145 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO), 3.99 (s, 2H), 6.67 (d, J=8.2 Hz, 1H), 6.92 (dd, J=8.2, 2.2 Hz, 2H), 7.10(d, J=2.2 Hz, 1H), 7.15 (d, J=8.4 Hz, 1H), 7.44 (d, J=8.4 Hz, 1H), 8.96 (s, 1H), 9.09 (s, 1H), 11.10 (s, 1H); ^{13}C NMR (500 MHz, [D$_6$]DMSO): δ=30.4, 113.5, 115.7, 118.1, 119.4, 127.4, 131.1, 131.6, 137.6, 145.5, 146.9, 154.6 ppm; ESIMS: 321.00([*M*+H]$^+$); Anal. calcd for $C_{14}H_{12}BrNO_3$: C 52.20, H 3.75, Br, 24.80, N 4.35%, found: C 52.11, H 3.70, Br 24.89, N 4.38%.

Biological assays

Animals

Six- to eight-week-old female BALB/c mice were purchased from the Experimental Animal Center of Jiangsu Province (Jiangsu, China). Mice were maintained with free access to pellet food and water in plastic cages at 21±2 °C and kept on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of Nanjing University. All efforts were made to minimize animal's suffering and to minimize the number of animals used.

Cells and reagents

Lymph node cells isolated from BALB/c mice were incubated in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, under a humidified atmosphere of 5.0% CO_2 at 37 °C. Anti-mouse antibodies against CD3 (clone 145-2C11) and CD28 (clone 37.51), were purchased from BD Pharmingen (Becton Dickinson, San Diego, CA, USA). The Annexin V–FITC/PI Kit was purchased from Jingmei Biotech (Nanjing, China). Anti-(cleaved PARP) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-(cleaved caspase-3) antibody and anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell proliferation assay

Cells were incubated in a 96-well plate at a density of 5×105 cells per well and stimulated with anti-CD3/anti-CD28 in the presence of various concentrations of compounds for 72 h. For the proliferation assay, 20 μL MTT (Sigma, 4 mg mL^{-1} in PBS) was added per well 4 h before the end of incubation. After removing the supernatant, 200 μL DMSO was added to dissolve the formazan crystals. The absorbance at λ 540 nm (OD_{540}) was read on an ELISA reader (Tecan, Austria).

Cytotoxicity test

Cells were incubated in a 96-well plate at a density of 5×10^5 cells per well with various concentrations of compounds for 48 h. For the cytotoxicity assay, 20 μL of MTT (Sigma, 4 mg mL^{-1} in PBS) was added per well 4 h before the end of the incubation. MTT formazan production was terminated by replacing the medium with 200 mL DMSO. The absorbance (OD_{540}) was read on an ELISA reader (Tecan, Austria).

Apoptosis assay

Lymph node cells were stimulated with anti-CD3/anti-CD28 in the presence of various concentrations of compounds for 48 h and then stained with both Annexin V–FITC (fluorescein isothiocyanate) and propidium iodide (PI). Samples were then analyzed by a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Western blot analysis

After incubation, cells were washed with PBS and lysed using lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). After centrifugation at 10,000 *g* for 10 min, the protein content of the supernatant was determined by a BCATM protein assay kit (Pierce, Rockford, IL, USA). The protein lysates were separated by 10% SDS-PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat milk for 2 h at room temperature. The blocked membrane was probed with the indicated primary antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase (HRP)-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

Acute oral toxicity of compound 31

Acute oral toxicity studies were performed according to the method of Vasudevan et al.[27] Briefly, mice of either sex, selected using the random sampling technique, were employed in this study. Mice ($n=3$) in each group were fasted for 4 h with free access to water only. Compound **31** (suspended with carboxymethyl cellulose [CMC, 0.5% *w*/*v*]) was administered orally at a doses of 50, 250, and 1000 mg kg^{-1}. Appearance and mortality of test animals were observed for 14 days.

Statistical analysis

All experiments were repeated 3–5 times with a similar outcome. The *P*-values between two experimental groups were tested by a two-tailed Student's *t*-test. The level of significance was set at a *P*-value of 0.05. Where applicable, data are reported as the mean ± SD

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