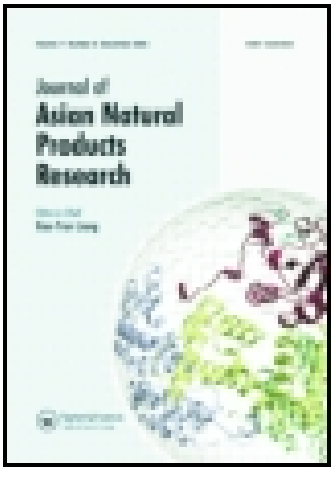



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# Synthesis and biological evaluation of pseudolaric acid B derivatives as potential immunosuppressive agents

Shou-Qiang Chen[ab], Jie Wang[ab], Chuan Zhao[a], Qiang-Wen Sun[ab], Yi-Teng Wang[b], Ting Ai[b], Tan Li[b], Ying Gao[b], Huo Wang[ac]* and Hong Chen[b]*

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Pseudolaric acid B (PB) derivatives with immunosuppressive activity were found by our group. In order to find potential immunosuppressive agents with high efficacy and low toxicity, a series of novel PB derivatives were synthesized and evaluated on their immunosuppressive activities. Most of the synthesized compounds were tested *in vitro* on murine T and B proliferation. In particular, compound **11** exhibited excellent inhibitory activity toward murine T cells (up to 19-fold enhancement compared to that of mycophenolatemofetil) and little cytotoxicity toward normal murine spleen cells. These experimental data demonstrated that some of these PB derivatives have great potential for future immunosuppressive studies.

**Keywords:** pseudolaric acid B derivatives; immunosuppressive activity; cytotoxicity



## 1. Introduction

Immunosuppressant is 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]. The major immunosuppressive drugs in clinical use today are cyclosporin A (CsA), sirolimus (rapamycin), tacrolimus (FK506), and mycophenolatemofetil (MMF) [2]. In clinical, most compounds influence both the innate and adaptive immune system, and interact with pathways of lymphocyte activation and proliferation [3]. It is well known that T-lymphocyte plays an important role in transplant rejection and autoimmune diseases. CsA and FK506 are calcineur inhibitors, which exert their immunosuppressive effects through the $Ca^{2+}$-sensitive T-cell signal transduction pathway, thereby preventing the activation of specific transcription factors involved in lymphokine gene expression [4]. Sirolimus is an inhibitor of the mammalian target of rapamycin while MMF is an antimetabolite.

Although immunosuppressive drugs have been successfully used for organ transplantation and treatment of autoimmune diseases in clinic, their side effects cannot be neglected, such as secondary infection, inducing cancer, kidney toxicity, and liver toxicity [5–7]. Furthermore, chronic rejection is not satisfactorily controlled with current immunosuppressants. Therefore, there is a clinical need for new therapeutic agents capable of modulating immune responses with high efficacy and low toxicity [7–10].

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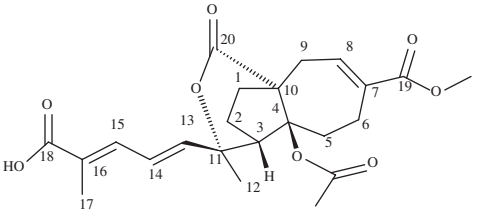
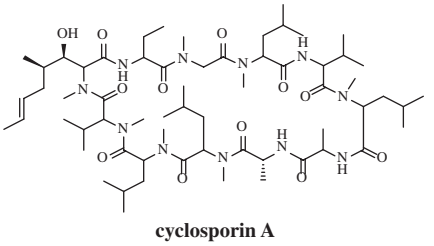
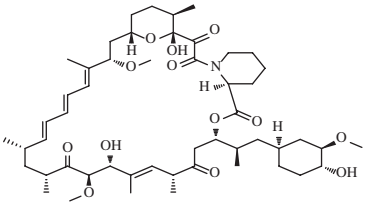
It is known that a majority of drugs in clinical use derive from natural products, including drugs for the treatment of cancer and infections as well as immunosuppressants [11]. Some traditional Chinese medicines have been used for centuries in China to treat various immune system disorders, and represent a valuable resource to find new immunosuppressive agents. In view of the facts mentioned above, we turned our attention to the natural product pseudolaric acid B (PB; shown in Figure 1), which was isolated from the root and trunk bark of *Pseudolarix kaempferi* Gordon (Pinaceae). The barks of this tree, known as "Tu-Jin-Pi" in traditional Chinese herbal medicine, have been used to treat skin diseases caused by fungal infections as early as the seventeenth century [12]. PB has a variety of biological activities, such as anti-fertile, antitumor, and anti-angiogenic properties [13,14]. PB served as the anticancer drug lead and a series of structurally modified PB derivatives showed significant and specific inhibition on HMEC-1 cell migration *in vitro* anticancer tests [15]. The 7, 8-carbon–carbon double bond, 13, 14, 15, 16-conjugated double bonds, 4-acetoxy and 19-methyl ester of PB are the essential groups of PB for its antitumor activity, which has been confirmed by Chen et al. [16,17]. The *in vitro* antifungal activities of structurally diversified analogues of pseudolaric acids tested against the major pathogenic fungus have led to the establishment of a very clear structure–activity relationship of pseudolaric acids derivatives [18–20]. In addition, recent reports



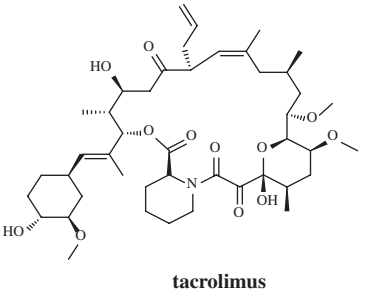
**pseudolaric acid B**
**PB**



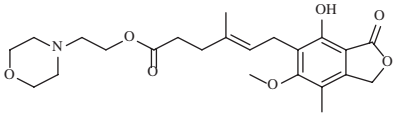
**cyclosporin A**
**CsA**



**sirolimus (rapamycin)**



**tacrolimus**
**FK506**



**mycophenolate mofetil**
**MMF**

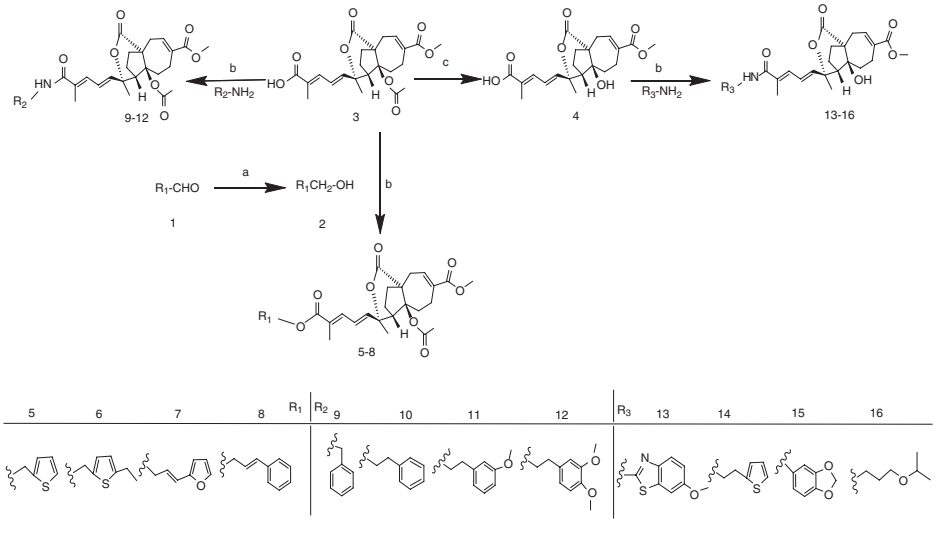
Figure 1. Structures of PB, CsA, sirolimus, FK506, and MMF.

showed that PB can suppress human T lymphocytes activation [21], indicating that it might have immunosuppressive activity. PB contains an unique backbone of a polyhydroazulene with a trans-substitution pattern at the junction sites [22]. In order to investigate PB derivatives for possible application as immunosuppressants, 12 PB derivatives were synthesized and their immunosuppressive activities were evaluated with murine T and B lymphocytesassays, and their cytotoxicities were tested using MTT method. Most of the synthetic compounds exhibited potent immunosuppressive activities.

## 2. Results and discussion

In this study, 12 novel PB derivatives were synthesized to screen for immunosuppressive activity. The route that enabled the syntheses of the PB derivatives **5**–**16** is outlined in Scheme 1. The syntheses of these derivatives utilized three structural derivatizations. The first derivatization was the syntheses of the PB esters compounds. Four kinds of thiophenealdehydes were firstly reduced in anhydrous methanol by $NaBH_4$ at 0°C for 1 h to generate the alcohols. And then in the presence of 1-hydroxy-1H-benzotriazole (HOBt), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and triethylamine ($Et_3N$), the alcohols from the first step were reacted with PB in anhydrous $CH_2Cl_2$ to get the compounds **5**–**8**. The second derivatization was the synthesis of PB amides compounds. In the presence of HOBt, EDCI, and $Et_3N$, a series of different aromatic amines were reacted with PB in anhydrous $CH_2Cl_2$ to get the compounds **9**–**12**. The third derivatization was the synthesis of PB amides in which the acetyl group at the 4th position was hydrolyzed. The selective hydrolysis of the acetyl group was achieved by stirring the methanol solution of PB in the presence of $CH_3ONa$. And then the standard amide coupling conditions were applied to get the compounds **13**–**16**.

The inhibitory activity on ConA-induced murine T and LPS-induced murine B cell proliferation of all the synthesized compounds was tested with MMF as the control. To exclude the possibility that the




Scheme 1. The synthesis route of target compounds. Conditions and reagents: (a) $NaBH_4$, anhydrous $CH_3OH$, 0°C, 84–93% yield; (b) HOBt, EDCI, $Et_3N$, anhydrous $CH_2Cl_2$, rt, 8 h, 85–93% yield; (c) $CH_3ONa$, anhydrous $CH_3OH$, rt, 93–97% yield.

observed inhibitory activity originates from the toxic effects, we evaluated the cytotoxicity of these compounds on murine spleen cells with MTT assay with MMF as the control *in vitro*. The pharmacological results of these compounds are summarized in Table 1. The data showed that most of these compounds were low toxic to normal cells. As can be seen from the immunosuppressive activity of all the synthesized compounds (Table 1), the activities of PB amides are better than PB esters against T and B cells, except for the PB amides in which the 4-acetyl group has been hydrolyzed.

Compounds **5** and **6** containing the thiophene motif showed inferior activities while compounds **7** and **8** showed moderate activities with $IC_{50}$ values of 10.12 and 15.25 $\mu mol\,l^{-1}$ respectively, compared to PB with an $IC_{50}$ value of 10.12 $\mu mol\,l^{-1}$ against T cell. Although the amide derivative **9** showed very low activity ($IC_{50}$ 41.73 $\mu mol\,l^{-1}$) against T cells, amides **10**–**12** exhibit higher inhibitory activities against T cells than PB. For compounds **10** ($IC_{50}$ 13.49 $\mu mol\,l^{-1}$), adding one more carbon between the nitrogen and the benzene ring compared to **9** significantly improved the activity against T cells. Notably, compound **11** displayed the most outstanding immunosuppressive effects *in vitro* with $IC_{50}$ values of 0.35 and 11.12 $\mu mol\,l^{-1}$ against T and B cells, respectively. Its activity against T cells is 19-fold more potent than MMF. This result indicated that the introduction of electron-donating group ($-OCH_3$) on the phenyl ring are beneficial for the activity. Compound **12**, installed with two electron-donating groups on the $R_3$-position and $R_4$-position of phenyl ring showed decreased activity ($IC_{50}$ 3.25 $\mu mol\,l^{-1}$) compared to compound **11**. This result indicated that the position of substituent on benzene ring significantly influenced the activities. Derivatives **13**–**16**, without the acetyl group generally exhibit poor activities against both T and B cells.

Table 1. *In vitro* inhibitory effects and cytotoxicities of novel PB derivatives (**5**–**16**).

| | $IC_{50}$[a] ($10^{-6}$ mol $l^{-1}$) | | |
|---|---|---|---|
| Compounds | T Cell | B Cell | Cytotoxicity |
| **5** | 40.26 | >100 | 36.56 |
| **6** | 53.34 | >100 | >100 |
| **7** | 10.12 | >100 | >100 |
| **8** | 15.25 | 16.15 | >100 |
| **9** | 41.73 | >100 | 13.61 |
| **10** | 13.49 | >100 | 32.07 |
| **11** | 0.35 | 11.12 | >100 |
| **12** | 3.25 | >100 | 22.23 |
| **13** | 6.85 | >100 | >100 |
| **14** | >100 | >100 | >100 |
| **15** | >100 | >100 | 35.56 |
| **16** | >100 | >100 | >100 |
| PB | 10.12 | 40.35 | >10 |
| MMF[b] | 6.71 | 11.83 | 41.00 |

Note: $IC_{50}$: concentration that causes a 50% reduction of cell growth. Values are the mean of two independent experiments.
[a] $IC_{50} > 100$ was defined as inactive.
[b] Mycophenolatemofetil.

For the assays with B cells, all the compounds (**5**–**16**) except **8** ($IC_{50}$ 16.15 $\mu mol\,l^{-1}$) and **11** ($IC_{50}$ 11.12 $\mu mol\,l^{-1}$) showed poor activities. Therefore, it was difficult to get a clear structure–activity relationship on the immunosuppressive activity of B lymphocytes.

In summary, a series of PB derivatives have been synthesized and tested for immunosuppressive activities. We found that the amide derivatives are the most potent species, especially for compound **11**, which has the potential to be developed into an immunosuppressive drug. The further study of the structure–activity relationship is ongoing in our group.

## 3. Experimental

### 3.1 General experimental procedures

Melting points (uncorrected) were determined on a XT4MP apparatus (Taike Corp., Beijing, China). $^1H$ NMR and $^{13}C$ NMR spectra were taken on Bruker Advance 2B/400 (Bruker Technologies, Bremen,

Germany) spectrometer (400 MHz) in $CDCl_3$, using TMS an internal standard (chemical shifts in $\delta$). HR-ESI-MS were recorded using an Agilent-6210LC/TOF-MS spectrometer (Agilent Technologies, Waldbronn, Germany). PB was extracted from the "Tu-Jin-Pi" by our group. All chemicals used were purchased from J&K Chemical (Beijing, China). The reaction was monitored by thin layer chromatography on E. Merck (Darmstadt, Germany) precoated silica GF254 plates.

### 3.2 *Synthesis of pseudolaricesters compounds 5–8*

2-Thiophenecarboxaldehyde (0.224 g, 2.0 mmol) (as shown in Scheme 1) was dissolved with anhydrous $CH_3OH$ (10 ml), and then the mixture was moved into an ice bath at 0°C. $NaBH_4$ (0.152 g, 4.0 mmol) was added to the solution slowly and stirred at 0°C for 1 h. After the reaction was accomplished, some water was added to the mixture, and 5% HCl was added to adjusted pH 7. The resulting mixture was extracted with EtOAc, washed with water and brine, and the organic phase was evaporated under vacuum. The crude product **2** was isolated as a solid (0.179 g, 80% yield). The corresponding alcohols were added to a solution of PB (0.432 g, 1 mmol), HOBt (0.136 g, 1.2 mmol), EDCI (0.229 g, 1.2 mmol) and $Et_3N$ (0.1 ml) in anhydrous $CH_2Cl_2$ (10 ml) at 0°C. The reaction mixture was stirred at room temperature for 10 h, then washed with 5% HCl ($3 \times 15$ ml), saturated $NaHCO_3$ (20 ml), $H_2O$ (20 ml) and dried with anhydrous $Na_2SO_4$. The crude was purified by chromatography on silica gel using petroleum ether/ethyl acetate (2:1) as the eluent and afforded **5–8** (0.376 g, 87% yield as a brown oil).

#### 3.2.1 *Compound* ***5***

White powder; m.p. 142–143°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.32 (1H, d, $J = 5.2$ Hz, H-4′), 7.17–7.21 (2H, m, H-8, 15), 7.11 (1H, d, $J = 3.2$ Hz, H-6′), 6.99 (1H, dd, $J = 5.1$, 3.5 Hz, H-5′), 6.53 (1H, dd, $J = 15.1$, 11.5 Hz, H-14), 5.87 (1H, d, $J = 15.1$ Hz, H-13), 5.35 (2H, s, H-1′), 3.72 (3H, s, $H_3$-19), 3.29 (1H, d, $J = 5.0$ Hz, H-3), 3.07 (1H, dd, $J = 14.3$, 5.6 Hz, H-5a), 2.89 (1H, dd, $J = 15.6$, 6.3 Hz, H-6a), 2.75 (1H, dd, $J = 15.0$, 8.8 Hz, H-9a), 2.58–2.63 (1H, m, H-9b), 2.14–2.18 (1H, m, H-6b), 2.12 (3H, s, $H_3$-OAC), 1.97 (3H, s, $H_3$-17), 1.69–1.89 (5H, m), 1.58 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 172.8 (C-18), 169.3 (C-20), 167.9 (—$\underline{C}OCH_3$), 167.8 (C-19), 143.8 (C-13), 141.6 (C-8), 138.2 (C-2′), 137.2 (C-15), 134.5 (C-7), 128.3 (C-16), 127.9 (C-4′), 126.8 (C-5′), 126.7 (C-6′), 121.7 (C-14), 90.1 (C-4), 83.7 (C-11), 60.8 (C-1′), 55.3 (C-10), 51.9 (—$CO\underline{C}H_3$), 49.3 (C-3), 33.3 (C-1), 30.7 (C-5), 28.5 (C-12), 27.8 (C-9), 24.3 (C-2), 21.7 (—$CO\underline{C}H_3$), 20.2 (C-6), 12.9 (C-17); HR-ESI-MS: *m/z* 551.1703 $[M + Na]^+$ (calcd for $C_{28}H_{32}O_8SNa$, 551.1716).

#### 3.2.2 *Compound* ***6***

White powder; m.p. 139–140°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.16–7.21 (2H, m, H-8, 15), 6.91 (1H, d, $J = 3.4$ Hz, H-5′), 6.65 (1H, d, $J = 3.4$ Hz, H-6′), 6.52 (1H, dd, $J = 15.1$, 11.5 Hz, H-14), 5.86 (1H, d, $J = 15.1$ Hz, H-13), 5.27 (2H, s, H-1′), 3.72 (3H, s, $H_3$-19), 3.29 (1H, d, $J = 5.1$ Hz, H-3), 3.07 (1H, dd, $J = 13.9$, 6.0 Hz, H-5a), 2.72–2.92 (4H, m, H-6a, 9a, 7′), 2.58–2.63 (1H, m, H-9b), 2.14–2.18 (1H, m, H-6b), 2.12 (3H, s, $H_3$-OAc), 1.96 (3H, s, $H_3$-17), 1.80 (5H, m), 1.58 (3H, s, $H_3$-12), 1.30 (3H, t, $J = 7.5$ Hz, H-8′); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 172.8 (C-20), 169.4 (C-18), 168.0 (—$\underline{C}OCH_3$), 167.9 (C-19), 149.3 (C-13), 143.6 (C-2′), 141.7 (C-8), 137.1 (C-15), 135.4 (C-4′), 134.5 (C-7), 128.5 (C-16), 127.9 (C-5′), 122.9 (C-6′), 121.8 (C-14), 90.2 (C-4), 83.7 (C-11), 61.2 (C-1′), 55.3 (C-10), 51.9 (—$O\underline{C}H_3$), 49.4 (C-3), 33.3 (C-1), 30.7 (C-

5), 28.5 (C-12), 27.8 (C-9), 24.3 (C-2), 23.5 (C-7′), 21.7 (—$COCH_3$), 20.2 (C-6), 15.8 (C-8′), 12.9 (C-17); HR-ESI-MS: *m/z* 579.2016 $[M + Na]^+$ (calcd for $C_{30}H_{36}O_8SNa$, 579.2029).

### 3.2.3 Compound 7

White powder; m.p. 144–145°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.36 (1H, d, $J = 1.5$ Hz, H-6′), 7.19–7.22 (2H, m, H-8, 15), 6.46–6.58 (2H, m, H-14, 3′), 6.38 (1H, dd, $J = 3.2$, 2.0 Hz, H-7′), 6.28 (1H, d, $J = 3.2$ Hz, H-8′), 6.24 (1H, m, H-2′), 5.88 (1H, d, $J = 15.1$ Hz, H-13), 4.79 (2H, d, $J = 7.4$ Hz, H-1′), 3.72 (3H, s, $H_3$-19), 3.30 (1H, d, $J = 5.2$ Hz, H-3), 3.08 (1H, dd, $J = 14.2$, 5.6 Hz, H-5a), 2.90 (1H, dd, $J = 15.6$, 6.3 Hz, H-6a), 2.75 (1H, dd, $J = 15.0$, 8.8 Hz, H-9a), 2.58–2.64 (1H, m, H-9b), 2.18 (1H, overlap, H-6b), 2.12 (3H, s, $H_3$-OAc), 1.98 (3H, s, $H_3$-17), 1.72–1.87 (5H, m), 1.59 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 172.9 (C-20), 169.4 (C-18), 168.1 (—$COCH_3$), 167.9 (C-19), 151.8 (C-4′), 143.6 (C-13), 142.4 (C-6′), 141.7 (C-8), 136.9 (C-15), 134.5 (C-7), 128.5 (C-16), 122.1 (C-2′), 121.9 (C-3′), 121.8 (C-14), 111.4 (C-7′), 108.9 (C-8′), 90.1 (C-4), 83.7 (C-11), 64.9 (C-1′), 55.2 (C-10), 52.1 (—$OCH_3$), 49.3 (C-3), 33.3 (C-1), 30.7 (C-5), 28.5 (C-12), 27.8 (C-9), 24.3 (C-2), 21.8 (—$COCH_3$), 20.1 (C-6), 12.9 (C-17); HR-ESI-MS: *m/z* 561.2061 $[M + Na]^+$ (calcd for $C_{30}H_{34}O_9Na$, 561.2101).

### 3.2.4 Compound 8

White powder; m.p. 139–140°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.20–7.41 (7H, m, H-8, 15, 5′, 6′, 7′, 8′, 9′), 6.68 (1H, d, $J = 15.9$ Hz, H-3′), 6.55 (1H, dd, $J = 15.1$, 11.5 Hz, H-14), 6.33 (1H, dt, $J = 15.9$, 6.4 Hz, H-2′), 5.88 (1H, d, $J = 15.1$ Hz, H-13), 4.82 (2H, d, $J = 6.4$ Hz, H-1′), 3.72 (3H, s, $H_3$-19), 3.30 (1H, d, $J = 5.1$ Hz, H-3), 3.08 (1H, dd, $J = 14.1$, 6.0 Hz, H-5a), 2.90 (1H, dd, $J = 15.5$, 6.3 Hz, H-6a), 2.75 (1H, dd, $J = 15.0$, 8.8 Hz, H-9a), 2.58–2.63 (1H, m, H-9b), 2.15–2.18 (1H, m, H-6b), 2.13 (3H, s, $H_3$-OAC), 1.99 (3H, s, $H_3$-17), 1.70–1.87 (5H, m), 1.59 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 172.8 (C-20), 169.4 (C-18), 168.0 (—$COCH_3$), 167.9 (C-19), 143.6 (C-13), 141.7 (C-8), 136.9 (C-4′), 136.3 (C-15), 134.5 (C-7), 134.2 (C-3′), 128.6 (C-6′, C-8′), 128.1 (C-5′, C-9′), 126.6 (C-7′), 123.4 (C-16), 121.8 (C-14, C-2′), 90.2 (C-4), 83.7 (C-11), 65.4 (C-1′), 55.3 (C-10), 52.0 (—$OCH_3$), 49.3 (C-3), 33.3 (C-1), 30.7 (C-5), 28.5 (C-12), 27.8 (C-9), 24.3 (C-2), 21.8 (—$COCH_3$), 20.2 (C-6), 12.9 (C-17); HR-ESI-MS: *m/z* 571.2310 $[M + Na]^+$ (calcd for $C_{32}H_{36}O_8Na$, 571.2308).

## 3.3 Synthesis of pseudolaricamides compounds 9–12

To a solution of PB (0.432 g, 1 mmol) in anhydrous $CH_2Cl_2$ (10 ml) were added corresponding amines (as shown in Scheme 1), HOBt (0.136 g, 1.2 mmol), EDCI (0.229 g, 1.2 mmol), and $Et_3N$ (0.1 ml) at 0°C. The reaction mixture was stirred at room temperature for 10 h, then washed with 5% HCl (3 × 15 ml), saturated $NaHCO_3$ (20 ml), $H_2O$ (20 ml), and dried with anhydrous $Na_2SO_4$. The crude product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (3:2) as the eluent to afford **9–12** (0.367 g, 85% yield as a brown oil).

### 3.3.1 Compound 9

White powder; m.p. 142–143°C; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.26–7.35 (5H, m, H-3′,4′, 5′, 6′, 7′), 7.18–7.20 (1H, m, H-8), 6.97 (1H, d, $J = 11.3$ Hz, H-15), 6.50 (1H, dd, $J = 15.0$, 11.4 Hz, H-14), 6.07 (1H, t, $J = 5.3$ Hz, N-H), 5.80 (1H, d, $J = 15.0$ Hz, H-13), 4.52 (2H, d, $J = 6.0$ Hz, H-1′), 3.71 (3H, s, $H_3$-19), 3.28 (1H, d, $J = 5.6$ Hz, H-3), 3.07 (1H, dd, $J = 14.1$, 6.1 Hz, H-5a), 2.88 (1H, dd, $J = 15.5$, 6.2 Hz, H-6a), 2.73

(1H, dd, $J = 15.0, 8.8$ Hz, H-9a), 2.58–2.61 (1H, m, H-9b), 2.13–2.16 (1H, m, H-6b), 2.12 (3H, s, $H_3$-OAc), 1.98 (3H, s, $H_3$-17), 1.68–1.84 (5H, m), 1.57 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 172.9 (C-20), 169.4 (C-18), 168.4 (—$COCH_3$), 168.0 (C-19), 142.3 (C-13), 141.8 (C-8), 138.2 (C-15), 134.4 (C-7), 132.8 (C-2′), 130.7 (C-4′, C-6′), 128.7 (C-3′, C-7′), 127.9 (C-5′), 127.6 (C-16), 121.4 (C-14), 90.1 (C-4), 83.7 (C-11), 55.2 (C-10), 52.0 (—$OCH_3$), 49.3 (C-3), 43.9 (C-1′), 33.2 (C-1), 30.7 (C-5), 28.6 (C-12), 27.8 (C-9), 24.3 (C-2), 21.8 (—$COCH_3$), 20.1 (C-6), 13.2 (C-17); HR-ESI-MS *m/z*: 522.2480 $[M + H]^+$ (calcd for $C_{30}H_{36}NO_7$, 522.2486).

### 3.3.2 Compound **10**

White powder; m.p. 139–140°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.30 (2H, m, H-5′, 7′), 7.17–7.23 (4H, m, H-8, 4′, 6′, 8′), 6.89 (1H, d, $J = 11.5$ Hz, H-15), 6.46 (1H, dd, $J = 15.1$, 11.5 Hz, H-14), 5.84 (1H, t, $J = 5.5$ Hz, N-H), 5.77 (1H, d, $J = 15.0$ Hz, H-13), 3.70 (3H, s, $H_3$-19), 3.57 (2H, q, $J = 7.0$ Hz, H-1′), 3.27 (1H, d, $J = 5.5$ Hz, H-3), 3.05 (1H, dd, $J = 15.0$, 6.5 Hz, H-5a), 2.83–2.90 (3H, m, H-6a, 2′), 2.72 (1H, dd, $J = 15.5$, 9.5 Hz, H-9a), 2.57–2.61 (1H, m, H-9b), 2.14 (1H, overlap, H-6b), 2.11 (3H, s, $H_3$-OAc), 1.87 (3H, s, $H_3$-17), 1.70–1.81 (5H, m), 1.56 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 173.0 (C-20), 169.4 (C-18), 168.4 (—$COCH_3$), 168.0 (C-19), 142.2 (C-13), 141.7 (C-8), 138.9 (C-15), 134.5 (C-7), 132.6 (C-3′), 131.6 (C-5′, 7′) 130.8 (C-4′, 8′), 128.7 (C-16), 126.6 (C-6′), 121.4 (C-14), 90.1 (C-4), 83.6 (C-11), 55.2 (C-10), 52.0 (—$OCH_3$), 49.4 (C-3), 41.0 (C-1′), 35.6 (C-2′), 33.3 (C-1), 30.7 (C-5), 28.6 (C-12), 27.8 (C-9), 24.3 (C-2), 21.8 (—$COCH_3$), 20.1 (C-6), 13.1 (C-17); HR-ESI-MS *m/z*: 536.2652 $[M + H]^+$ (calcd for $C_{31}H_{38}NO_7$, 536.2702).

### 3.3.3 Compound **11**

White powder; m.p. 145–146°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.19–7.25 (2H, m, H-8, 7′), 6.90 (1H, d, $J = 11.2$ Hz, H-15), 6.74–6.80 (3H, m, H-4′, 6′, 8′), 6.47 (1H, dd, $J = 15.0$, 11.0 Hz, H-14), 5.78 (1H, d, $J = 15.1$ Hz, H-13), 3.79 (3H, s, H-9′), 3.72 (3H, s, $H_3$-19), 3.59 (2H, q, $J = 4.8$ Hz, H-1′), 3.28 (1H, d, $J = 5.5$ Hz, H-3), 3.07 (1H, dd, $J = 14.0$, 6.0 Hz, H-5a), 2.89 (1H, dd, $J = 16.0$, 6.5 Hz, H-6a), 2.83 (2H, t, $J = 5.2$ Hz, H-2′), 2.74(1H, dd, $J = 15.0$, 9.0 Hz, H-9a), 2.58–2.62 (1H, m, H-9b), 2.14–2.17 (1H, m, H-6b), 2.12 (1H, s, $H_3$-OAc), 1.90 (3H, s, $H_3$-17), 1.71–1.82 (5H, m), 1.57 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 172.9 (C-20, C-18), 169.4 (—$COCH_3$), 168.0 (C-19), 159.8 (C-5′), 142.2 (C-13), 141.7 (C-8), 140.5 (C-3′), 134.5 (C-15), 132.6 (C-7), 129.7 (C-7′, C-16), 121.4 (C-14), 121.1 (C-8′), 114.5 (C-4′), 112.0 (C-6′), 90.1 (C-4), 83.7 (C-11), 55.2 (C-9′, C-10), 52.0 (—$OCH_3$), 49.4 (C-3), 40.9 (C-1′), 35.7 (C-2′), 33.3 (C-1), 30.7 (C-5), 28.6 (C-12), 27.8 (C-9), 24.3 (C-2), 21.8 (—$COCH_3$), 20.1 (C-6), 13.1 (C-17); HR-ESI-MS *m/z*: 566.2751 $[M + H]^+$ (calcd for $C_{32}H_{40}NO_8$, 566.2754).

### 3.3.4 Compound **12**

White powder; m.p. 144–145°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.19–7.21 (1H, m, H-8), 6.91 (1H, d, $J = 11.5$ Hz, H-15), 6.82 (1H, d, $J = 8.0$ Hz, H-7′), 6.72–6.75 (2H, m, H-4′, 8′), 6.48 (1H, dd, $J = 15.0$, 11.3 Hz, H-14), 5.79 (1H, d, $J = 15.0$ Hz, H-13), 3.87 (6H, s, $H_3$-9′, $H_3$-10′), 3.72 (3H, s, $H_3$-19), 3.57 (2H, m, H-1′), 3.29 (1H, d, $J = 6.0$ Hz, H-3), 3.07 (1H, dd, $J = 14.0$, 6.0 Hz, H-5a), 2.89 (1H, dd, $J = 15.5$, 6.5 Hz, H-6a), 2.80 (2H, t, $J = 7.0$ Hz, H-2′), 2.74 (1H, dd, $J = 15.0$, 8.5 Hz, H-9a), 2.58–2.63 (1H, m, H-9b), 2.14–2.16 (1H, m, H-6b), 2.12 (3H, s, $H_3$-OAc), 1.90 (3H, s, $H_3$-17), 1.71–1.82 (5H, m), 1.58 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 173.0 (C-20), 169.4 (C-18), 168.4 (—$COCH_3$), 168.0 (C-19), 149.1 (C-5′), 147.7 (C-6′), 142.2 (C-13), 141.7 (C-8), 134.5 (C-15), 132.6 (C-7), 131.4 (C-3′), 130.7 (C-16), 121.4 (C-14),

120.6 (C-8′), 111.9 (C-7′), 111.3 (C-4′), 90.1 (C-4), 83.7 (C-11), 55.9 (C-9′), 55.2 (C-10′, C-10), 52.0 (—$OCH_3$), 49.3 (C-3), 41.1 (C-1′), 35.2 (C-2′), 33.3 (C-1), 30.7 (C-5), 28.6 (C-12), 27.8 (C-9), 24.3 (C-2), 22.8 (—$COCH_3$), 20.1 (C-6), 13.1 (C-17); HR-ESI-MS: *m/z* 596.2866 $[M + H]^+$ (calcd for $C_{33}H_{42}NO_9$, 596.2860).

### *3.4 Synthesis of pseudolaricamides without 4-acetyl 13–16*

The $CH_3ONa$ (0.065 g, 1.2 mmol) was added to a solution of PB (0.432 g, 1 mmol) in anhydrous $CH_3OH$ (10 ml) at 0°C slowly. Ten minutes later, it was moved to room temperature for 4 h. Then, the pH value of the mixture was adjusted to 6 with 5% HCl, extracted with EtOAc (3 × 15 ml), and washed with saturated NaCl. The combined organic layers were dried with anhydrous $Na_2SO_4$ and the solvents were evaporated *in vacuo*. The crude product **4** was isolated as a solid (0.397 g, 92% yield). Then, compounds **13**–**16** were synthesised via the method b (as shown in Scheme 1).

#### *3.4.1 Compound **13***

White powder; m.p. 142–143°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.49 (1H, d, $J = 9.2$ Hz, H-7′), 7.27–7.29 (1H, m, H-4′), 7.21–7.23 (1H, m, H-8), 6.70–6.97 (2H, m, H-15, 6′), 6.48 (1H, dd, $J = 15.0$, 11.1 Hz, H-14), 5.42 (1H, d, $J = 15.0$ Hz, H-13), 3.85 (3H, s, H-10′), 3.72 (3H, s, $H_3$-19), 2.86 (1H, dd, $J = 15.1$, 5.8 Hz, H-5a), 2.62 (3H, m, H-3, 6a, 9a), 2.43 (1H, s, N-H), 2.13 (3H, s, $H_3$-17), 2.05–2.09 (1H, m, H-6b), 1.59–2.01 (5H, m), 1.37 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 174.1 (C-1′), 168.2 (C-20), 167.2 (C-18), 157.9 (C-19), 156.9 (C-5′), 144.9 (C-13), 142.7 (C-8), 141.7 (C-8′), 135.8 (C-15), 134.2 (C-7), 133.0 (C-3′), 129.5 (C-16), 120.9 (C-14), 120.8 (C-7′), 115.3 (C-6′), 104.4 (C-4′), 83.3 (C-11), 80.4 (C-4), 55.8 (C-10), 55.0 (C-10′), 53.7 (—$OCH_3$), 52.0 (C-3), 35.1 (C-5), 33.4 (C-1), 28.4 (C-12), 27.3 (C-9), 24.4 (C-2), 19.8 (C-6), 13.0 (C-17); HR-ESI-MS: *m/z* 553.2032 $[M + H]^+$ (calcd for $C_{29}H_{33}N_2O_7S$, 553.2008).

#### *3.4.2 Compound **14***

White powder; m.p. 145–146°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.20–7.23 (1H, m, H-8), 7.17 (1H, d, $J = 5.2$ Hz, H-5′), 6.94–6.98 (2H, m, H-15, 6′), 6.84 (1H, d, $J = 3.2$ Hz, H-7′), 6.48 (1H, dd, $J = 15.0$, 11.4 Hz, H-14), 6.10 (1H, t, $J = 5.7$ Hz, N-H), 5.81 (1H, d, $J = 15.0$ Hz, H-13), 3.70 (3H, s, $H_3$-19), 3.60 (2H, q, $J = 6.4$ Hz, H-1′), 3.08 (1H, t, $J = 6.4$ Hz, H-2′), 2.83 (1H, dd, $J = 14.6$, 5.7Hz, H-5a), 2.58–2.65 (3H, m, 6a), 2.24 (1H, d, $J = 6.2$ Hz, H-3), 2.08–2.17 (3H, m, 9a, 9b, H-6b), 1.92 (3H, s, $H_3$-17), 1.64–1.89 (4H, m), 1.53 (3H, s, $H_3$-12); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 174.5 (C-20), 168.7 (C-18), 168.3 (C-19), 143.3 (C-13), 142.8 (C-8), 141.2 (C-3′), 134.3 (C-15), 133.2 (C-7), 130.2 (C-7′), 127.1 (C-16), 125.4 (C-6′), 124.0 (C-5′), 121.1 (C-14), 83.6 (C-11), 80.0 (C-4), 55.1 (C-10), 54.0 (C-3), 51.9 (—$OCH_3$), 41.1 (C-1′), 35.2 (C-5), 33.5 (C-1), 29.8 (C-2′), 28.6 (C-12), 27.3 (C-9), 24.5 (C-2), 19.9 (C-6), 13.0 (C-17); HR-ESI-MS: *m/z* 500.2112 $[M + H]^+$ (calcd for $C_{27}H_{34}NO_6S$, 500.2107).

#### *3.4.3 Compound **15***

White powder; m.p. 140–141°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.44 (1H, brs, N-H), 7.29 (1H, d, $J = 2.0$ Hz, H-2′), 7.22–7.24 (1H, m, H-8), 7.03 (1H, d, $J = 11.3$ Hz, H-15), 6.82 (1H, d, $J = 8.4$ Hz, H-5′), 6.75 (1H, d, $J = 8.4$ Hz, H-6′), 6.53 (1H, dd, $J = 15.0$, 11.1 Hz, H-14), 5.96 (2H, s, 7′), 5.87 (1H, d, $J = 15.0$ Hz, H-13), 3.72 (3H, s, $H_3$-19), 2.88 (1H, dd, $J = 15.0$, 5.9 Hz, H-5a), 2.50–2.70 (3H, m, H-3, 6a, 9a), 2.20 (1H, m, H-9b), 2.09–2.16 (1H, m, H-6b), 2.07 (3H, s, $H_3$-17), 1.68–2.00 (5H, m), 1.55 (3H, s, $H_3$-12); $^{13}$C NMR

(100 MHz, $CDCl_3$) $\delta$ 174.2 (C-20), 168.2 (C-18), 166.7 (C-19), 147.9 (C-3′), 144.5 (C-4′), 143.4 (C-13), 142.7 (C-8), 134.2 (C-15), 133.4 (C-7), 132.1 (C-1′), 131.0 (C-16), 121.2 (C-6′), 113.5 (C-14), 108.1 (C-5′), 103.2 (C-2′), 101.3 (C-7′), 83.5 (C-11), 80.4 (C-4), 55.1 (C-10), 54.1 (C-3), 51.9 (—$OCH_3$), 35.2 (C-5), 33.4 (C-1), 28.6 (C-12), 27.3 (C-9), 24.5 (C-2), 19.8 (C-6), 13.3 (C-17); HR-ESI-MS: *m/z* 510.2176 $[M + H]^+$ (calcd for $C_{28}H_{32}NO_8$, 510.2128).

#### 3.4.4 Compound **16**

White powder; m.p. 141–142°C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.21–7.24 (1H, m, H-8), 7.00 (1H, d, $J = 11.3$ Hz, H-15), 6.92 (1H, t, $J = 4.6$ Hz, N-H), 6.50 (1H, dd, $J = 15.0$, 11.4 Hz, H-14), 5.80 (1H, d, $J = 15.0$ Hz, H-13), 3.72 (3H, s, $H_3$-19), 3.59 (3H, m, H-3′, 5′), 3.46 (1H, q, $J = 5.2$ Hz, H-1′), 2.85 (1H, dd, $J = 14.9$, 5.9 Hz, H-5a), 2.55–2.70 (3H, m, H-3, 6a, 9a), 2.24 (1H, m, H-9b), 2.11 (2H, m, H-2′), 1.95 (3H, s, $H_3$-17), 1.69–1.88 (5H, m), 1.54 (3H, s, $H_3$-12), 1.17 (6H, s, H-6′, 7′); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 174.3 (C-20), 168.3 (C-18,C-19), 142.8 (C-13), 142.7 (C-8), 134.2 (C-15), 132.7 (C-7), 130.5 (C-16), 121.3 (C-14), 83.5 (C-11), 80.3 (C-4), 72.0 (C-5′), 68.3 (C-3′), 55.0 (C-10), 54.1 (C-3), 51.9 (—$OCH_3$), 39.8 (C-1′), 35.2 (C-5), 33.4 (C-1), 28.9 (C-2′), 28.7 (C-12), 27.3 (C-9), 24.5 (C-2), 22.2 (C-6′, 7′), 19.8 (C-6), 13.0 (C-17); HR-ESI-MS: *m/z* 490.2867 $[M + H]^+$ (calcd for $C_{27}H_{40}NO_7$, 490.2805).

### 3.5 Immunoactivity

#### 3.5.1 Cell preparation

Mice were killed by cervical dislocation, and their spleens were removed aseptically. Cell debris and clumps were removed, and erythrocytes were lysed with ammonium chloride buffer solution. Mononuclear cell suspensions were resuspended in RPMI 1640 medium supplemented with 2 mmol l$^{-1}$ L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, and 100 U ml$^{-1}$ penicillin–streptomycin. T cells were purified from the auricular and mandibular lymphnodes using immunomagnetic negative selection as described by Wei et al. [23]. The purity of T cells was consistently more than 95% as assessed by flow cytometric antibody (BD Biosciences, New Jersey, USA).

#### 3.5.2 Cytotoxicity assay

The cytotoxicity of PB and its derivatives on T cells was assessed by MTT assay as described previously [23]. The optical density (OD) values were recorded at 570 nm wavelength on a microplate reader (Model 680, Bio-Rad, California, USA). Cell viability was calculated according to the following formula: viability rate (%) = OD treated/OD control × 100% [21].

#### 3.5.3 T and B cell proliferation assay

Splenocytes ($5 \times 10^5$/well) were cultured with 5 μg ml$^{-1}$ of ConA/LPS in triplicate for 48 h. Cell proliferation was determined by the WST-1 assay, a colorimetric assay for the quantification of cell proliferation, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells [24]. At the end of incubation, WST-1 (1:10) was added to each well and the absorption of samples was measured at 450 nm. The inhibitory rate was calculated as follows: inhibitory rate (%) = [(OD control–OD treated)/OD control] × 100%.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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