JOURNAL OF NATURAL PRODUCTS

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Article

Immunomodulatory Potential of a Bibenzyl-dihydrophenanthrene Derivative Isolated from *Calanthe cardioglossa*

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Cite This: https://doi.org/10.1021/acs.jnatprod.4c01394

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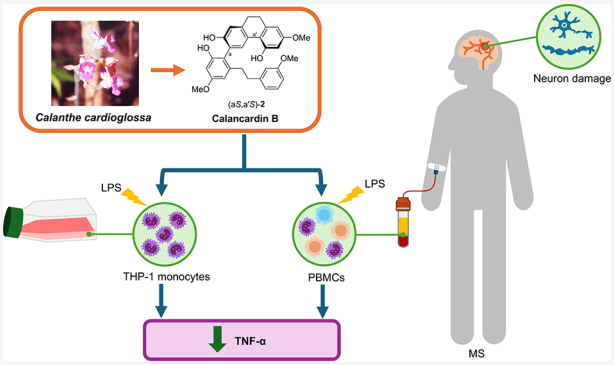
ABSTRACT: Bibenzyls and dihydrophenanthrenes exhibit promising immunomodulatory effects in various human diseases. In this study, we isolated one new dihydrophenanthrene derivative (**1**), two new bibenzyl-dihydrophenanthrene derivatives (**2, 3**) along with 12 known compounds (**4**−**15**) from the methanol extract of *Calanthe cardioglossa*. These compounds were identified by using physicochemical analyses and spectroscopic methods. The three new compounds possess enantiomeric forms, and their configurations were determined by comparing the experimental electronic circular dichroism (ECD) spectra to data from the literature. The immunomodulatory activity of the isolated compounds was assessed in THP-1 monocytes and human peripheral blood mononuclear cells (PBMCs) derived from multiple sclerosis (MS) patients. Notably, five of the isolated compounds significantly reduced the TNF-α levels in LPS-stimulated THP-1 monocytes. Furthermore, calancardin B (**2**) exhibited a significant reduction in TNF-α levels in both THP-1 monocytes and CD14$^+$ monocytes from MS PBMCs.

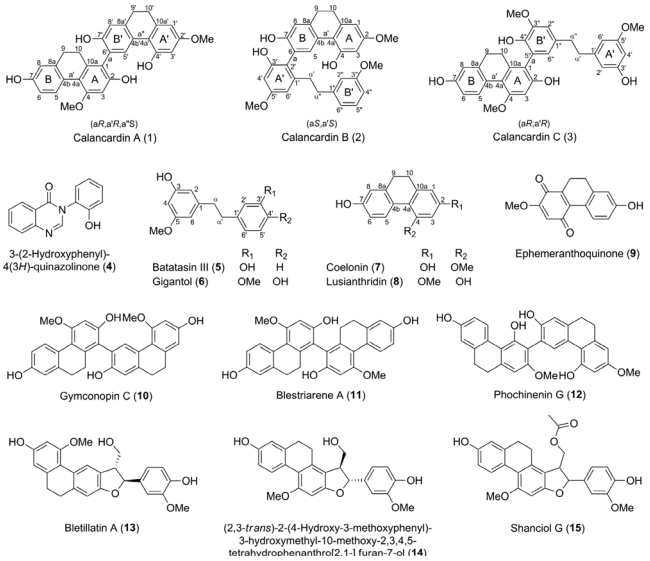


The Orchidaceae family is the second largest among angiosperms, encompassing approximately 850 genera and 20,000 species. This family plays a significant role in the commercial cut flower industry, and many of its species have documented uses as remedies for various diseases.[1] The genus *Calanthe* has a rich history of use in traditional medicine across China (Traditional Chinese Medicine, TCM), Thailand, India (Ayurveda), Indonesia, and Nepal for treating a range of ailments. In TCM, around 14 species of *Calanthe* are utilized in phytotherapy to address health issues such as traumatic injuries, edema, and pain relief for conditions such as joint pain, toothaches, and abdominal discomfort.[2−4] Moreover, various bioactive compounds isolated from *Calanthe* species including phenanthrenes, phenanthrenequinones, calanthoside, and 6′-*O*-β-D-apiofuranosylindican have demonstrated significant biological activities, including antiplatelet aggregation, anticancer effects, and hair restoration.[5−9] *Calanthe cardioglossa* Schltr. is a terrestrial orchid distributed throughout Thailand, Laos, and Vietnam. This plant has been used as a tonic in Thai ethnomedicine[2,10] and has not yet been reported in any phytochemical studies. Therefore, investigating the compounds present in this plant for their biological activities is particularly intriguing.

Inflammation is a natural process that helps the body heal and defend itself from harm. A dynamic balance between pro-inflammatory and anti-inflammatory processes is essential for controlling disease and limiting pathology. However, when inflammation persists and becomes chronic, it can be detrimental. Chronic inflammation is a key factor in many diseases, and prolonged periods of covert or subclinical inflammation may precede organ damage, leading to clinical manifestations, such as obesity, cardiovascular disease (CVD), type 2 diabetes (T2D), and neurological diseases. Strengthening the human immune system is a potential strategy for addressing disease pathogenesis and reducing mortality. Immunomodulatory treatments that utilize phytochemicals derived from plants are gaining attention as promising approaches to combat various diseases such as cancer,[11] neuroinflammation[12] and infections.[13] Medicinal plants are excellent sources of bioactive compounds; they are affordable, widely accessible, and generally associated with fewer side effects, making them ideal candidates for drug development. Notable immune-active bioactive molecules from plants include bibenzyls,[11,14] phenanthrenes,[12] alkaloids,[15,16] and terpenoids.[17] Additionally, active compounds derived from orchids have been reported to exhibit immunomodulatory effects.[11,12,14] This study aimed to isolate compounds from *C. cardioglossa* and to evaluate their immunomodulatory activity

Received: December 5, 2024
Revised: January 16, 2025
Accepted: January 18, 2025

in neuroinflammation, i.e., multiple sclerosis (MS), an autoimmune inflammatory disease of the central nervous system (CNS) that is a leading cause of chronic neurological disability.[18,19]



RESULTS AND DISCUSSION

Compound **1** was obtained as a brown, amorphous solid. The molecular formula was determined to be $C_{30}H_{26}O_6$ based on its $[M-H]^-$ ion at m/z 481.1637 (calcd 481.1651 for $C_{30}H_{25}O_6$) in the HR-ESI-MS. The IR spectrum showed the absorption bands at 3338 (hydroxyl groups), 1551 and 1465 (aromatic rings) cm^{-1}, and UV maxima at 217, 279, and 298 nm, which corresponded to dihydrophenanthrene skeleton.[20,21] The ^1H NMR spectrum presented characteristics of dimeric dihydrophenanthrene with signals of four methylene groups at δ_H 2.52 (4H, m, H_2-9 and H_2-10) and δ_H 2.75 (4H, m, H_2-9′ and H_2-10′).

For the first dihydrophenanthrene moiety (rings A and B), ^1H NMR showed signals for a singlet aromatic proton at δ_H 6.59 (1H, s, H-3), the ABX spin system at δ_H 6.66 (1H, br s, H-8), δ_H 6.68 (1H, dd, J = 8.4, 2.8 Hz, H-6), δ_H 8.06 (1H, d, J = 8.4 Hz, H-5), a methoxy group at δ_H 3.87 (3H, s, MeO-4) and a hydroxyl proton at δ_H 8.17 (1H, s, HO-7) (Table 1). The locations of H-3 and H-5 were assigned by their HMBC connection with C-4a (δ_C 116.9). The HMBC correlation between H-8 and C-9 (δ_C 30.5) was also observed (Figure 1). The methoxy group was attached at C-4 (δ_C 157.8) confirmed by its NOESY cross-peak with H-3 and H-5 (Figure 2). Therefore, the first phenanthrene moiety was 2,7-dihydroxy-4-methoxy-9,10-dihydrophenanthrene with C-1 substitution.

For the second dihydrophenanthrene moiety (rings A′ and B′), the ^1H NMR spectrum displayed two *meta*-coupled aromatic protons at δ_H 6.39 (1H, d, J = 2.8 Hz, H-1′) and δ_H 6.40 (1H, d, J = 2.8 Hz, H-3′), two singlet aromatic protons at δ_H 6.84 (1H, s, H-8′) and δ_H 8.15 (1H, s, H-5′), a methoxy group at δ_H 3.74 (3H, s, MeO-2′) and one hydroxyl proton at δ_H 8.56 (1H, s, HO-4′) (Table 1). The assignments of H-1′ and H-8′ were deduced from their HMBC correlations to C-10′ (δ_C 31.5) and C-9′ (δ_C 30.4), respectively. The assignments of H-3′ and H-5′ were confirmed by their HMBC correlations with C-4a′ (δ_C 115.7) (Figure 1). The location of the methoxy group at C-2′ was supported by its NOESY interactions with H-1′ and H-3′ (Figure 2). So, the second moiety of dihydrophenanthrene was 4′,7′-dihydroxy-2′-methoxy-9′,10′-dihydrophenanthrene with C-6′ substitution.

The connection between the two moieties of dihydrophenanthrene at C-1 (δ_C 117.3) and C-6′ (δ_C 121.3) was deduced by the HMBC correlations from H-5′ to C-1. The experimental ECD spectrum of compound **1** exhibited negative Cotton effects at 248 and 314 nm, and a positive Cotton effect

Table 1. ^1H (400 M Hz) and ^{13}C NMR (100 MHz) Spectral Data of Compound 1 in Acetone-d_6 (δ in ppm, J in Hz)

	1	
Position	δ_H (J in Hz)	δ_C, type
1		117.3, C
2		154.9, C
3	6.59, s	99.0, CH
4		157.8, C
4a		116.9, C
4b		126.0, C
5	8.06, d (8.4)	130.1, CH
6	6.68, dd (8.4, 2.8)	113.3, CH
7		156.0, C
8	6.66, br s	114.6, CH
8a		140.2, C
9	2.52, m	30.5, CH_2
10	2.52, m	28.5, CH_2
10a		140.8, C
1′	6.39, d (2.8)	106.0, CH
2′		159.4, C
3′	6.40, d (2.8)	101.5, CH
4′		155.9, C
4a′		115.7, C
4b′		126.2, C
5′	8.15, s	132.6, CH
6′		121.3, C
7′		153.9, C
8′	6.84, s	115.6, CH
8a′		139.3, C
9′	2.75, m	30.4, CH_2
10′	2.75, m	31.5, CH_2
10a		141.4, C
MeO-4	3.87, s	55.7, CH_3
MeO-2′	3.74, s	55.3, CH_3
HO-7	8.17, s	
HO-4′	8.56, s	

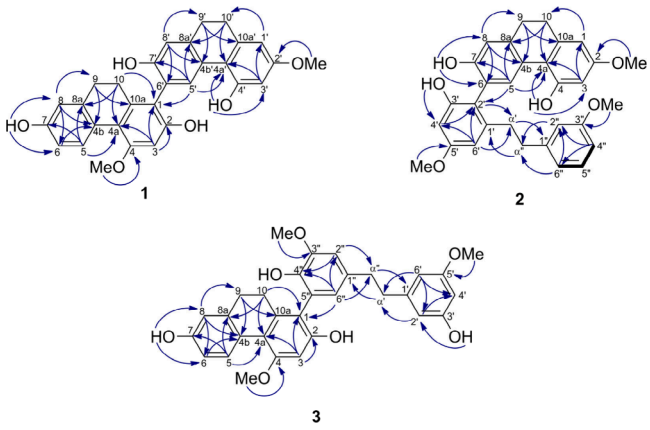


Figure 1. Key HMBC (arrow) and COSY (bold) correlations of compounds **1**–**3**.

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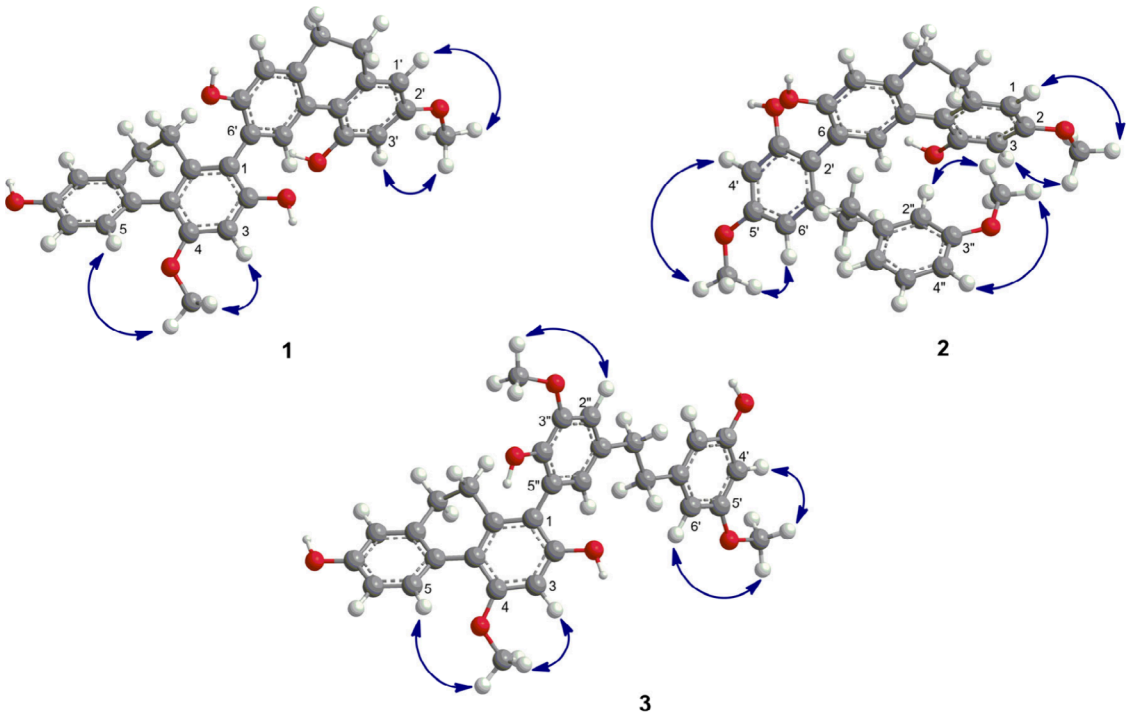

Figure 2. Key NOESY correlations of compounds **1**−**3**.

at 271 nm (Figure 3). This spectral feature is closely matched with the calculated ECD spectrum of the blestrianol B from

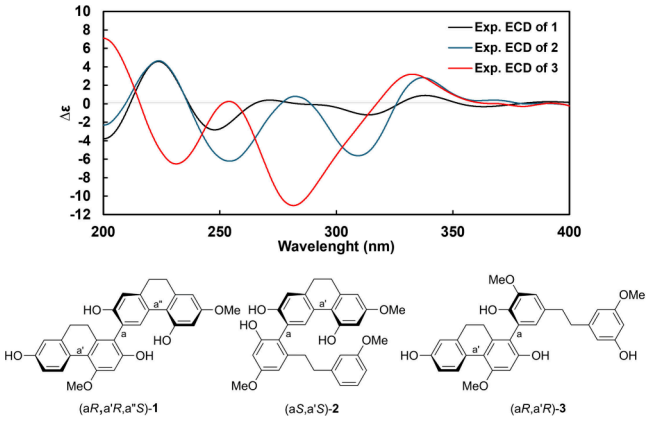

Figure 3. Experimental ECD spectra in MeOH and enantiomer structure of compounds **1**−**3**.

Bletilla striata,[22] therefore the absolute configuration of compound **1** was assigned as (a*R*,a′*R*,a″*S*)-configuration. From the evidence mentioned above, it leads to the structure compound **1** as shown and is named calancardin A.

Compound **2** was isolated as a brown amorphous solid. The formula was determined to be $C_{31}H_{30}O_6$ based on its $[M-H]^-$ ion at *m*/*z* 497.1914 (calcd 497.1964 for $C_{31}H_{29}O_6$) in the HR-ESI-MS. The IR spectrum exhibited the absorption bands at 3337 (hydroxyl groups), 1576 and 1444 (aromatic rings) cm^{-1}. The UV spectrum showed maximum absorption at 203, 279, and 298 nm, suggesting a bibenzyl-dihydrophenanthrene skeleton.[20,22] This was confirmed by ^1H, ^{13}C NMR, and HSQC spectra. Two methylene groups at δ_H 2.76 (4H, m, H_2-9 and H_2-10) with HSQC correlations to the carbon atom at δ_C 29.6 (C-9) and δ_C 30.6 (C-10) and two methylene groups at δ_H 2.63 (2H, m, H_2-α') and 2.69 (2H, m, H_2-α'') with HSQC correlations to the carbon at δ_C 37.0 (C-α') and δ_C 37.3 (C-α'') exhibited characteristics of dihydrophenanthrene and bibenzyl, respectively.[23]

For the dihydrophenanthrene moiety (rings A and B), the ^1H and ^{13}C NMR spectra (Table 2) closely resembled that of the second dihydrophenanthrene moiety of compound **1**. The ^1H NMR spectrum displayed two doublet aromatic protons at δ_H 6.39 (1H, d, *J* = 2.8 Hz, H-1) and δ_H 6.41 (1H, d, *J* = 2.8 Hz, H-3), two singlet aromatic protons at δ_H 6.85 (1H, s, H-8) and δ_H 8.24 (1H, s, H-5), one methoxy group at δ_H 3.74 (3H, s, MeO-2) and two hydroxyl protons at δ_H 7.59 (1H, s, HO-7) and δ_H 8.58 (1H, s, HO-4). A NOESY interaction of MeO-2 with H-1 and H-3 places the methoxy group at C-2 (Figure 2). From the above evidence, the first moiety of compound **2** was 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene with a C-6 substitution.

For bibenzyl moiety (rings A′ and B′), the ^1H NMR signals revealed two *meta*-coupled aromatic protons at δ_H 6.37 (1H, d, *J* = 2.4 Hz, H-4′) and δ_H 6.44 (1H, d, *J* = 2.4 Hz, H-6′), a set of aromatic proton for *meta*-substituted benzene at δ_H 6.49 (1H, t, *J* = 2.0 Hz, H-2″), δ_H 6.55 (1H, br d, *J* = 8.0 Hz, H-6″), δ_H 6.57 (1H, t, *J* = 8.0 Hz, H-5″), and δ_H 6.59 (1H, ddd, *J* = 8.0, 2.8, 0.8 Hz, H-4″), two methoxy groups at δ_H 3.31 (3H, s, MeO-3″) and δ_H 3.76 (3H, s, MeO-5′) and one hydroxyl proton (δ_H 7.31, 1H, s, HO-3′) (Table 2). The assignment of H-6′ was referenced from the HMBC correlation to C-α' and C-2′ (δ_C 117.5). The position of H-2″ and H-6″ was confirmed by three-bond correlation to C-α'' in the HMBC spectrum. In addition, the $^1H-^1H$ COSY of H-5″ with H-4″ and H-6″ was observed (Figure 1). The location of MeO-5′ at C-5′ (δ_C = 159.9) was confirmed by its NOESY correlations with H-4′ and H-6′. The attachment of MeO at C-3″ (δ_C 159.7) was inferred by its NOESY correlations with H-2″ and H-4″ (Figure 2). Thus, this bibenzyl moiety was 3′-hydroxy-5′,3″-dimethoxybibenzyl with C-2′ substitution.

https://doi.org/10.1021/acs.jnatprod.4c01394
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Table 2. ^{1}H (400 M Hz) and ^{13}C NMR (100 MHz) Spectral Data of Compounds 2−3 in Acetone-d_6 (δ in ppm)

	2		3	
Position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
1	6.39, d (2.8)	105.2, CH		117.3, C
2		158.5, C		154.5, C
3	6.41, d (2.8)	100.6, CH	6.58, s	99.0, CH
4		155.1, C		157.7, C
4a		114.9, C		116.8, C
4b		125.3, C		126.0, C
5	8.24, s	132.0, CH	8.06, d (8.8)	130.1, CH
6		120.2, C	6.69, dd (8.8, 2.0)	113.3, CH
7		153.1, C		156.0, C
8	6.85, s	114.7, CH	6.68, br s	114.6, CH
8a		138.5, C		140.2, C
9	2.76, m	29.6, CH_2	2.52, t (7.2)	30.6, CH_2
10	2.76, m	30.6, CH_2	2.39, m	28.3, CH_2
10a		140.5, C		140.3, C
1′		143.4, C		145.1, C
2′		117.5, C	6.32, br t (2.0)	108.9, CH
3′		156.1, C		159.2, C
4′	6.37, d (2.4)	99.1, CH	6.24, t (2.0)	99.7, CH
5′		159.9, C		161.8, C
6′	6.44, d (2.4)	106.4, CH	6.31, br t (2.0)	106.3, CH
1″		144.1, C		133.4, C
2″	6.49, t (2.0)	113.5, CH	6.87, d (2.0)	111.7, CH
3″		159.7, C		148.3, C
4″	6.59, ddd (8.0, 2.8, 0.8)	111.3, CH		143.6, C
5″	6.57, t (8.0)	128.9, CH		123.5, C
6″	6.55, br d (8.0)	120.4, CH	6.54, d (2.0)	124.8, CH
α'	2.63, m	37.0, CH_2	2.84, br s	39.0, CH_2
α''	2.69, m	37.3, CH_2	2.84, br s	38.1, CH_2
MeO-2	3.74, s	54.4, CH_3		
MeO-4			3.87, s	55.7, CH_3
MeO-5′	3.76, s	54.4, CH_3	3.71, s	55.2, CH_3
MeO-3″	3.31, s	54.2, CH_3	3.89, s	56.2, CH_3
HO-4	8.58, s			
HO-7	7.59, s		8.16, s	
HO-3′	7.31, s		8.21, s	

Compound **2** was derived from dihydrophenanthrene and bibenzyl moieties which connected at C-6 (δ_C 120.2) and C-2′ by the HBMC correlation of H-5 with C-2′. The experimental ECD spectrum of this compound showed a negative Cotton effect at 254 nm and a positive Cotton effect at 282 nm (Figure 3). The absolute configuration of compound **2** was assessed as (a*S*,a′*S*)-configuration when compared to (a*S*,a′*S*)-blestanol H from previous reported.[22] We named compound **2** calancardin B.

Compound **3** was obtained as a brown amorphous solid. The molecular formula was $C_{31}H_{30}O_7$ based on its $[M-H]^-$ ion at m/z 513.1859 (calcd 513.1913 for $C_{31}H_{29}O_7$) in the HR-ESI-MS. The IR spectrum exhibited the absorption bands at 3305 cm^{-1} (hydroxyl groups), 2952 and 1638 cm^{-1} (aromatic rings). This compound was proposed to be a bibenzyl-dihydrophenanthrene skeleton based on the maximum UV absorption peaks at 204 and 281 nm,[23] along with NMR spectra of dihydrophenanthrene characteristic [two signals of methylene protons at δ_H 2.39 (2H, m, H_2-10) and at δ_H 2.52 (2H, t, J = 7.2 Hz, H_2-9) with two methylene carbon signals at δ_C 28.3 (C-10) and at δ_C 30.6 (C-9)] and bibenzyl characteristic [four methylene proton signals at δ_H 2.84 (4H, br s, H_2-α' and H_2-α'') with two methylene carbon signals at δ_C 38.1 (C-α'') and at δ_C 39.0 (C-α')]. The ^{1}H and ^{13}C NMR data (Table 2) of dihydrophenanthrene moiety in compound **3** (rings A and B) was strikingly similar to the first dihydrophenanthrene moiety of compound **1**. The ^{1}H NMR exhibited the following signals: a singlet aromatic proton at δ_H 6.58 (1H, s, H-3), an ABX system proton at δ_H 6.68 (1H, br s, H-8), δ_H 6.69 (1H, dd, J = 8.8, 2.0 Hz, H-6), δ_H 8.06 (1H, d, J = 8.8 Hz, H-5), a methoxy group at δ_H 3.87 (3H, s, MeO-4) and a hydroxyl proton (δ_H 8.16, 1H, s, HO-7). The positions of H-3 and H-5 were confirmed by their HMBC correlations with C-4a (δ_C 116.8). H-8 was assigned by its HMBC correlation with C-9. The attachment of the methoxy group to C-4 (δ_C 157.7) was referenced from its NOESY correlation with H-3 and H-5 (Figure 2). Consequently, the structure of the dihydrophenanthrene moiety of compound **3** was 2,7-dihydroxy-4-methoxy-9,10-dihydrophenanthrene with C-1 substitution.

In addition, the ^{1}H NMR spectrum displayed three *meta*-coupled aromatic protons at δ_H 6.24 (1H, t, J = 2.0 Hz, H-4′), δ_H 6.31 (1H, br t, J = 2.0 Hz, H-6′), and δ_H 6.32 (1H, br t, J = 2.0 Hz, H-2′), a pair of *meta*-coupled aromatic proton at δ_H 6.54 (1H, d, J = 2.0 Hz, H-6″) and δ_H 6.87 (1H, d, J = 2.0 Hz, H-2″), together with three singlet protons at δ_H 3.71 (3H, s, MeO-5′), δ_H 3.89 (3H, s, MeO-3″), and δ_H 8.21 (1H, s, HO-3′) (Table 2). The position of H-2′ and H-6′ was assigned by the HMBC correlation to C-α' and C-4′ (δ_C 99.7). The assignment of H-2″ and H-6″ was referenced from the HMBC cross-peak to C-α'' and C-4″ (δ_C 143.6) (Figure 1). Moreover, two methoxy groups were placed at C-5′ (δ_C 161.8) and C-3″ (δ_C 148.3) based on their NOESY correlation from MeO-5′ to H-4′ and H-6′ and MeO-3″ to H-2″ (Figure 2). Therefore, the second moiety of compound **3** was 3′,4″-dihydroxy-5′,3″-dimethoxybibenzyl with C-5″ substitution.

The connection at C-1 (δ_C 117.3) and C-5″ (δ_C 123.5) of the two moieties was deduced by the HMBC correlation from H-6″ to C-1. Furthermore, the experimental ECD spectrum of compound **3** displayed a positive Cotton effect at 254 nm and a negative Cotton effect at 282 nm (Figure 3), for which the absolute configuration of compound **3** was assessed as (a*R*,a′*R*)-configuration by comparison with (a*R*,a′*R*)-blestanol F from literature data.[22] From the aforementioned evidence, the structure of compound **3** was finally determined and named calancardin C.

Calancardin A (**1**) is a dimer of dihydrophenanthrene. Calancardins B (**2**) and C (**3**) were dimers of dihydrophenanthrene and bibenzyl, which were less common than dimers formed between dihydrophenanthrene and dihydrophenanthrene/phenanthrene.[24] Bibenzyl-dihydrophenanthrene derivatives have mostly been reported to be found in orchids including *Bletilla formosana*,[25] *Bletilla striata*,[22,26] *Dendrobium parishii*,[27] *Dendrobium signatum*,[28] *Pholidota chinensis*,[23] *Pholidota yunnanensis*,[20] and *Pleione bulbocodioides*.[29,30] Additionally, 12 known compounds were isolated from the whole plant of *C. cardioglossa*: 3-(2-hydroxyphenyl)-4(3*H*)-quinazolinone (**4**),[31] batatasin III (**5**),[32] gigantol (**6**),[33] coelonin (**7**),[34] lusianthridin (**8**),[20] ephemeranthoquinone (**9**),[35] gymconopin C (**10**),[36] blestriarene A (**11**),[37] phochinenin G (**12**),[23] bletillatin A (**13**),[26] (2,3-*trans*)-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-10-methoxy-2,3,4,5-

https://doi.org/10.1021/acs.jnatprod.4c01394
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tetrahydrophenanthro[2,1-] furan-7-ol (**14**),[38] and shanciol G (**15**).[39] Their structures were identified by comparing physicochemical data with the literature data.

To investigate the immunomodulatory effects, all isolated compounds from *C. cardioglossa*, except calancardin C (**3**) and phochinenin G (**12**) due to insufficient quantities, were tested for their activity in THP-1 cells, a human monocytic cell line. Toxicity tests in THP-1 cells (Figure 4A) indicated that most

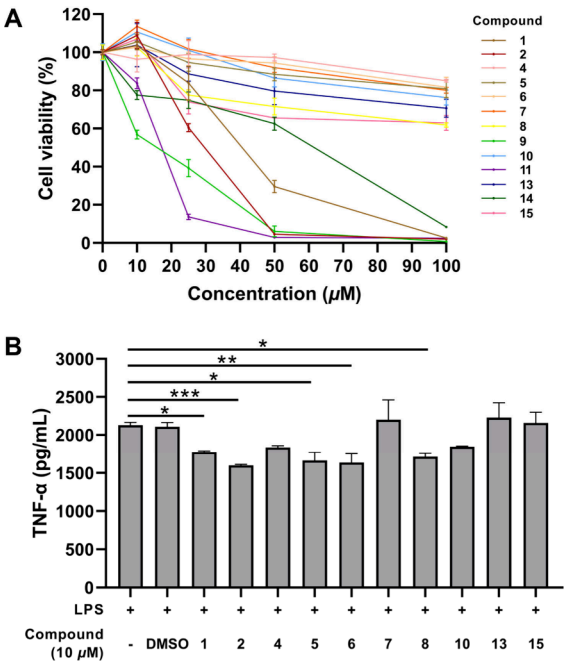

Figure 4. (A) Cell viability of isolated compounds at 10, 25, 50, and 100 μM in LPS-stimulated THP-1 monocytes. (B) TNF-α level of isolated compounds at 10 μM in LPS-stimulated THP-1 monocytes. The results are shown as mean ± SD. One-way ANOVA followed the correction of multiple comparisons (Tukey test), ***P < 0.001, **P < 0.01, *P < 0.05.

compounds (compounds **1**, **2**, **4**, **5**, **6**, **7**, **8**, **10**, **13**, **15**) were not cytotoxic at a concentration of 10 μM. In our previous study,[14] a concentration at 10 μM of bibenzyls has been shown to have good immunomodulatory effects in an *in vitro* model of LPS-induced inflammation in human primary peripheral blood mononuclear cells (PBMCs). In this study, we also used the concentration of 10 μM to further evaluate the immunomodulatory effects under LPS stimulation of the purified compounds in the THP-1 cell line as well as in human PBMCs. Utilizing the THP-1 cell line, five compounds including calancardin A (**1**), calancardin B (**2**), batatasin III (**5**), gigantol (**6**), and lusianthridin (**8**) demonstrated a significant reduction in LPS-induced TNF-α production compared to the control group (Figure 4B). Among these five active compounds, calancardin B (**2**) exhibited the most pronounced activity in reducing the level of TNF-α production following LPS stimulation. Consequently, we further investigated its immunomodulatory effects on primary human PBMCs. To achieve this, we conducted *in vitro* assessments using PBMCs isolated from five patients with MS (pwMS).

In brief, PBMCs were stimulated with LPS following a previously described method.[12,14] Four hours after LPS stimulation, we determined the expression of cytokines IFN-γ, IL-2, and TNF-α in multiple cell types, including $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^-HLADR^+$ and $CD19^-HLADR^-$ myeloid cells, as well as $CD19^+$ B cells using flow cytometry (Figure 5). Unstimulated PBMCs served as negative controls. No changes in IFN-γ and IL-2 could be observed in all cell populations after LPS stimulation. As expected, we observed an increased expression of TNF-α in $CD14^+$ monocytes but not in other immune cell population (Figure 5). This specificity of monocytes in responding to LPS is attributed to the binding of LPS to TLR4 receptors, which facilitates TNF-α expression with the assistance of CD14, a marker unique to monocytes.[40]

PBMCs from pwMS that were coincubated with calancardin B (**2**) (PBMCs + LPS + calancardin B) demonstrated a significant reduction in LPS-induced TNF-α production in $CD14^+$ monocytes, as compared with LPS-stimulated PBMCs without calancardin B treatment (i.e., PBMCs + LPS) (Figure 6). It is well established that pwMS present both functional and compositional alterations in their monocyte populations, particularly an increased level of pro-inflammatory cytokines such as TNF-α during the active phases of the disease, which contribute to neuroinflammation.[41,42] Our findings suggest a correlation between monocyte activation and disease activity, underscoring the central role of monocytes in MS pathology. Consequently, modulating monocyte activity presents a promising therapeutic strategy for MS. Approaches aimed at enhancing the anti-inflammatory functions of monocytes or inhibiting their pro-inflammatory actions could lead to novel treatment options for this condition.

The most potent compound, calancardin B (**2**), is a dimer formed from 9,10-dihydrophenanthrene (monomer: lusianthridin, **8**) and bibenzyl (monomer: 3′-O-methylbatatasin III). It has demonstrated the ability to inhibit LPS-induced TNF-α expression in both THP-1 monocytes and PBMCs from pwMS. The effects of calancardin B may be enhanced by the synergistic action of its two monomers, both of which are capable of reducing TNF-α production following LPS stimulation in THP-1 monocytes and PBMCs. In examining the structure−activity relationships (SAR) of five active compounds (compounds **1**, **2**, **5**, **6**, **8**) within the THP-1 monocyte model, it was observed that two bibenzyl compounds (batatasin III (**5**) and gigantol (**6**)) and calancardin B (**2**), a bibenzyl-dihydrophenanthrene derivative (a dimer of lusianthridin (**8**) and 3′-O-methyl-batatasin III) exhibited low activity in inhibiting TNF-α production after LPS stimulation. These compounds share a core structure featuring a methoxy group at C-5 and a hydroxy group at C-3 within the bibenzyl moiety. Furthermore, it is plausible to suggest that a methoxy group at C-3′, combined with the absence of substitution at C-4′, would yield higher activity compared to that of having a hydroxy group at C-3′. This aligns with our previous findings, which indicated that bibenzyl compounds associated with the inhibition of LPS-induced TNF expression in PBMCs possess three methoxy groups located at C-3, C-5, and C-3′.[14] For dihydrophenanthrene derivatives, calancardin A (**1**) (a dimer of lusianthridin (**8**) and coelonin (**7**)) and lusianthridin (**8**) resulted in a slight decrease in TNF-α levels in THP-1 monocytes stimulated with LPS, while coelonin (**7**) did not demonstrate any inhibitory effect in these cells. The distinction between these 9,10-dihydrophenanthrene compounds lies in their structural features: lusianthridin (**8**) contains a methoxy group at C-2 and a hydroxy group at C-4, whereas calancardin A (**1**), which is derived from lusianthridin (**8**) and coelonin (**7**), along with

https://doi.org/10.1021/acs.jnatprod.4c01394
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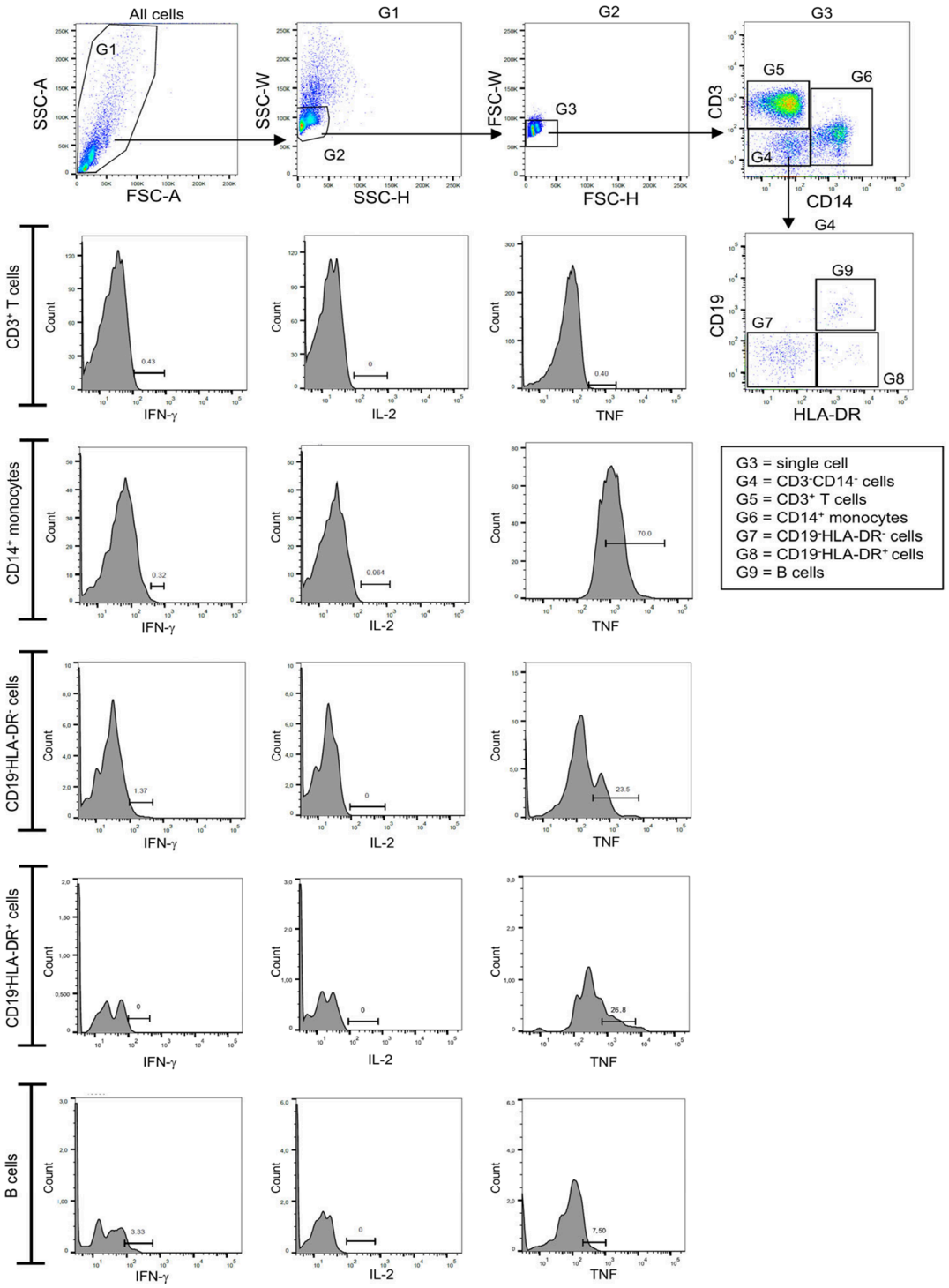

Figure 5. Dot plots and histograms display a flow cytometric gating strategy in LPS-induced inflammatory cytokine (IFN-γ, IL-2, and TNF-α) expression in human PBMCs of multiple sclerosis obtaining $CD3^+$ T cells (G5), $CD14^+$ monocytes (G6), and B cells (G9). $CD19^-HLADR^+$ (G8) and B cells (G9).

coelonin (**7**) itself, possesses a hydroxy group at C-2 and a methoxy group at C-4.

In summary, three newly elucidated dimers composed of dihydrophenanthrene and bibenzyl derivatives, alongside 12 known compounds, were isolated from *C. cardioglossa*. Notably, calancardin B (**2**), a dimer of dihydrophenanthrene and bibenzyl, displayed a substantial reduction in TNF-α levels in both THP-1 cells and monocytes isolated from pwMS. This finding indicates that calancardin B may exert beneficial immunomodulatory effects, thereby offering promising prospects for the development of treatments targeting immune-related inflammatory diseases.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco P-2000 polarimeter (Hachioji, Tokyo, Japan). UV spectra were recorded using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The IR spectra were obtained by using a

https://doi.org/10.1021/acs.jnatprod.4c01394
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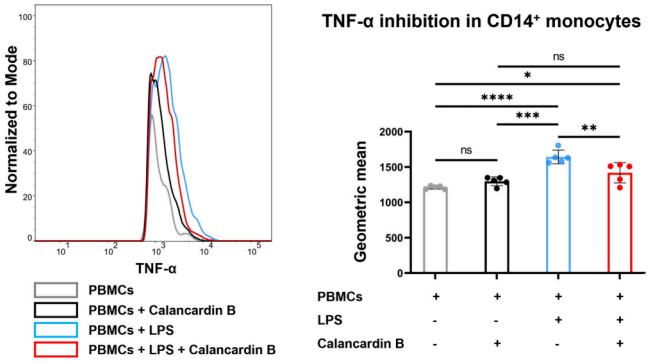

Figure 6. Determination of the immune modulatory effects. Bar graphs show the percentage of frequency of TNF-α expression in the immune cells of MS PBMCs (five biological replicates) after 4 h treatment with 10 μM of active compound from *C. cardioglossa with* or without LPS stimulation. One-way ANOVA followed the correction of multiple comparisons (Tukey test), ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; ns, not significant.

PerkinElmer FT-IR spectrophotometer (Waltham, MA, USA). NMR spectra were recorded on a Bruker Avance DPX-400 FT-NMR spectrometer (Billerica, MA, USA). High-resolution electrospray ionization mass spectra (HRESIMS) were recorded on an Agilent 6530 accurate-mass Q-TOF LC/MS instrument (Santa Clara, CA, USA). CD spectra were measured with a Jasco J-815 spectrophotometer (Hachioji, Tokyo, Japan). Semipreparative HPLC was performed on a Shimadzu SPD-20A UV detector with a Shimadzu LC-20AD pumping system (Kyoto, Japan) and VDSpher PUR 100 SIL (250 mm × 10 mm) column (Berlin, Germany). Dry column vacuum chromatography (DCVC) and column chromatography (CC) isolations were carried out on silica gel 60 (Merck, Darmstadt, Germany) with particle sizes of 40−63 and 63−200 μm. Sephadex LH-20 (Merck, Darmstadt, Germany) was used for separation by size exclusion chromatography. Thin layer chromatography (TLC) was run on aluminum sheets of silica gel 60 F_{254} (Merck, NJ, USA) and detected under UV light. Human monocytes (THP-1 cells) and RPMI-1640 medium were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). 2-Mercaptoethanol, dimethyl sulfoxide (DMSO), lipopolysaccharides (LPS), and Human Tumor Necrosis Factor (TNF-α) ELISA kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Gibco (Gaithersburg, MA, USA). PrestoBlue cell viability reagent was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

Plant Material. Plant material was purchased from the Chatuchak market in April 2020. Plant identification was performed by Yanyong Punpreuk, Department of Agriculture, Bangkok, Thailand. A voucher specimen (BS-CC-012563) was deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and Isolation. The dried whole plants of *C. cardioglossa* (2 kg) were extracted with MeOH (3 × 10 L) at room temperature. The MeOH extract (74.6 g) was subjected to dry column vacuum chromatography (DCVC) using silica gel with a hexane-acetone gradient to give 7 fractions (Fr.A-G). Fraction E (2.6 g) was isolated with column chromatography (CC) on silica gel (MeOH−CH_2Cl_2 gradient) to give 7 fractions (EI-EVII). Fraction EV (112.6 mg) was separated on Sephadex LH-20 (acetone) and then CC (silica gel, acetone/hexane 1:9) to yield gigantol (**6**) (22.1 mg) and ceolonin (**7**) (23.1 mg). Faction EVII (1.2 g) was isolated using Sephadex LH-20 (acetone) and then CC (silica gel, EtOAc-hexane 1:3) to obtain batatasin III (**5**) (509.1 mg) and 3-(2-hydroxyphenyl)-4(3*H*)-quinazolinone (**4**) (3.3 mg). Fraction EVI (151.0 mg) was purified by Sephadex LH-20 (acetone) and then CC (silica gel, MeOH−CH_2Cl_2 1.5:98.5) to yield lusianthridin (**8**) (5.0 mg). Fraction F (4.3 g) was isolated using CC on silica gel (MeOH−CH_2Cl_2 gradient) to obtain 7 fractions (FI−FVII). Faction FIII (60.3 mg) was purified using HPLC (silica gel, MeOH−CH_2Cl_2 1.5:98.5) to yield ephemeranthoquinone (**9**) (4.3 mg), gymconopin C (**10**) (2.0 mg), and blestriarene A (**11**) (7.7 mg). Fraction FIV (23.5 mg) was isolated with Sephadex LH-20 (MeOH) and then HPLC (silica gel, MeOH−CH_2Cl_2 1.5:98.5) to give compound **1** (3.5 mg), phochinenin G (**12**) (1.7 mg), bletillatin A (**13**) (2.1 mg), and (2,3-*trans*)-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-10-methoxy-2,3,4,5-tetrahydro-phenan (**14**) (5.0 mg). Fraction FVI (32.7 mg) was separated with Sephadex LH-20 (MeOH) and then HPLC (silica gel, EtOAc-hexane 1:3) to obtain compound **2** (3.3 mg), compound **3** (1.3 mg), and shanciol G (**15**) (2.2 mg).

*Calancardin A (**1**).* Brown amorphous solid; α_D^{20} − 7.1 (*c* 0.10, MeOH). UV (MeOH) λ_{max} (log ε) 217 (2.98), 279 (1.91), 298 (1.51) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 224 (+4.57), 248 (−2.82), 271 (+0.39), 314 (−1.20) nm; IR (ATR) ν_{max} 3338, 2935, 2853, 1647, 1617, 1551, 1465, 1247, 1211 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1. HRESIMS *m/z* 481.1637 $[M-H]^-$ (calcd 481.1651 for $C_{30}H_{25}O_6$).

*Calancardin B (**2**).* Brown amorphous solid; α_D^{20} + 5.8 (*c* 0.10, MeOH). UV (MeOH) λ_{max} (log ε) 203 (2.87), 279 (0.96), 298 (0.69) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 224 (+4.65), 254 (−6.20), 282 (+0.80), 309 (−5.62) nm; IR (ATR) ν_{max} 3337, 2941, 2853, 1620, 1576, 1444, 1258 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2. HRESIMS *m/z* 497.1914 $[M-H]^-$ (calcd 497.1964 for $C_{31}H_{29}O_6$).

*Calancardin C (**3**).* Brown amorphous solid; α_D^{20} − 4.0 (*c* 0.10, MeOH). UV (MeOH) λ_{max} (log ε) 204 (3.46), 281 (1.03) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 231 (−6.50), 254 (+0.25), 282 (−11.01) nm; IR (ATR) ν_{max} 3305, 2952, 2848, 1638, 1521, 1456, 1206, 1063 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2. HRESIMS *m/z* 513.1859 $[M-H]^-$ (calcd 513.1913 for $C_{31}H_{29}O_7$).

Cell Viability and TNF-α Screening. THP-1 cells were maintained in RPMI-1640 supplemented with 0.05 mM 2-mercaptoethanol, 10% FBS and 1% Pen/Strep at 5% CO_2 and 37 °C. For cell viability testing, the PrestoBlue cell viability reagent was used as in the previous study with slight modifications.[43] Cells were seeded at 1 × 10^6 cells/mL into 96-well plate (Corning, Jiangsu, Chaina) and treated with various concentrations of compounds from *C. cardioglossa* for 4 h, then PrestoBlue was added, and the cells were incubated for 30 min in dark conditions at 37 °C. After that, the absorbance was measured at a fluorescence excitation 560 nm and an emission 590 nm using a microplate reader (CLARIOstar, BMG Labtech, Germany). For TNF-α screening, the same method with previous study[44] was performed with some modifications. Briefly, THP-1 cells at 5 × 10^5 cells/mL were seeded into 24-well plate (Corning, ME, USA), then 100 μM of LPS and 10 μM of each compound were put into well. After 4 h, the medium was collected to measure the TNF-α with an ELISA kit.

Ethics and Human Peripheral Blood Mononuclear Cells (PBMCs). The Ethics for this study was approved by the Ethics Committee of Charite Universitätsmedizin Berlin (EA1/187/17). Buffy coats were obtained from five patients with multiple sclerosis. Human MS PBMCs were isolated and then cryopreserved in liquid nitrogen tank as described in a prior study.[12]

LPS Stimulation in PBMCs. Frozen human MS PBMCs were thawed and resuspended with RPMI 1640 medium with 10% FBS. Cells were seeded in an ultralow-attachment 96-well plate (Corning, New York, USA) at a density of 2 × 10^5 cells per well. Then, calancardin B (**2**) (10 μM) was added to the corresponding well. After that, cells were stimulated for inflammatory condition using LPS (100 ng/mL) and further incubated at 37 °C for 2 h. Subsequently, brefeldin A (10 μg/mL) was added to the wells and then incubated for 2 h. Eventually, all cells were harvested and washed with PBS then analyzed using flow cytometry as described below.

Cytokines in LPS-Treated MS PBMCs Measurement Using Flow Cytometry. LPS-treated MS PBMCs samples were thawed and washed twice with staining buffer (0.5% BSA in PBS with 2 mM EDTA). Cells were resuspended in FcR-blocking buffer (1:100; Miltenyi Biotec, Bergisch Gladbach, Germany) and then incubated at 4 °C for 10 min to block unspecific antibodies. After incubation with blocking buffer, cells were stained at 4 °C for 20 min with extracellular

https://doi.org/10.1021/acs.jnatprod.4c01394
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immunofluorescent antibodies including CD3 (APC, HIT3a, Biolegend), CD14 (FITC, RMO52, Beckman Coulter), CD19 (PE, HIB19, Biolegend) and HLA-DR-(APC/Cy7, L243, Biolegend) antibodies diluted in staining buffer. Subsequently, 2% methanol-free FA was added to fix the cells at 4 °C for 30 min. Cells were washed with staining buffer and incubated at 4 °C for 30 min with intracellular immunofluorescent antibodies including TNF (BV, MAb11, Biolegend), IL-2 (PerCP/Cy5.5, MQ1−17H12, Biolegend) and IFN-γ (PE/Cy7, 4S.B3, Biolegend) diluted in permeabilization buffer (eBioscience, California, USA). After that, stained cells were washed with staining buffer and centrifuged at 300g at 12 °C for 10 min. Finally, the supernatants were discarded, and pellets were resuspended in staining buffer. For evaluation of extracellular antibodies and intracellular cytokines, pellets were measured using BD CANTO II flow cytometer (BD Biosciences, New Jersey, USA) with software BD DIVA version 8.1. Gating strategy and data analysis were performed by FlowJo software, version 10.1 (Ashland, OR, USA).

Statistical Analysis. Data are indicated as mean ± standard deviation (SD). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by Tukey's test for comparisons between groups ($P < 0.05$). The analysis was performed with GraphPad Prism software version 8.0 (San Diego, CA, USA).

■ ASSOCIATED CONTENT

Data Availability Statement

The NMR data for Calancardins A−C (**1**−**3**) have been deposited in nmrXiv at https://nmrxiv.org/P89 and can be found at Calancardin A (#NMRXIV:S741), Calancardin B (#NMRXIV:S743) and Calancardin C (#NMRXIV:S742).

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.4c01394.

Spectrum data (UV, IR, HR-ESI-MS, and NMR (1D and 2D)) of three new compounds (**1**−**3**) (PDF)

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Author Contributions

B.S. and C.B. conceived and designed the project. C.P. performed extraction and isolation. C.P. and B.S. performed structure elucidation. W.C. performed spectroscopy data. C.P. performed *in vitro* experiment in THP-1 monocytes. A.D. performed *in vitro* experiment in MS PBMCs. V.K. and C.B. analyzed flow cytometry data. C.B. designed the antibody panels for mass cytometry. C.C. and P.R. performed in data curation. C.P., V.K., C.B., and B.S. wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research is supported by the 90th Anniversary of Chulalongkorn University, Rachadapisek Sompote Fund (GCUGR1125672001D). C.P. under the supervision of B.S. was funded by the Second Century Fund (C2F) for PhD Scholarship, Chulalongkorn University. We are grateful to Mr. Yanyong Punpreuk, Department of Agriculture, Bangkok, Thailand, for plant identification.

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https://doi.org/10.1021/acs.jnatprod.4c01394
J. Nat. Prod. XXXX, XXX, XXX−XXX