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Curcumane C and (±)-curcumane D, an unusual *seco*-cadinane sesquiterpenoid and a pair of unusual *nor*-bisabolane enantiomers with significant vasorelaxant activity from *Curcuma longa*

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ABSTRACT

A new *seco*-cadinane sesquiterpenoid (curcumane C, **1**) and a pair of new *nor*-bisabolene enantiomers [(+)- and (−)-curcumane D, **2a** and **2b**] were isolated from *C. longa*. Compound **1** possesses an unusual 4,5-*seco*-cadinane skeleton with a tetrahydrophthalide moiety, while **2a** and **2b** contain an unusual 15-*nor*-bisabolene skeleton with a chromone core. All compounds exhibited significant vasorelaxant effects against KCl-induced contraction of rat aortic rings. Compound **1** also exhibited a vasorelaxant effect against phenylephrine-induced contraction of rat aortic rings. Meanwhile, compound **1** showed a stronger vasorelaxant effect in endothelium-intact rat aortic rings compared with endothelium-denuded rat aortic rings, indicating that vasodilation by **1** involved both endothelium-dependent and endothelium-independent pathways. Furthermore, compound **1** increased the NO content in human umbilical vein endothelial cells and its vasorelaxant effect could be attenuated by treatment with L-NAME, an endothelium NO synthase inhibitor. Thus, the underlying vasodilatory mechanisms of **1** may be mediated via abrogation of extracellular Ca^{2+} influx and regulation of NO release in vascular endothelial cells.

1. Introduction

Curcuma longa L. (Zingiberaceae) is a perennial herb widely distributed and cultivated in tropical and subtropical areas of the world. It has been commonly used as a natural flavoring agent and colorant in the food industry [1–3]. Due to its widespread applications as a food additive, the chemical constituents and effects of *C. longa* have attracted extensive attention. Until now, studies investigating the chemical composition of *C. longa* rhizomes have resulted in the isolation of more than 200 compounds, most of which are curcuminoids and sesquiterpenoids [4]. As a food additive, the use of *C. longa* leads to antiplatelet aggregation [5], anticoagulation [6], antiatherosclerosis [7], and cardiovascular protection [8]. Cardiovascular disease (CVD) is a diet-related chronic disease, and is the number one cause of death worldwide. According to the World Health Organization, approximately 17.9 million people were died of CVD in 2016, accounting for 31% of all deaths worldwide [9]. In our ongoing endeavor to explore bioactive natural products with vasorelaxant, antiplatelet, and angiogenic activities that are related to the prevention and treatment of CVD, we have investigated a series of natural plant extracts, such as patchouli oil (an essential oil derived from *Pogostemon cablin*) [10,11] and ethanolic extracts of *Leonurus japonicus* [12–14], *Ligusticum striatum* [15], and *C. longa* [16]. In our previous study of *C. longa*, two novel sesquiterpenoids with unprecedented carbon skeletons and significant vasorelaxant activity were isolated [16]. Thus, this study continues to focus on sesquiterpenoids and their vasorelaxant ability. A new 4,5-*seco*-cadinane sesquiterpenoid (**1**) and a pair of new *nor*-bisabolene enantiomers (**2a** and **2b**) were isolated (Fig. 1). Their vasorelaxant effects were evaluated, and the preliminary vasodilatory mechanisms of compound **1** were investigated.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured using an Anton Paar MCP 200 automatic polarimeter. An Agilent cary 600 FT-IR microscope instrument was used to measure IR spectra. ECD spectra were recorded using an Applied photophysics Chirascan and Chirascan-plus circular dichroism spectrometer. NMR spectra were recorded on a Bruker Avance

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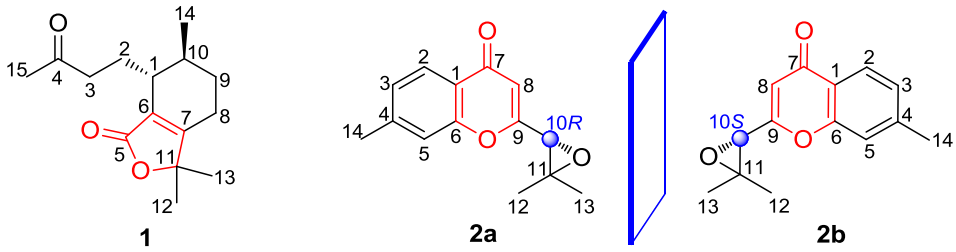

Fig. 1. Structures of compounds **1**, **2a**, and **2b**.

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **1** and **2** in acetone-d_6 (δ in ppm, J in Hz).

Position	Compound **1**		Compound **2**	
	δ_H	δ_C	δ_H	δ_C
1	2.03 m	37.9		122.8
2	1.82 m	27.1	7.96 d (7.8)	126.0
3	2.51 m	41.2	7.30 d (7.8)	127.6
4		207.9		146.2
5		172.6	7.38 s	118.6
6		126.8		157.3
7		168.9		176.8
8	2.28 m	19.7	6.09 s	109.7
9	1.82 m, 1.54 m	25.9		164.9
10	1.84 m	31.1	3.80 s	60.7
11		85.4		62.8
12	1.40 s	25.2	1.50 s	24.4
13	1.38 s	24.9	1.32 s	18.1
14	0.92 d (7.2)	18.8	2.49 s	21.6
15	2.08 s	29.7		

III 600 NMR spectrometer (Bruker Corporation, Billerica, MA, USA) with solvent peaks as internal standards. HRESIMS spectra were measured using a Synapt G2 HDMS instrument (Waters Corporation Milford, MA, USA). TLC was performed using glass-precoated silica gel GF254 plates (Qingdao Marine Chemical Inc., Qingdao, China). Silica gel (200–300 mesh, Yantai Institute of Chemical Technology, Yantai, China) and Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) were used for column chromatography. HPLC separations were carried out using an Agilent 1100 instrument. A Zorbax SB-C18 (250 × 9.4 mm, 5 μm) was employed for reversed-phase (RP) semipreparative HPLC, and a Daicel Chiralpak AD-H (250 × 4.6 mm, 5 μm) was used for normal-phase (NP) enantioseparation. Assays for vasorelaxant activity were conducted with a PL3508B6/C-V Panlab 8 Chamber Organ Bath System (including Stimulating Electrodes, Panlab eight Chamber Organ Baths, Organ Chambers, Tissue Hooks, Labchart Pro software). NO (nitric oxide) content was measured using the 50T/48 NO detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.2. Plant material

The rhizomes of *Curcuma longa* L. (Zingiberaceae) were purchased from Sichuan Neautus Traditional Chinese Medicine Co., Ltd. (Chengdu, China) and identified by Prof. Min Li (Chengdu University of TCM, Chengdu, China). A voucher specimen (CL-20160803) was deposited at the Institute of Innovative Medicine Ingredients of Southwest Specialty Medicinal Materials, Chengdu University of TCM.

2.3. Extraction and isolation

The dried rhizomes of *C. longa* (50 kg) were extracted under reflux with 95% EtOH for three times (3 h, 2 h, and 1.5 h, respectively). The EtOH extract was evaporated under reduced pressure to obtain a yellow residue (7 kg), which was suspended in H_2O and partitioned sequentially with petroleum ether and EtOAc. The EtOAc extract (3 kg) was subjected to silica gel column using a gradient elution of petroleum ether–EtOAc (1:0, 7:3, and 4:6) and EtOAc–MeOH (1:0, 1:1, and 0:1). The fraction eluted by petroleum ether–EtOAc (7:3) was chromatographed over a silica gel column with a gradient of increasing CH_2Cl_2–EtOAc (1:0–0:1) to yield 16 fractions (F_1–F_{16}). Fraction F_6 was further separated via RP-MPLC chromatography with a gradient solvent system (30–100% MeOH in H_2O) to obtain subfractions $F_{6\text{-}1}$–$F_{6\text{-}12}$. Subfraction $F_{6\text{-}6}$ was chromatographed via Sephadex LH-20 column chromatography (petroleum ether–CH_2Cl_2–MeOH, 5:5:1) to obtain $F_{6\text{-}6\text{-}1}$–$F_{6\text{-}6\text{-}6}$. Further purification of $F_{6\text{-}6\text{-}2}$ via preparative TLC (CH_2Cl_2–EtOAc, 20:1) and RP semipreparative HPLC (58% MeOH in H_2O) afforded **1** (7.8 mg). Subfraction $F_{6\text{-}10}$ was further separated into five subfractions $F_{6\text{-}10\text{-}1}$–$F_{6\text{-}10\text{-}5}$ by Sephadex LH-20 column chromatography (petroleum ether–CH_2Cl_2–MeOH, 5:5:1). The $F_{6\text{-}10\text{-}3}$ subfraction was purified by preparative TLC (petroleum–Me_2CO, 2:1), followed by RP semipreparative HPLC (61% MeOH in H_2O) to yield **2** (3.2 mg). Racemic compound **2** was subjected to chiral separation on a Daicel Chiralpak AD-H column (Hexane–EtOH, 1:1) to afford enantiomers **2a** (1.4 mg, t_R = 5.9 min) and **2b** (1.5 mg, t_R = 7.6 min).

Curcumane C **(1)**: Colorless oil; $[\alpha]_D^{25}$ + 43 (*c* 0.15, MeOH); ECD (MeCN) λ_{max} ($\Delta\varepsilon$) 216 (+7.1), 242 (−4.9) nm; UV (MeCN) λ_{max} (log ε) 216 (3.98) nm; IR (ATR) ν_{max} 2979, 2932, 1743, 1712, 1673, 1459, 1366, 1292, 1240, 1203, 1161, 1042, 954, 902, 788 cm^{-1}; ^{1}H NMR (acetone-d_6, 600 MHz) and ^{13}C NMR (acetone-d_6, 150 MHz) data, see Table 1. (+)-HRESIMS *m/z* 273.1477 $[M + Na]^+$ (calcd. for $C_{15}H_{22}O_3Na$, 273.1467).

Curcumane D **(2)**: Colorless oil; {$[\alpha]_D^{25}$ + 54 (*c* 0.02, MeOH); ECD (MeCN) λ_{max} ($\Delta\varepsilon$) 207 (+18.1), 241 (+0.5), 263 (+16.7) nm; **2a**}; {$[\alpha]_D^{25}$ − 50 (*c* 0.02, MeOH); ECD (MeCN) λ_{max} ($\Delta\varepsilon$) 213 (−6.2), 236 (+3.2), 263 (−15.5) nm; **2b**}; UV (MeCN) λ_{max} (log ε) 302 (3.41), 293 (3.39), 266 (3.52), 227 (3.75) nm; IR (ATR) ν_{max} 2979, 1679, 1642, 1453, 1424, 1380, 1303, 1203, 1179, 1137, 968, 868, 828, 804, 721 cm^{-1}; ^{1}H NMR (acetone-d_6, 600 MHz) and ^{13}C NMR (acetone-d_6, 150 MHz) data, see Table 1. (+)-HRESIMS *m/z* 253.0851 $[M + Na]^+$ (calcd. for $C_{14}H_{14}O_3Na$, 253.0841).

2.4. Animals and cell culture

Male Sprague-Dawley rats (200–220 g) were obtained from Da Shuo Biotechnology Co., Ltd (Chengdu, Sichuan, China). All rats were maintained on a 12 h light/dark cycle under a controlled temperature at 25 ± 1 °C and a relative humidity of 50 ± 5%. All the experimental procedures were performed in accordance with the guidelines of the Management Committee for Experimental Animals, China.

Human umbilical vein endothelial cells (HUVECs) were purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). HUVECs were grown at 37 °C in a humidified atmosphere with 5% CO_2. The cells were seeded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.5. Effects of compounds 1 and 2 on KCl- or PHE-induced contractions of rat aortic rings

As described previously [11,12], the rat was anesthetized by intraperitoneal injection with chloral hydrate. Its thoracic aorta was carefully dissected and immediately transferred into oxygenated Krebs-Henseleit (K-H) solution [composition (mM): NaCl, 120; KCl, 4.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 10; $CaCl_2$, 2.5] at 4 °C. The aorta was cleaned of surrounding fat and connective tissues and cut into rings approximately 3–5 mm in length. The aortic rings were suspended in K-H solution at a constant temperature of 37 °C and bubbled with a gas mixture of 95% O_2 and 5% CO_2. All the rings were equilibrated for 1 h under an initial tension of 1 g in normal K-H solution before the start of the experiment. The aortic rings were pre-contracted with 60 mM KCl or 1 μM phenylephrine (PHE) solution. When the rings were stably contracted, the cumulative concentrations of the test compounds (0.25, 0.75, 2.5, 7.5, and 25 μM) were added into the organ bath, and the effects were recorded. Methoxyverapamil and phentolamine mesylate were used as positive controls in KCl- and PHE-induced contraction models, respectively. The EC_{50} and E_{max} (maximal vasorelaxation) values of the test compounds and the positive drugs were calculated from the cumulative concentration–tension curves via linear regression analysis.

2.6. Effects of compound 1 on rat aortic rings without endothelium [11]

Vasorelaxation occurs via endothelium-dependent and -independent pathways, and therefore, the vasorelaxant effects of **1** on endothelium-intact (E+) and endothelium-denuded (E−) aortic rings were compared. The endothelium of aortic rings was mechanically removed by gently rubbing inside the lumen of the rings using a pair of small forceps. The endothelium was removed successfully based on less than 10% relaxation to 10 μM acetylcholine (Ach) in response to pre-treatment of the aortic rings with 1 μM PHE. Cumulative concentrations of **1** (0.25, 0.75, 2.5, 7.5, and 25 μM) were used to detect the contractile tension of E+ and E− aortic rings, when the contractions of E+ and E− arterial rings were stimulated by 1 μM PHE and reached a plateau. The EC_{50} and E_{max} values of **1** on E+ and E− aortic rings were calculated.

2.7. Effects of compound 1 on NO production in HUVECs [17]

NO is a key factor in the vasodilation of vascular endothelium. To investigate the effect of **1** on NO production of HUVECs, cells were incubated with **1** for 1 h. The level of NO in the supernatant of HUVECs was measured using a NO detection kit at 550 nm according to the manufacturer's instructions.

2.8. Effects of compound 1 on rat aortic rings incubated with an eNOS inhibitor (L-NAME) [11,17]

NO in vascular endothelial cells is mainly produced by endothelial nitric oxide synthase (eNOS). To analyze the vasorelaxant effect of **1** via the eNOS/NO pathway, the aortic rings were pre-incubated with 100 μM of an eNOS inhibitor, L-NAME [N(ω)-nitro-L-arginine methyl ester] for 30 min to block eNOS activity before the PHE-induced contraction. The cumulative concentration of **1** (0.25, 0.75, 2.5, 7.5, and 25 μM) was added after the aortic rings were stably contracted. The EC_{50} and E_{max} values of **1** on the aortic rings pre-incubated with L-NAME were calculated.

2.9. Statistical analysis

All data are presented as mean ± SEM. Relaxant responses were expressed relatively as tension percentages based on KCl- or PHE-induced maximal contractile tension considered as 100%. The drug concentrations for 50% maximal effects (EC_{50}) and maximal vasorelaxation (E_{max}) values were analyzed via non-linear curve fitting using GraphPad Prism 5. Statistical analysis was performed using Student's *t*-test and $P < 0.05$ was considered to be statistical significance.

3. Results and discussion

Compound **1** was obtained as a colorless oil with a molecular formula ($C_{15}H_{22}O_3$), suggesting five degrees of unsaturation based on a quasi-molecular ion at *m/z* 273.1477 $[M + Na]^+$ (calcd. for $C_{15}H_{22}O_3Na$, 273.1467) in its HRESIMS. The IR spectrum of **1** exhibited characteristic absorption bands for carbonyl (1743 and 1712 cm^{-1}) and olefinic (1673 cm^{-1}) functionalities. The ^1H NMR spectrum of **1** showed signals of three tertiary methyl groups (δ_H 1.38, 1.40, and 2.08) and one secondary methyl group (δ_H 0.92, d, J = 7.2 Hz) (Table 1). The ^{13}C NMR and DEPT data (Table 1) revealed the presence of 15 carbons, including four methyls (δ_C 18.8, 24.9, 25.2, and 29.7), four methylenes (δ_C 19.7, 25.9, 27.1, and 41.2), two methines (δ_C 31.1 and 37.9), and five quaternary carbons (one oxygenated carbon at δ_C 85.4, two olefinic carbons at δ_C 126.8 and 168.9, and two carbonylic carbons at δ_C 172.6 and 207.9). The above NMR spectroscopic data suggested that **1** was an analogue of phacadinane D [18], an unusual 4,5-*seco*-cadinane sesquiterpenoid with a phthalide moiety identified in *C. phaeocaulis*. The two compounds differed by the absence of the hydroxy group at C-8 and the reduction of double bonds ($\Delta^{8,9}$ and $\Delta^{1,10}$) in **1**. This deduction was further confirmed by the ^1H–^1H COSY correlations of H_2-3/H_2-2/H-1/H-10/H_2-9/H_2-8, and H-10/H_3-14, as well as HMBC correlations of H_3-14 with C-1, C-9, and C-10, of H-1 with C-6, C-7, C-9, and C-14, of H_2-8 with C-6, C-7, C-9, and C-10, of H_3-15 with C-3 and C-4, of H-3 with C-1, C-2, and C-4, and of H_3-13 with C-7, C-11, and C-12 (Fig. 2). In the 1D-NOE experiment of **1**, H_3-14 was enhanced when H-1 was irradiated. Thus, H_3-14 and H-1 were located in the same orientation. Furthermore, the absolute configuration of **1** was assigned as 1*R*,10*S* via comparison of its calculated electronic circular dichroism (ECD) data with the experimental ECD data (Fig. 3).

Compound **2** was also obtained as a colorless oil. It had the molecular formula $C_{14}H_{14}O_3$, as indicated by HRESIMS at *m/z* 253.0851 $[M + Na]^+$ (calcd. for $C_{14}H_{14}O_3Na$, 253.0841). The ^1H NMR spectrum of **2** displayed resonances attributed to three tertiary methyl groups (δ_H 1.32, 1.50, 2.49), one oxymethine (δ_H 3.80, s), a set of aromatic ABX

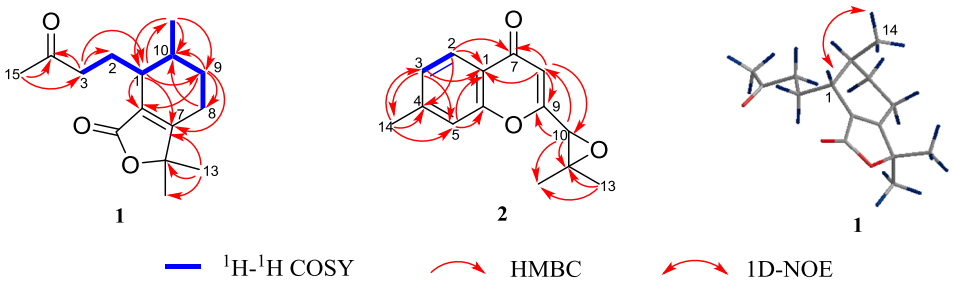

Fig. 2. Key $^1H-^1H$ COSY, HMBC, and 1D-NOE correlations of **1** and **2**.

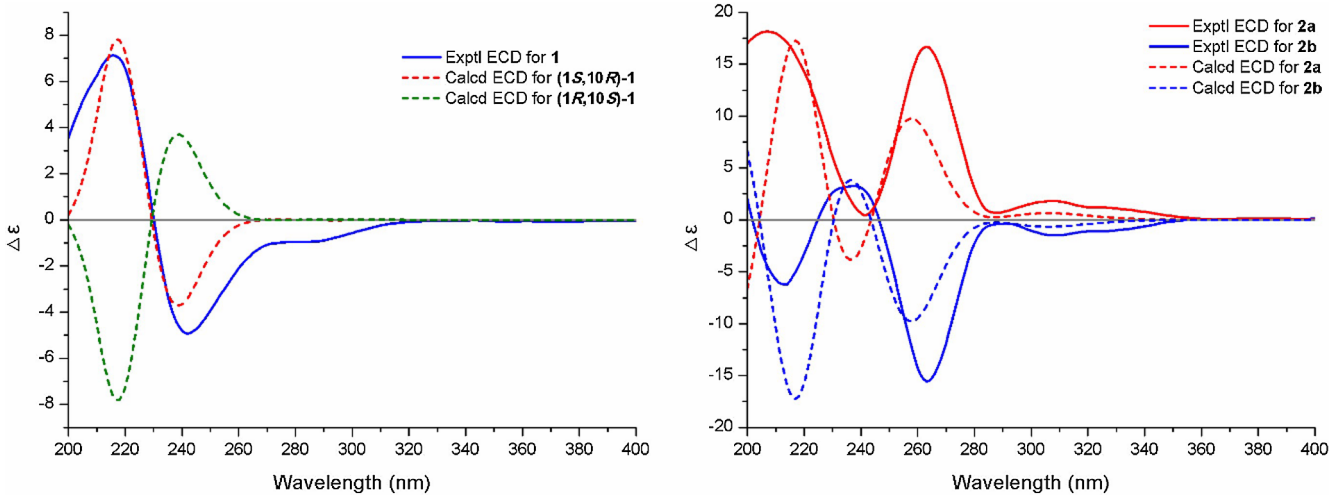

Fig. 3. Experimental and calculated ECD spectra of **1** and **2**.

system protons [δ_H 7.38 (s), 7.30 (d, J = 7.8 Hz), and 7.96 (d, J = 7.8 Hz)], and one olefinic proton (δ_H 6.09, s) (Table 1). The ^{13}C NMR and DEPT spectra of **2** exhibited 14 carbon resonances (Table 1) corresponding to the above protonated units and six quaternary carbons (one carbonylic carbon, δ_C 176.8; three aromatic carbons, δ_C 122.8, 146.2, and 157.3; one olefinic carbon, δ_C 164.9; and one oxygenated carbon, δ_C 62.8). These spectroscopic data suggested that **2** was a *nor*-bisabolane [19]. The HMBC correlations (Fig. 2) from H-2 to C-4, C-6, and C-7; from H-3 to C-1 and C-5; from H-5 to C-1, C-3, and C-6; from H_3-14 to C-3, C-4, and C-5; and from H-8 to C-1, C-7, and C-9, together with the chemical shifts of C-6 (δ_C 157.3) and C-9 (δ_C 164.9) [20] and the degrees of unsaturation, indicated the presence of a chromone core with a methyl group at C-4 in compound **2**. The side chain at C-9 was determined by the HMBC correlations from H-10 to C-8, C-9, C-11, and C-12. The chemical shifts of C-10 (δ_C 60.7) and C-11 (δ_C 62.8), combined with the remainder of one degree of unsaturation revealed a 10,11-epoxy unit in **2**. Thus, the planar structure of compound **2** was determined, as shown in Fig. 2. Surprisingly, the ECD spectrum of **2** showed no Cotton effect, suggesting that it may be isolated as a racemic mixture. A subsequent chiral HPLC separation yielded a pair of enantiomers (**2a** and **2b**). Their absolute configurations were determined by the same calculated ECD method as described above. It was found that the calculated ECD data of 10*R*-**2** agreed well with the experimental ECD of **2a**, naturally elucidating the absolute configuration of **2b** as 10*S* (Fig. 3).

Curcuma longa is an attractive food additive and phytomedicine, which can be used to promote blood circulation and remove blood stasis [4]. Since two new sesquiterpenoids isolated from *C. longa* were found to manifest significant vasorelaxant activity in our previous study [16], compounds **1**, **2a**, and **2b** were assayed for their vasorelaxant effects on rat aorta rings. As shown in Fig. 4, all three compounds exhibited a concentration-dependent relaxation in rat aorta rings pre-contracted with KCl, without any significant difference in vasorelaxation effect with enantiomers **2a** and **2b**. The EC_{50} values of **1**, **2a**, **2b**, and the positive control (methoxyverapamil) against KCl-induced contractions were 6.61 ± 0.24, 14.56 ± 1.03, 16.03 ± 1.34, and 0.51 ± 0.09 μM, respectively. The E_{max} values of **1**, **2a**, **2b**, and methoxyverapamil against KCl-induced contractions were 70.66 ± 5.39%, 62.17 ± 5.51%, 60.42 ± 4.23%, and 100.00%, respectively. Although curcumanes C (**1**) and D (**2a** and **2b**) isolated in this study and curcumanes A and B reported previously [16] are different types of sesquiterpenoids with large structural differences, they all showed significant vasorelaxant activity. The effects of curcumanes C and D were weaker than those of curcumanes A and B. The above two studies indicate that sesquiterpenoids are the main active compounds of

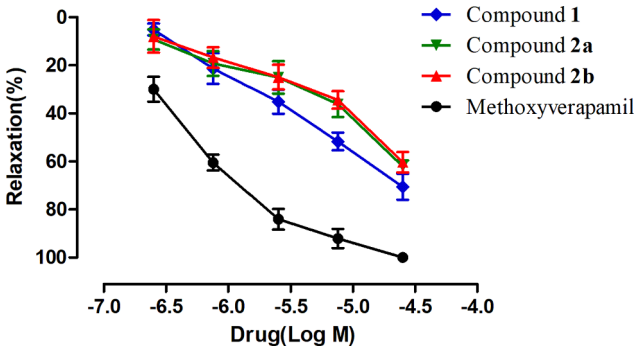

Fig. 4. Vasorelaxant effects of **1**, **2a**, **2b**, and methoxyverapamil on rat aortic rings pre-contracted with KCl (n = 5).

C. longa for the vasorelaxant effect, and the effect of sesquiterpenoids may be closely related to their subtypes and oxidation degree.

Compound **1** with the optimal vasorelaxant effect was selected for further investigation of the underlying vasodilatory mechanism in aortic rings. It showed a concentration-dependent relaxation in rat aorta rings pre-contracted with PHE (Fig. 5), and the vasorelaxant activity against PHE-induced contractions was better than in KCl-induced contractions. The EC_{50} values for **1** and the positive control (phentolamine mesylate) were 4.26 ± 0.43 μM and 66.09 ± 1.33 nM, respectively, and the E_{max} values were 76.70 ± 4.75% and

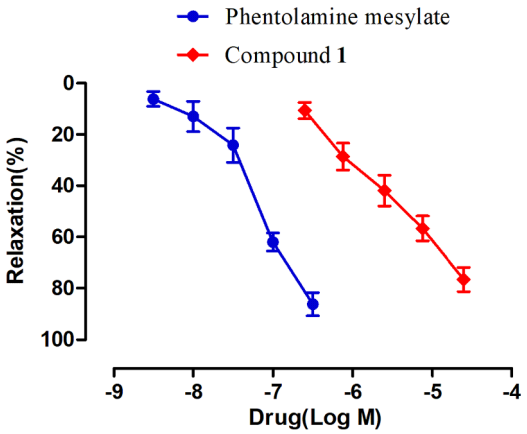

Fig. 5. Vasorelaxant effect of **1** on rat aortic rings pre-contracted with PHE (n = 5).

86.22 ± 1.56%. It is well known that the contraction of vascular smooth muscle is modulated by the concentration variation in Ca^{2+}. Elevation of the Ca^{2+} level occurs via intracellular Ca^{2+} release and/or extracellular Ca^{2+} influx, and extracellular Ca^{2+} influx results from the opening of voltage-dependent calcium channels (VDCCs) and receptor-operated calcium channels (ROCCs) [21,22]. K^+-depolarization (KCl)-activated Ca^{2+} entry facilitated sustained force maintenance in vascular smooth muscle via activation of VDCCs [23], while the PHE-induced contraction was induced by the activation of ROCCs via stimulation of the α_1 adrenoceptor [24]. Thus, the results suggest that compound **1** possibly inhibited extracellular Ca^{2+} influx via both VDCCs and ROCCs, resulting in vasorelaxation.

Vascular endothelial cells usually produce and release a series of crucial vascular endothelium-derived relaxing factors, such as nitric oxide (NO) [25], prostacyclin [26], and endothelium-derived hyperpolarizing factor [27,28], which regulate the vasorelaxant effect by controlling the level of Ca^{2+} [29,30]. Depending on whether the drug affects vascular endothelial cells, the mechanisms of vasorelaxation are classified into endothelium-dependent and endothelium-independent pathways. Therefore, the vasorelaxant effects of **1** on endothelium-intact (E+) and endothelium-denuded (E−) rat aortic rings pre-contracted with PHE were compared. As shown in Fig. 6, the relaxant effect of **1** on E+ was significantly stronger compared with E–, and the E_{max} values of **1** for E+ and E– were 76.70 ± 4.75% and 41.44 ± 5.89%, respectively. These findings suggest that the mechanisms of vasorelaxation by **1** are mediated via both endothelium-dependent and endothelium-independent pathways.

NO synthesized and secreted by vascular endothelial cells is an endothelium-derived relaxing factor, which plays a crucial role in regulating the vascular tone [31]. In normal vascular endothelial cells, NO is principally generated by endothelial nitric oxide synthase (eNOS) [32]. Since the vasorelaxant effect of **1** was found to be endothelium dependent, our further investigations focused on the effect of **1** on NO production in HUVECs. At a concentration of 50 μM, the compound **1** increased NO production in HUVECs significantly (Fig. 7).

To further confirm that the vasorelaxant effect of compound **1** is associated with NO production and release, the endothelium-intact rat aortic rings were pre-incubated with an endothelium NO synthase inhibitor, L-NAME. The results showed that the vasorelaxant effect against PHE-induced contraction decreased significantly in the group pre-treated with L-NAME (E_{max} = 17.35 ± 3.81%), when compared with the group exposed to compound **1** alone (E_{max} = 76.70 ± 4.75%) (Fig. 8). The results indicated that compound **1** induced vasorelaxation via NO release by vascular endothelial cells. NO in organisms is catalyzed by nitric oxide synthase (NOS) [33], which is divided into

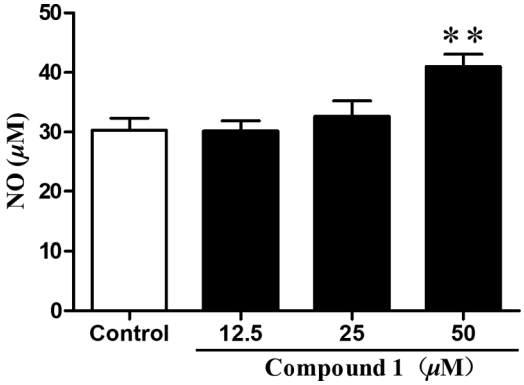

Fig. 7. Effects of **1** on NO production in HUVECs (**P < 0.01 vs. control group, n = 5).

neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) [34]. In normal vascular endothelial cells, NOS is mainly expressed as eNOS [32]. Since the PI3K/AKT signaling pathway is the main important factor regulating eNOS [35], we speculated that **1** may promote the synthesis of NO by eNOS through activating PI3K/AKT pathway. Further experimental verification will be performed in the following study.

4. Conclusion

In conclusion, a new 4,5-*seco*-cadinane sesquiterpenoid (**1**) and a pair of new *nor*-bisabolene enantiomers (**2a** and **2b**) were isolated from the rhizomes of *C. longa*, a natural flavoring agent and colorant manifesting cardiovascular protective effects. Interestingly, all compounds exhibited a potent vasorelaxant effect on rat aortic rings pre-contracted by KCl. Moreover, the compound **1** had a vasorelaxant effect against PHE-induced contraction in rat aortic rings. The underlying mechanisms of action of **1** may involve inhibition of extracellular Ca^{2+} influx via both VDCCs and ROCCs and stimulation of NO release in vascular endothelial cells. Thus, it can be speculated that **1** and **2** are pharmacodynamic compounds of *C. longa* that can be used to activate blood circulation and prevent CVD.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

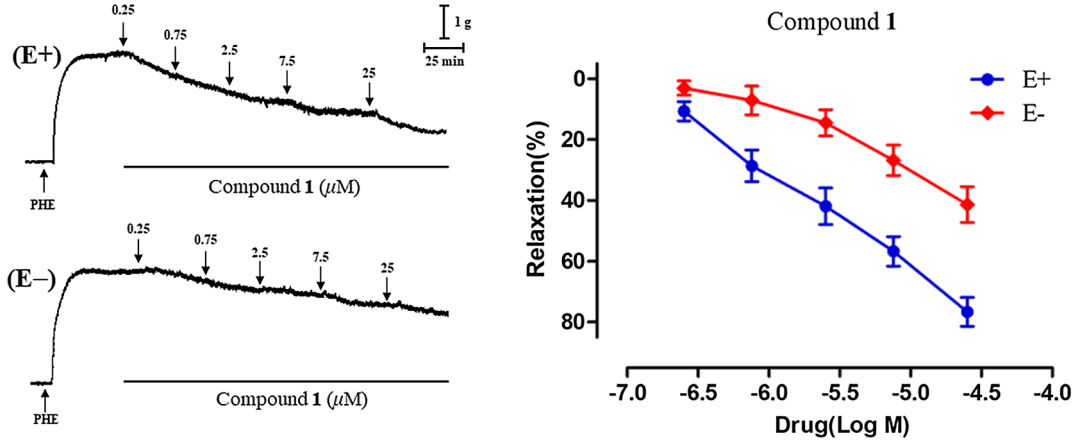

Fig. 6. Vasorelaxant effects of **1** on endothelium-intact (E+) and endothelium-denuded (E−) rat aortic rings (n = 5).

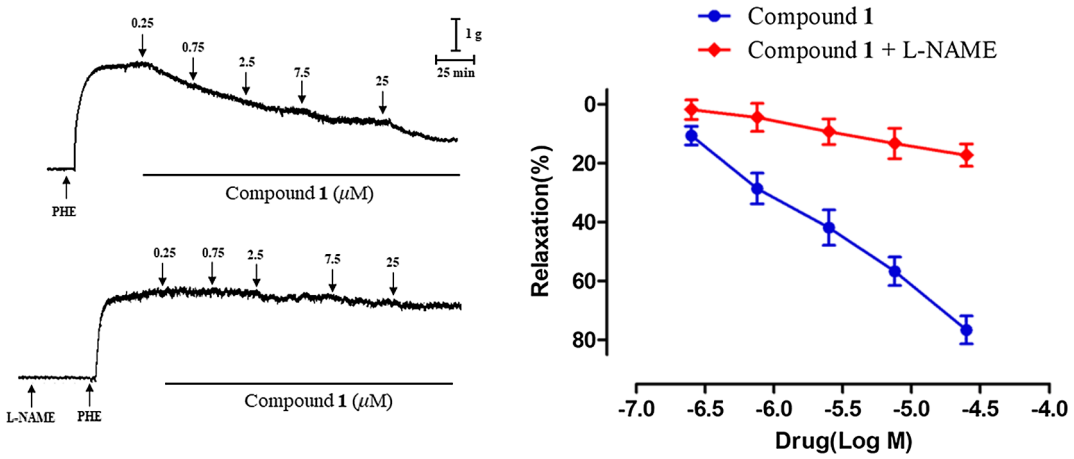

Fig. 8. Vasorelaxant effects of **1** on PHE-contracted rat aortic rings incubated with or without L-NAME (n = 5).

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