Bioorganic & Medicinal Chemistry 18 (2010) 175–181

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Anti-inflammatory diterpenes from the seeds of *Vitex negundo*

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ARTICLE INFO

*Article history:*
Received 22 September 2009
Revised 31 October 2009
Accepted 3 November 2009
Available online 12 November 2009

*Keywords:*
Anti-inflammatory
Diterpenes
*Vitex negundo* seeds

ABSTRACT

Phytochemical investigation of a dichloromethane-soluble extract of *Vitex negundo* seeds led to the isolation of five labdane diterpenes, negundoins A–E (**1**–**5**), a 9,10-*seco*-abietane diterpene, negundoin F (**6**), a sandaracopimara-7,15-diene diterpene, negundoin G (**7**), and two known diterpene derivatives (**8**, **9**). Their chemical structures were elucidated by detailed spectroscopic analyses on the basis of NMR, IR, and MS data. The anti-inflammatory effects of metabolites **1**–**7** were also evaluated in vitro. Compounds **3** and **5** were among the most potent inhibitors on nitric oxide production by LPS-stimulated RAW 264.7 macrophages, with $IC_{50}$ values of 0.12 and 0.23 μM, respectively. Further studies revealed that compounds **3** and **5** (5 μM) significantly reduced the levels of the iNOS protein to 0.40 ± 0.13% and 41.02 ± 6.02%, respectively, and COX-2 protein to 2.06 ± 0.53% and 26.40 ± 7.43%, respectively.



## 1. Introduction

*Vitex negundo* L. (Verbenaceae) flourishes abundantly in wastelands and is widely distributed in tropical to temperate regions, being a native of South Asia, China, Japan, Indonesia, East Africa, and South America. The seeds of this plant have been widely used in folk medicine for anti-inflammatory,[1] analgesic,[2] and antioxidant[3] purposes. The presence of lignans,[3,5] flavonoids,[4,6] and terpenoids[1,3] has been previously reported. In the course of our investigations for bioactive constituents from *V. negundo* seeds, we have recently isolated six new lignans, named vitexdoins A–E and vitedoamine B, which exhibited significant anti-inflammatory activity in vitro.[7] Our continuous chemical examination of this medicinal seeds led to the isolation of seven new diterpenes, negundoins A–G (**1**–**7**), together with two known diterpenes (**8**, **9**). We report herein the details of isolation, structure elucidation, and anti-inflammatory effects of the isolated compounds.

## 2. Results and discussion

The dichloromethane-soluble extract of *V. negundo* seeds was successively subjected to silica gel, Sephadex LH20, and Chromatorex ODS column chromatography as well as preparative TLC to afford **1**–**7**, negundoins A–G. Compounds **8** and **9** were identified as vitedoin B[3] and 3β-hydroxy-abieta-8,11,13-trien-7-one,[8] respectively, on the basis of their spectroscopic data.

Compound **1**, trivially named negundoin A, was obtained as colorless syrup and analyzed for the molecular formula $C_{22}H_{34}O_5$ by positive HRESIMS $[M+Na]^+$ at *m*/*z* 401.2307 (calcd 401.2304), which was supported by its NMR data. The $^1H$ and $^{13}C$ NMR spectra of **1** (Table 1) together with DEPT and $^1H$–$^{13}C$ COSY experiments indicated the presence of an oxygenated trisubstituted olefin ($\delta_H$ 5.30; $\delta_C$ 178.5, 87.3), two carboxyl groups ($\delta_C$ 170.8, 169.3), a methylene ($\delta_H$ 3.12, 3.04; $\delta_C$ 31.5) adjacent to the olefinic group, an oxygenated methane ($\delta_H$ 4.47, dd, $J$ = 4.6, 11.6 Hz; $\delta_C$ 80.2), and a methoxyl group ($\delta_H$ 3.65; $\delta_C$ 50.5). A one-proton multiplet at $\delta_H$ 1.78 was assigned to H-8 and signal at $\delta_H$ 1.58 assigned to H-5. In addition, four methyl singlets at $\delta_H$ 2.04, 0.95, 0.89, and 0.86 were assigned to methyl groups at C-2′, C-19, C-17, and C-18, respectively, and a methyl doublet signal ($\delta_H$ 0.77, d, $J$ = 6.6 Hz) was due to the secondary methyl group at C-16. The positions of a methoxyl group and an acetoxyl group, at C-15 and C-3, respectively, were established by HMBC experiments. A conjugation of the oxygenated olefinic group with the carboxyl group (C-15, $\delta_C$ 169.3) was determined with the aid of HSQC and HMBC techniques. The geometry of $\Delta^{13}$ was determined to be *E* on the basis of NOESY spectra. The NOSEY correlations were detected between H-14/H-16, H-8/H-19, H-11/H-19, H-18/H-19, H-5/H-16, and H-3/H-17, indicating a β-orientation for H-8, H-18, and H-19 and an α-orientation for H-3, H-5, H-16, and H-17. These data suggested **1** was a labdane-type diterpene possessing a spiro-tetrahydrofuran ring and an α,β-unsaturated carboxyl system, characterized as illustrated in Figure 1. The structure of **1** was therefore defined as (3*S**, 5*S**, 8*R**, 9*R**, 10*S**)-3-acetoxy-9,13-epoxy-16-norlabda-13*E*-en-15-oic acid methyl ester.

Compound **2**, trivially named negundoin B, was obtained as colorless syrup and gave the molecular formula $C_{21}H_{32}O_5$ as determined by positive HRESIMS $[M+Na]^+$ at *m*/*z* 387.2150 (calcd 387.2147), which was supported by its NMR data. The $^{13}C$ NMR spectra of **2**

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doi:10.1016/j.bmc.2009.11.004

**Table 1**
$^{1}$H NMR and $^{13}$C NMR data of compounds **1**–**3**

| No. | **1** | | **2** | | **3** | |
|---|---|---|---|---|---|---|
| | $\delta_H$ ($J$ in Hz) | $\delta_C$ | $\delta_H$ ($J$ in Hz) | $\delta_C$ | $\delta_H$ ($J$ in Hz) | $\delta_C$ |
| 1 | 1.38 (2H, m) | 29.3 t | 1.37 (2H, m) | 29.3 t | 1.40 (2H, m) | 29.3 t |
| 2 | 1.71 (2H, m) | 23.3 t | 1.70 (2H, m) | 23.3 t | 1.73 (2H, m) | 23.1 t |
| 3 | 4.47 (1H, dd, $J$ = 4.6, 11.6) | 80.2 d | 4.48 (1H, dd, $J$ = 4.2, 12.0) | 80.1 d | 4.46 (1H, dd, $J$ = 4.2, 12.0) | 79.9 d |
| 4 | | 37.7 s | | 37.7 s | | 37.7 s |
| 5 | 1.58 (1H, m) | 46.2 d | 1.58 (1H, m) | 46.2 d | 1.59 (1H, m) | 46.2 d |
| 6a | 1.63 (1H, m) | 20.9 t | 1.63 (1H, m) | 20.9 t | 1.63 (1H, m) | 20.8 t |
| 6b | 1.38 (1H, m) | | 1.40 (1H, m) | | 1.42 (1H, m) | |
| 7a | 1.55 (1H, m) | 31.0 t | 1.56 (1H, m) | 31.0 t | 1.60 (1H, m) | 30.9 t |
| 7b | 1.37 (1H, m) | | 1.35 (1H, m) | | 1.43 (1H, m) | |
| 8 | 1.78 (1H, m) | 36.5 d | 1.79 (1H, m) | 36.6 d | 1.85 (1H, m) | 36.5 d |
| 9 | | 97.7 s | | 98.3 s | | 99.4 s |
| 10 | | 42.0 s | | 41.9 s | | 41.9 s |
| 11a | 2.08 (1H, m) | 26.7 t | 2.09 (1H, m) | 26.6 t | 2.16 (1H, m) | 26.3 t |
| 11b | 1.80 (1H, m) | | 1.80 (1H, m) | | 1.87 (1H, m) | |
| 12a | 3.12 (1H, m) | 31.5 t | 3.14 (1H, m) | 31.9 t | 3.11 (1H, m) | 30.1 t |
| 12b | 3.04 (1H, m) | | 3.02 (1H, m) | | 3.05 (1H, m) | |
| 13 | | 178.5 s | | 180.4 s | | 182.2 s |
| 14 | 5.30 (1H, s) | 87.3 d | 5.29 (1H, s) | 87.0 d | 5.61 (1H, d, $J$ = 7.8) | 100.7 d |
| 15 | | 169.3 s | | 173.3 s | 9.54 (1H, d, $J$ = 7.8) | 190.4 d |
| 16 | 0.77 (3H, d, $J$ = 6.5) | 15.5 q | 0.78 (3H, d, $J$ = 6.6) | 15.5 q | 0.78 (3H, d, $J$ = 6.6) | 15.5 q |
| 17 | 0.89 (3H, s) | 27.9 q | 0.89 (3H, s) | 27.9 q | 0.89 (3H, s) | 27.9 q |
| 18 | 0.86 (3H, s) | 16.5 q | 0.87 (3H, s) | 16.5 q | 0.87 (3H, s) | 16.5 q |
| 19 | 0.95 (3H, s) | 16.8 q | 0.96 (3H, s) | 16.8 q | 0.98 (3H, s) | 16.8 q |
| 1′ | | 170.8 s | | 170.8 s | | 170.8 s |
| 2′ | 2.04 (3H, s) | 21.3 q | 2.05 (3H, s) | 21.3 q | 2.04 (3H, s) | 21.2 q |
| 15-$OCH_3$ | 3.65 (3H, s) | 50.5 q | | | | |

Multiplicity was determined by DEPT experiments (s = quaternary, d = methine, t = methylene, q = methyl).

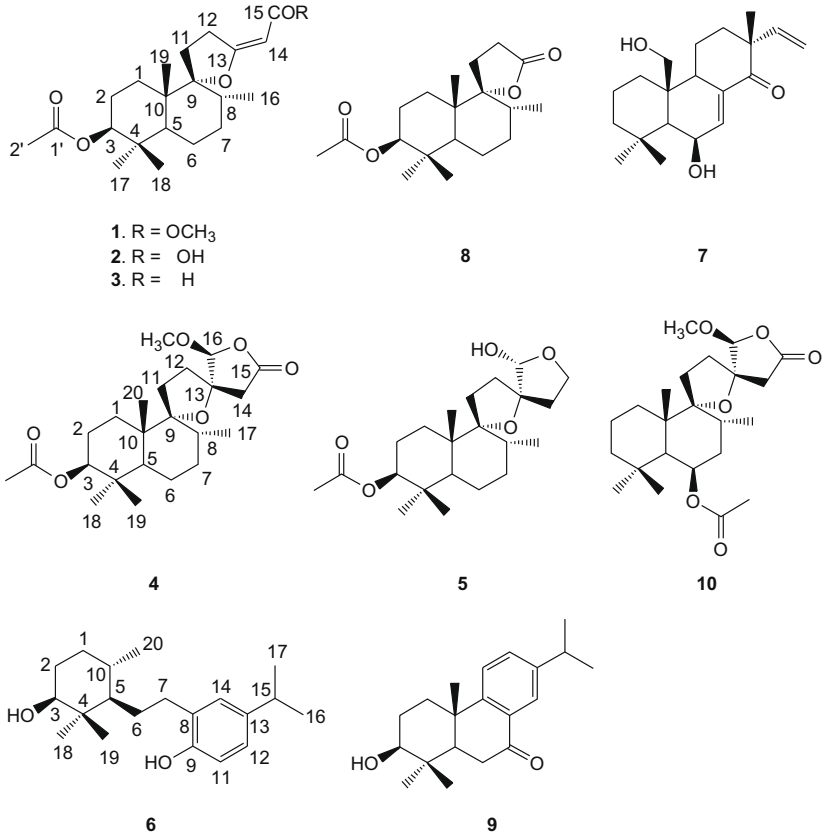


**Figure 1.** Diterpenes from the seeds of *Vitex negundo*.

are summarized in Table 1 and suggested the presence of most structural features in common with compound **1**, apart from three slightly shifted signals due to the α,β-unsaturated carboxyl group (C-13 at $\delta_C$ 180.4, C-14 at $\delta_C$ 87.0, and C-15 at $\delta_C$ 173.3 for **2**, in contrast to 178.5, 87.3, and 169.3 for **1**, respectively) and the disappearance of the signal corresponding to a methoxyl group. Considering the molecular

weights of **2** and **1**, compound **2** was deduced to be a 15-*O*-demethyl derivative of **1**. Furthermore, all of the correlations supporting the structure of **1** were also observed in the 2D NMR spectroscopic data of **2**, suggesting that the configuration of **2** was the same as **1**. Accordingly, compound **2** was identified as (3*S**, 5*S**, 8*R**, 9*R**, 10*S**)-3-acetoxy-9,13-epoxy-16-norlabda-13*E*-en-15-oic acid.

Compound **3**, trivially named negundoin C, was obtained as colorless syrup and the molecular formula was established as $C_{21}H_{32}O_4$ by positive HRESIMS $[M+Na]^+$ at *m*/*z* 371.2199 (calcd 371.2198) and confirmed by $^{13}C$ NMR and DEPT analysis. The IR spectrum revealed absorption bands of an α,β-unsaturated aldehyde group (1655 and 1626 $cm^{-1}$). Its $^1H$ and $^{13}C$ NMR spectra were closely analogous to that of **2**, except for the appearance of a new doublet due to an aldehyde proton ($\delta_H$ 9.54, d, *J* = 7.8 Hz) coupled with an oxygenated trisubstituted olefinic proton ($\delta_H$ 5.61, d, *J* = 7.8 Hz). Inspection of HSQC and DEPT spectra of **3** confirmed the presence of five methyls, six methylenes, five methines, and five quaternary carbons. The molecular weight of **3** is only one less oxygen atom than that of **2**. Therefore, compound **3** was considered to be a 15-dehydroxy derivative of **2**. The NOSEY correlations were detected between H-12/H-15, H-8/H-19, H-11/H-19, H-18/H-19, H-5/H-16, and H-3/H-17, indicating a β-orientation for H-8, H-18, and H-19 and an α-orientation for H-3, H-5, H-16, and H-17. On the basis of these data, compound **3** was finally deduced as (3*S**, 5*S**, 8*R**, 9*R**, 10*S**)-3-acetoxy-9,13-epoxy-16-norlabda-13*E*-en-15-al.

Compound **4**, trivially named negundoin D, was obtained as colorless syrup and gave the molecular formula $C_{23}H_{36}O_6$, as determined by positive HRESIMS $[M+Na]^+$ at *m*/*z* 431.2407 (calcd 431.2410). The NMR spectra of **4** suggested the presence of general structural features in common with (*rel* 5*S*, 6*R*, 8*R*, 9*R*, 10*S*, 13*S*, 16*S*)-6-acetoxy-9,13-epoxy-16-methoxy-labdan-15,16-olide (**9**), which was previously isolated from the fruits of *Vitex rotundifolia*.[9] The only differences were the splitting pattern and chemical shift of an oxygenated methane proton ($\delta_H$ 4.46, dd, *J* = 4.4, 11.4 Hz in **4**; $\delta_H$ 5.38, ddd, *J* = 3.0, 3.0, 3.0 Hz in **10**) and the chemical shift corresponding to the oxygenated methane carbon signal ($\delta_C$ 80.7 in **4**; $\delta_C$ 70.4 in **10**). These data led to the assumption that **4** was a regioisomer of **10** with an acetoxyl group at C-3 instead of C-6, which was confirmed by detailed HMBC and $^1H$–$^1H$ COSY spectroscopic analysis. In the NOESY spectra of **4**, correlations were observed between H-8/H-20, H-19/H-20, H-3/H-18, H-5/H-3, H-14/H-17 and H-1/H-16, indicating a β-orientation for H-8, H-19, and H-20 and an α- orientation for H-3, H-5, H-17, and H-18. The β-configuration for the methoxyl group at C-16 was confirmed from the low-field resonance of Ha-12 ($\delta_H$ 2.44), which was deshielded by the methoxyl group at C-16.[10,11] Compound **4** was therefore characterized as (3*S**, 5*S**, 8*R**, 9*R**, 10*S**, 13*S**, 16*S**)-3-acetoxy-9,13-epoxy-16-methoxy-labda-15,16-olide.

Compound **5**, trivially named negundoin E, was obtained as colorless syrup and analyzed for the molecular formula $C_{22}H_{36}O_5$ by positive HRESIMS $[M+Na]^+$ at *m*/*z* 403.2463 (calcd 403.2460), which was supported by its NMR data. The $^1H$ NMR spectrum of **5** indicated signals due to three tertiary methyl groups ($\delta_H$ 0.85, 0.80, 0.79), one secondary methyl group ($\delta_H$ 0.81, d, *J* = 6.6 Hz), one acetyl group ($\delta_H$ 1.97), two oxygenated methine protons ($\delta_H$ 4.40, dd, *J* = 4.4, 11.4 Hz; $\delta_H$ 4.68, s), and two oxygenated methylene protons ($\delta_H$ 4.04, m; $\delta_H$ 3.71, m). The $^{13}C$ NMR spectrum of **5** gave 22 carbon signals, including one carboxyl carbon ($\delta_C$ 170.7), one acetal carbon ($\delta_C$ 98.9), one oxygenated methylene carbon ($\delta_C$ 64.9), one oxygenated methine carbon ($\delta_C$ 80.5) and two oxygenated quaternary carbons ($\delta_C$ 93.7, 90.8). These data suggested that **5** was a labdane-type diterpene having a hydroxyl group, an acetoxy group, and two spiro-tetrahydrofuran rings. Further comparison of the $^1H$ and $^{13}C$ NMR data of **5** with those of **4** led to the assumption that **5** is a 15-deoxy-16-*O*-demethyl derivative of **4**. The configuration of the hydroxyl group at C-16 was determined to be α on the basis of NOESY spectra, which was further evidenced by the non-deshielded signal of H-12 ($\delta_H$ 1.87, 2H) compared to that of **4** ($\delta_H$ 2.44, Ha; 1.81, Hb).[11] Key NOSEY correlations were detected between H-16/H-1, H-16/H-12, H-8/H-20, H-18/H-3, H-3/H-5, H-5/H-18, and H-14/H-17, indicating a β-orientation for H-8, H-16, H-19, and H-20 and an α-orientation for H-3, H-5, H-17, and H-18. Compound **5** was therefore defined as (3*S**, 5*S**, 8*R**, 9*R**, 10*S**, 13*S**, 16*R**)-3-acetoxy-9,13;15,16-diepoxy-labda-16-ol.

Compound **6**, trivially named negundoin F, was obtained as yellowish syrup and the molecular formula was established as $C_{20}H_{32}O_2$ by positive HRESIMS $[M+Na]^+$ at *m*/*z* 327.2301 (calcd 327.2300), indicating five degrees of unsaturation. The $^1H$ NMR spectrum of **6** exhibited signals corresponding to two tertiary methyl groups ($\delta_H$ 0.98, 0.77), one secondary methyl group ($\delta_H$ 1.01, d, *J* = 8.5 Hz), one isopropyl group with a six-proton doublet ($\delta_H$ 1.21, d, *J* = 7.0 Hz) and a methine proton heptet ($\delta_H$ 2.82, hepta, *J* = 7.0 Hz), one oxygenated methine proton ($\delta_H$ 3.22, dd, *J* = 4.2, 11.6 Hz), and two benzylic methylene protons ($\delta_H$ 2.68, m; $\delta_H$ 2.51, m) as well as three aromatic protons appeared as an ABX spin system. The $^{13}C$ NMR and DEPT analysis revealed 20 carbon signals, including five methyls, four methylenes, seven methines, and four quaternary carbons, of which the typical signals were six aromatic carbons and an oxygenated methine carbon. All these data suggest that compound **6** possess an abietane-type diterpene framework similar to that of **9**. Furthermore, two degrees of unsaturation less than **9** led to the assumption that **6** is a 7-deoxy-9,10-*seco*-derivative of **9**, which was confirmed by detailed HSQC, HMBC, $^1H$–$^1H$ COSY, and NOESY analysis. The $^1H$–$^1H$ COSY spectrum revealed the existence of fragment –$CH_2$–$CH_2$–CH–, from C-1 to C-3, fragment –CH–$CH_3$–, from C-10 to C-20, and the fragment –CH–$CH_2$–$CH_2$– from C-5 to C-7 (Fig. 2). The NOSEY correlations were detected between H-5/H-18, H-18/H-3, H-3/H-5, and H-10/H-19, indicating a β-orientation for H-10 and H-19 and an α-orientation for H-3, H-5, H-18, and H-20. Compound **6** was therefore concluded to be (3*S**, 5*R**, 10*S**)-3,9-dihydroxy-9,10-*seco*-abieta-8,11,13-triene.

Compound **7**, trivially named negundoin G, was obtained as colorless syrup and analyzed for the molecular formula $C_{20}H_{30}O_3$ by positive HRESIMS $[M+Na]^+$ at *m*/*z* 341.2095 (calcd 341.2093), which confirms a diterpene skeleton. The $^1H$, $^{13}C$, and DEPT NMR spectra of **7** indicated the presence of a vinyl group ($\delta_H$ 6.16, dd, *J* = 17.6, 10.8; $\delta_H$ 5.10, dd, *J* = 10.8, 0.8; $\delta_H$ 5.05, dd, *J* = 17.6, 0.8; $\delta_C$ 143.3, 112.7), a trisubstituted olefin ($\delta_H$ 6.67, dd, *J* = 5.0, 3.0; $\delta_C$ 136.1, 137.9) conjugated with a carbonyl group ($\delta_C$ 204.1), an oxygenated methane ($\delta_H$ 4.60, m; $\delta_C$ 63.9), and a secondary alcohol group ($\delta_H$

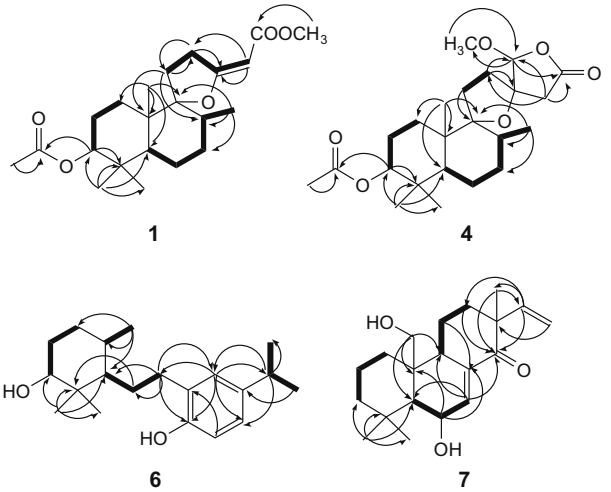


**Figure 2.** $^1H$–$^1H$ COSY (—) and key HMBC (→) correlations of compounds **1**, **4**, **6**, and **7**.

3.67, 4.28, d, $J$ = 12.1; $\delta_C$ 64.9). An one-proton multiplet at $\delta_H$ 2.13 was assigned to H-9 and signal at $\delta_H$ 1.39 assigned to H-5; in addition, three methyl singlet resonances at 1.25, 1.10, and 1.36 were assigned to methyl groups at C-17, C-18, and C-19, respectively. The long-range $^1H$–$^{13}C$ correlations observed from an olefinic proton ($\delta_H$ 6.67, dd, $J$ = 5.0, 3.0) to C-5 ($\delta_C$ 52.9) and C-9 ($\delta_C$ 51.4) led to the assignment of a double bond at C-7, which was conjugated with the carbonyl group, at C-14, established by detailed HMBC measurements. All these data supported that **7** was a sandaracopimara-7,15-dien diterpene having a hydroxymethyl group and an α,β-unsaturated carbonyl system, which was confirmed by the absorption bands at 3362 and 1686, 1625 $cm^{-1}$ in its FTIR spectrum. The detailed analysis of the 2D NMR experiments of **7** allowed the unequivocal assignment of all proton and carbon resonances. The NOSEY correlations were detected between H-20/H-19, H-20/H-11, H-17/H-11, H-18/H-9, H-18/H-6, H-18/H-5, H-9/H-5, and H-6/H-5, indicating a β-orientation for H-20, H-19, and H-17 and an α-orientation for H-18, H-9, H-6, and H-5. Compound **7** was therefore characterized and identified as (5$S^*$, 6$S^*$, 9$R^*$, 10$S^*$, 13$R^*$)-6,20-dihydroxy-sandaracopimara-7,15-dien-14-one.

The absolute configurations of these diterpenes remain to be determined because of the scarcity of material or the absence of secondary hydroxyl groups. However, the absolute configurations of **1**–**5** are probably the same as that of viteoside A, a labdane diterpene previously isolated from the fruits of *Vitex rotundifolia*,[12] from a biogenetic point of view.

Diterpene metabolites have been claimed to possess a variety of biological properties such as antibacterial, antifungal, cytotoxic, and anti-inflammatory activities,[13,14] and additionally, recent studies reported the inhibitory effects of diterpenes on nitric oxide production[15] and on iNOS (inducible nitric oxide synthetase) and/or COX-2 (cyclooxygenase-2)[16,17] protein expressions, which prompted us to evaluate the anti-inflammatory effect of these isolated compounds. The in vitro anti-inflammatory activity of compounds **1**–**7** was tested using LPS-stimulated RAW 264.7 cells. As shown in Table 4, compounds **3** and **5** were among the most potent inhibitors of NO production with $IC_{50}$ values of 0.12 and 0.23 μM, respectively, much less than that of the positive control indomethacin ($IC_{50}$ 45.51 μM). Though possessing the same 16-norlabdane framework, compound **3** ($IC_{50}$ 0.12 μM) exhibited much stronger activity than compounds **1** ($IC_{50}$ 9.83 μM) and **2** ($IC_{50}$ 23.43 μM), indicating that the presence of an α,β-unsaturated aldehyde group may significantly enhance the NO production inhibitory activity of these diterpenes. In addition, compound **5** ($IC_{50}$ 0.23 μM) showed better activity than compound **4** ($IC_{50}$ 4.39 μM), suggesting that a hydroxyl group at C-16 may increase the activity. Compounds **6** and **7** also demonstrated significant NO inhibitory potential, with $IC_{50}$ values of 1.16 and 0.70 μM, respectively. Since the cytotoxic effect was not observed in RAW 264.7 cells after compounds **1**–**7** treatment (up to 50 μM, data not shown), this result implied that compounds **1**–**7** inhibited nitrite release without causing cell death.

To further delineate the possible mechanism of these bioactive compounds as potential anti-inflammatory compounds, we examined their inhibitory effects on the protein expressions of COX-2 and iNOS in activated RAW 264.7 cells, by a western blot analysis. As shown in Figure 3, compounds **3** and **5** significantly reduced the levels of the iNOS protein to 0.40 ± 0.13% and 41.02 ± 6.02%, respectively, and COX-2 protein to 2.06 ± 0.53% and 26.40 ± 7.43%, respectively, compare with the control cells (LPS alone) at a concentration of 5 μM. Under the same concentration, compounds **1** and **4** did not inhibit the COX-2 protein expression, but moderately inhibited iNOS protein expression to 68.21 ± 5.11% and 62.03 ± 4.53%, respectively. Compound **6** showed modest inhibition on both iNOS and COX-2 protein expressions, with inhibitions of 58.18 ± 3.40% and 59.74 ± 5.84%, respectively, whereas compounds **2** and **7** did not seem to affect the protein expressions of iNOS and COX-2. Additionally, the housekeeping protein β-actin was not changed by the presence of all the tested compounds. Under the same experimental conditions, indomethacin (50 μM) reduced the levels of the iNOS and COX-2 protein to 86.48 ± 4.13% and 59.86 ± 5.67%, respectively, relative to the control cells stimulated with LPS. Taken together, our findings indicated that the reduction in the expression of iNOS protein contributed to the inhibitory effect of the tested diterpenes on LPS-induced NO production (with the exception of compounds **2** and **7**) and provides the possibility that these diterpenes isolated from *V. negundo* seeds might have promising therapeutic potential as anti-inflammatory agents.

## 3. Experimental

### 3.1. General experimental procedures

Optical rotations were acquired with a Perkin–Elmer 341 polarimeter. UV spectra were run on a Varian Cary Eclipse 300 spectrophotometer. IR spectra were recorded on a Bruker Vector 22

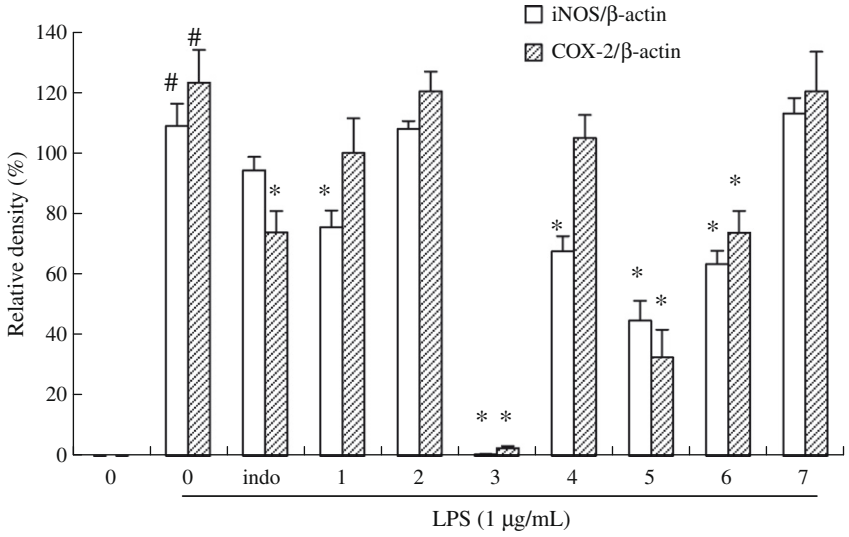


**Figure 3.** Effect of compounds **1**–**7** at 5 μM on the LPS-induced pro-inflammatory iNOS and COX-2 protein expressions of RAW 264.7 macrophage cells by immunoblot analysis. Values are mean ± S.D. of three experiments with triplicate of each experiment. $^{\#}P$ <0.05 indicates statistically significant differences from control group. $^{*}P$ <0.05 indicates statistically significant differences from LPS-stimulated group.

spectrometer with KBr pellets. NMR spectra were recorded on a Bruker Avance 600 or Avance 400 NMR spectrometer with TMS as an internal standard. ESIMS were measured on an Agilent LC/MSD Trap XCT mass spectrometer, whereas HRESIMS were measured using a Q-TOF micro mass spectrometer (Waters, USA). Materials for CC were silica gel (100–200 mesh; Huiyou Silical Gel Development Co. Ltd. Yantai, China), silica gel H (10–40 μm; Yantai), Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and YMC-GEL ODS-A (50 μm; YMC, Milford, MA). Preparative TLC (0.4–0.5 mm) was conducted on glass plates precoated silica gel $GF_{254}$ (Yantai).

### 3.2. Plant material

The seeds of *V. negundo* (Chinese name 'Huang-Jing-Zi') were obtained from Wanglang National Nature Reserve, Sichuan Province, in October 2006, and were identified by Professor Han-Chen Zheng, Second Military Medical University. A voucher specimen (#2006-168) has been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University.

### 3.3. Extraction and isolation

The air-dried and powdered seeds of *V. negundo* (25.0 kg) were extracted with 80% EtOH (×3), each extraction period lasting 2 h. After removal of the solvent under reduced pressure, the residue was suspended in $H_2O$ and partitioned sequentially with petroleum ether, $CH_2Cl_2$, EtOAc and *n*-butanol, respectively.

The $CH_2Cl_2$-soluble part (259.8 g) was subjected to CC on silica gel (200–300 mesh, 1000 g) and eluted successively with gradient petroleum ether–EtOAc mixtures (50:1, 20:1, 10:1, 5:1, 3:1, 1:1, 0:1) to afford fractions A–G. Fraction B (5.9 g) was further subjected on ODS column chromatography employing a MeOH–$H_2O$ mixture (80%) as eluent to provide six fractions (B.1–B.6). Fraction B.2 (180.3 mg) was subjected on preparative TLC to afford **7** (10.2 mg). Fraction B.4 (229.6 mg) was rechromatographed on Sephadex LH-20 with MeOH–$H_2O$ (80%) to give **6** (4.2 mg). Fraction C (6.5 g) was subjected on ODS column chromatography using MeOH–$H_2O$ (80%) as eluent to give five fractions (C.1–C.5). Fraction C.2 (615.3 mg) was rechromatographed on Sephadex LH-20 with MeOH–$H_2O$ (80%) followed by preparative TLC to give **8** (5.1 mg). Fraction C.3 (159.7 mg) was subjected on preparative TLC to afford **1** (5.2 mg), **4** (4.9 mg), **5** (5.3 mg), and **9** (5.1 mg). Fraction D (8.6 g) was subjected on ODS column chromatography using the gradient MeOH–$H_2O$ from 40% to 80% as eluent to provide six fractions (D.1–D.6). Fraction D.2 (200.4 mg) was rechromatographed on Sephadex LH-20 with MeOH–$H_2O$ (80%) followed by preparative TLC to give **2** (6.2 mg) and **3** (11.8 mg).

#### 3.3.1. Negundoin A (1)

Colorless syrup; $[\alpha]_D^{25}$ +8.9 (*c* 0.2, MeOH); UV (MeOH) $\lambda_{max}$ 243 nm; IR $\nu_{max}$ 2926, 2854, 1736, 1631, 1384, 1236, 1127 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data (see Table 1); (+)-HRESIMS *m*/*z* $[M+Na]^+$ 401.2307 (calcd for $C_{22}H_{34}O_5Na$, 401.2304).

#### 3.3.2. Negundoin B (2)

Colorless syrup; $[\alpha]_D^{25}$ +5.0 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ 245 nm; IR $\nu_{max}$ 3000–3600, 2924, 2853, 1736, 1653, 1636, 1384, 1245 $cm^{-1}$; $^1H$ ($CDCl_3$, 600 MHz) and $^{13}C$ NMR ($CDCl_3$, 150 MHz) data (see Table 1); (+)-HRESIMS *m*/*z* $[M+Na]^+$ 387.2150 (calcd for $C_{21}H_{32}O_5Na$, 387.2147).

#### 3.3.3. Negundoin C (3)

Colorless syrup; $[\alpha]_D^{25}$ −20.0 (*c* 0.16, MeOH); UV (MeOH) $\lambda_{max}$ 250 nm; IR $\nu_{max}$ 2940, 2877, 1734, 1655, 1626, 1384, 1244 $cm^{-1}$; $^1H$ ($CDCl_3$, 600 MHz) and $^{13}C$ NMR ($CDCl_3$, 150 MHz) data (see Table 1); (+)-HRESIMS *m*/*z* $[M+Na]^+$ 371.2199 (calcd for $C_{21}H_{32}O_4Na$, 371.2198).

#### 3.3.4. Negundoin D (4)

Colorless syrup; $[\alpha]_D^{25}$ +25.1 (*c* 0.2, MeOH); IR $\nu_{max}$ 2938, 1736, 1384, 1230 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data (see Table 2); (+)-HRESIMS *m*/*z* $[M+Na]^+$ 431.2407 (calcd for $C_{23}H_{36}O_6Na$, 431.2410).

#### 3.3.5. Negundoin E (5)

Colorless syrup; $[\alpha]_D^{25}$ −17.0 (*c* 0.2, MeOH); IR $\nu_{max}$ 3439, 2926, 1736, 1462, 1383, 1246, 1030 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data (see Table 2); (+)-HRESIMS *m*/*z* $[M+Na]^+$ 403.2463 (calcd for $C_{22}H_{36}O_5Na$, 403.2460).

#### 3.3.6. Negundoin F (6)

Yellowish syrup; $[\alpha]_D^{25}$ −2.1 (*c* 0.2, MeOH); UV (MeOH) $\lambda_{max}$ 269, 246 nm; IR $\nu_{max}$ 3440, 2926, 1738, 1384, 1216 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data (see Table 3); (+)-HRESIMS *m*/*z* $[M+Na]^+$ 327.2301 (calcd for $C_{20}H_{32}O_2Na$, 327.2300).

#### 3.3.7. Negundoin G (7)

Colorless syrup; $[\alpha]_D^{25}$ −95.8 (*c* 0.26, MeOH); UV (MeOH) $\lambda_{max}$ 325, 268 nm; IR $\nu_{max}$ 3362, 2925, 2868, 1686, 1625, 1460, 923 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data (see Table 3); (+)-HRESIMS *m*/*z* $[M+Na]^+$ 341.2095 (calcd for $C_{20}H_{30}O_3Na$, 341.2093).

### 3.4. Measurement of NO production

This assay was performed as previously described.[18] Briefly, RAW 264.7 macrophages were cultured at 37 °C in 5% $CO_2$ in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were seeded in 96-well plates at an initial density of $2 \times 10^5$ cells/well and then co-incubated with the isolated compounds and LPS (1 μg/mL) for 24 h. The amount of NO was assessed by determining the nitrite concentration in the cultured RAW 264.7 macrophage supernatants with Griess reagent. Aliquots of supernatants (100 μL) were incubated, in sequence, with 50 μL of 1% sulfanilamide and 50 μL of 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution. The absorbance at 548 nm was read using a microplate reader (POLARstar).

### 3.5. Cell viability

Cell viability was determined using the mitochondrial respiration-dependent MTT reduction method as previously described.[18] After transferring the required supernatant to another plate for the Griess assay, the remaining supernatant was aspirated from the 96-well plates, and 100 μL of fresh medium containing 2 mg/mL of MTT was added to each well. The cells were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. After incubating for 3 h, the medium was removed and the violet crystals of formazan in viable cells were dissolved in DMSO. Absorbance at 570 nm was measured using a microplate reader.

### 3.6. Western blot analysis

The murine RAW 264.7 cell line was seeded at an initial density of $2 \times 10^6$ cells/well in 6-well tissue culture plates overnight. Cells were exposed to *Escherichia coli* LPS (1 μg/mL; Sigma) for 24 h in the presence or absence of the tested compounds, which were dissolved in DMSO at an initial concentration of 10 mM and diluted to an appropriate concentration using culture medium, and the final

**Table 2**
$^{1}H$ NMR and $^{13}C$ NMR data of compounds **4** and **5**

| No. | **4** | | **5** | |
|---|---|---|---|---|
| | $\delta_H$ ($J$ in Hz) | $\delta_C$ | $\delta_H$ ($J$ in Hz) | $\delta_C$ |
| 1a | 1.44 (2H, m) | 30.8 t | 1.59 (1H, m) | 29.7 t |
| 1b | | | 1.37 (1H, m) | |
| 2a | 1.73 (1H, m) | 23.8 t | 1.68 (1H, m) | 23.7 t |
| 2b | 1.61 (1H, m) | | 1.55 (1H, m) | |
| 3 | 4.46 (1H, dd, $J$ = 4.4, 11.4) | 80.7 d | 4.40 (1H, dd, $J$ = 4.4, 11.4) | 80.5 d |
| 4 | | 37.9 s | | 37.9 s |
| 5 | 1.47 (1H, m) | 46.5 d | 1.45 (1H, m) | 46.6 d |
| 6a | 1.55 (1H, m) | 21.3 t | 1.50 (1H, m) | 21.2 t |
| 6b | 1.34 (1H, m) | | 1.33 (1H, m) | |
| 7a | 1.41 (1H, m) | 31.3 t | 1.38 (2H, m) | 31.4 t |
| 7b | 1.35 (1H, m) | | | |
| 8 | 1.71 (1H, m) | 36.1 d | 1.68 (1H, m) | 36.1 d |
| 9 | | 94.7 s | | 93.7 s |
| 10 | | 42.3 s | | 42.2 s |
| 11a | 2.03 (1H, m) | 30.1 t | 2.02 (1H, m) | 29.7 t |
| 11b | 1.68 (1H, m) | | 1.71 (1H, m) | |
| 12a | 2.44 (1H, m) | 31.3 t | 1.87 (2H, m) | 36.3 t |
| 12b | 1.81 (1H, m) | | | |
| 13 | | 88.6 s | | 90.8 s |
| 14a | 2.85 (1H, d, $J$ = 17.2) | 42.1 t | 2.31 (1H, m) | 35.6 t |
| 14b | 2.64 (1H, d, $J$ = 17.2) | | 1.80 (1H, m) | |
| 15a | | 173.6 s | 4.04 (1H, m) | 64.9 t |
| 15b | | | 3.71 (1H, m) | |
| 16 | 5.35 (1H, s) | 109.6 d | 4.68 (1H, s) | 98.9 d |
| 17 | 0.82 (3H, d, $J$ = 6.6) | 17.8 q | 0.81 (3H, d, $J$ = 6.6) | 17.2 q |
| 18 | 0.86 (3H, s) | 27.9 q | 0.80 (3H, s) | 27.9 q |
| 19 | 0.84 (3H, s) | 16.6 q | 0.79 (3H, s) | 16.6 q |
| 20 | 0.91 (3H, s) | 17.6 q | 0.85 (3H, s) | 17.6 q |
| 1′ | | 170.9 s | | 170.7 s |
| 2′ | 2.05 (3H, s) | 21.3 q | 1.97 (3H, s) | 21.3 q |
| 16-$OCH_3$ | 3.54 (3H, s) | 57.3 q | | |

Multiplicity was determined by DEPT experiments (s = quaternary, d = methine, t = methylene, q = methyl).

**Table 3**
$^{1}H$ NMR and $^{13}C$ NMR data of compounds **6** and **7**

| No. | **6** | | **7** | |
|---|---|---|---|---|
| | $\delta_H$ ($J$ in Hz) | $\delta_C$ | $\delta_H$ ($J$ in Hz) | $\delta_C$ |
| 1a | 1.65 (1H, m) | 33.7 t | 1.90 (1H, m) | 38.7 t |
| 1b | 1.08 (1H, m) | | 1.05 (1H, m) | |
| 2a | 1.70 (1H, m) | 30.4 t | 1.54 (1H, m) | 19.2 t |
| 2b | 1.51 (1H, m) | | 1.48 (1H, m) | |
| 3a | 3.22 (1H, dd, $J$ = 4.2, 11.6) | 78.6 d | 1.44 (1H, m) | 44.4 t |
| 3b | | | 1.26 (1H, m) | |
| 4 | | 39.9 s | | 34.0 s |
| 5 | 0.67 (1H, m) | 52.2 d | 1.39 (1H, m) | 52.9 d |
| 6a | 1.75 (1H, m) | 29.9 t | 4.60 (1H, m) | 63.9 d |
| 6b | 1.39 (1H, m) | | | |
| 7a | 2.68 (1H, m) | 32.8 t | 6.67 (1H, dd, $J$ = 5.0, 3.0) | 136.1 d |
| 7b | 2.51 (1H, m) | | | |
| 8 | | 128.7 s | | 137.9 s |
| 9 | | 151.4 s | 2.13 (1H, m) | 51.4 d |
| 10 | 1.36 (1H, m) | 33.9 d | | 39.5 s |
| 11 | 6.67 (1H, d, $J$ = 8.0) | 115.0 d | 1.87 (2H, m) | 19.1 t |
| 12 | 6.92 (1H, dd, $J$ = 8.0, 1.8) | 124.6 d | 1.85 (2H, m) | 34.3 t |
| 13 | | 141.3 s | | 49.2 s |
| 14 | 6.94 (1H, d, $J$ = 1.8) | 128.4 d | | 204.1 s |
| 15 | 2.82 (1H, hepta, $J$ = 7.0) | 33.3 d | 6.16 (1H, dd, $J$ = 17.6, 10.8) | 143.3 d |
| 16a | 1.21 (3H, d, $J$ = 7.0) | 24.3 q | 5.10 (1H, dd, $J$ = 10.8, 0.8) | 112.7 t |
| 16b | | | 5.05 (1H, dd, $J$ = 17.6, 0.8) | |
| 17 | 1.21 (3H, d, $J$ = 7.0) | 24.3 q | 1.25 (3H, s) | 23.5 q |
| 18 | 0.98 (3H, s) | 25.4 q | 1.10 (3H, s) | 32.7 q |
| 19 | 0.77 (3H, s) | 13.3 q | 1.36 (3H, s) | 24.5 q |
| 20a | 1.01 (3H, d, $J$ = 8.5) | 20.7 q | 4.28 (1H, d, $J$ = 12.1) | 64.9 t |
| 20b | | | 3.67 (1H, d, $J$ = 12.1) | |

Multiplicity was determined by DEPT experiments (s = quaternary, d = methine, t = methylene, q = methyl).

concentration of DMSO was adjusted to $\leqslant$0.01%. Indomethacin, a COX-2 inhibitor, was used as a positive control in this experiment. Beta-actin (β-actin) protein was used to monitor that equal amounts of protein were in each lane. Protein samples were col-

**Table 4**
Effects of isolated diterpenes (**1**–**7**) from *Vitex negundo* seeds on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells ($n = 4$)[a]

| Compounds | $IC_{50}$ (μM) |
|---|---|
| **1** | 9.83 |
| **2** | 23.43 |
| **3** | 0.12 |
| **4** | 4.39 |
| **5** | 0.23 |
| **6** | 1.16 |
| **7** | 0.70 |
| **Indo** | 45.51 |

[a] LPS: negative control; Indo: indomethacin, positive control.

lected and prepared as described previously,[18] and the iNOS and COX-2 expression levels were investigated using western blot analysis. Briefly, samples containing equal quantities of protein (50 μg) were subjected to SDS/20%–polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to nitrocellulose (NC) membranes. The resultant NC membranes were incubated with blocking solution and probed using antibodies specific to inducible nitric oxide synthase (iNOS; 1:1000 dilution; Cell Signaling) and cyclooxygenase-2 (COX-2; 1:1000 dilution; Cell Signaling) protein and visualised using an ECL detection kit (Perkin–Elmer, Western Lightning Chemiluminescence Reagent Plus).

## Acknowledgment

We thank professor Yang Genjin, Center of Analysis and Measurement, Second Military Medical University, for the NMR analysis and technical assistance.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.004.

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