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Novel Guaiane Endoperoxides, Nardoguaianone A–D, from *Nardostachys chinensis* Roots and their Antinociceptive and Antimalarial Activities

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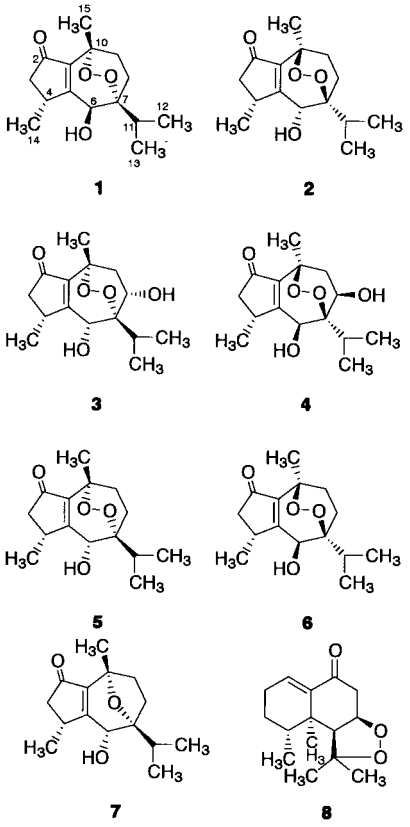
Abstract—Four novel guaianoids, nardoguaianone A, B, C and D, were isolated from *Nardostachys chinensis* roots. The structures were elucidated by spectral means and chemical transformation. Antinociceptive activities of the constituents of *N. chinensis*, including some of the above novel compounds, were investigated by the formalin test. As a result, it was revealed that nardoguaianone A and D showed activity at the second phase of pain induced by formalin injection. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

The crude drug 'Kanshoko', the rhizomes and roots of *Nardostachys chinensis* Batalin and its relatives, *N. grandiflora* DC. and *N. jatamansi* DC. (Valerianaceae), has been employed as a sedative and an analgesic in Oriental medicines. This plant is well known to be rich in terpenoids.[1–4] In our previous report, nardoperoxide (**5**), isonardoperoxide (**6**) and nardoxide (**7**) were introduced as the first guaiane-type sesquiterpenoids isolated from the plant.[4] On the other hand, the formalin test in mice is a valid and convenient method to investigate antinociceptive activity.[5,6] The pain induced by 2% formalin is biphasic, showing a first phase (from 0 to 5 min) and a second phase (from 15 to 20 min after formalin injection). The former is due to a direct effect on nociceptors, and the latter is an inflammatory response with inflammatory pain. Against these pains, mice lick or bite their paw where formalin is injected, and the time for this behavior is recorded in the formalin test. From the caused response by the injection of a sample, the site of action of analgesics can be easily distinguished. We describe herein the isolation and structure elucidation of four novel guaiane endoperoxides, nardoguaianone A–D (**1**–**4**), their antimalarial activity and the antinociceptive activities of the constituents of *N. chinensis* examined by the formalin test.



Keywords: *Nardostachys chinensis*; sesquiterpenoid; guaiane; formalin test; antinociceptive activity; antimalarial activity.

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Results and Discussion

Extraction and fractionation

Dried rhizome of *N. chinensis* (3.0 kg) was extracted three times with methanol. The extracts (357 g) were concentrated in vacuo and partitioned between ethyl acetate and water to yield ethyl acetate solubles (230 g). The aqueous layer was further extracted with *n*-butanol to afford 25 g of *n*-butanol solubles. The ethyl acetate solubles were fractionated by silica-gel column chromatography with stepwise gradient of *n*-hexane and ethyl acetate (10:0 to 0:10).

Antinociceptive activities of the fractions from *N. chinensis* extract

Prior to the study of compounds in each fraction, antinociceptive activities of the methanol extract and its fractionated samples were investigated by the formalin test using mice at a dose corresponding to 5 g crude drug/kg. Though all samples did not suppress the licking and biting time of the first phase, the methanol extract (106.9 ± 14.1 s at 124.0 mg/kg, $P<0.05$), the ethyl acetate solubles (88.0 ± 16.6 s at 87.9 mg/kg, $P<0.01$) and *n*-hexane–ethyl acetate (7:3) fraction (108.4 ± 13.5 s at 28.9 mg/kg, $P<0.05$) shortened the time at the second phase compared to control (147.0 ± 4.9 s).

Isolation of nardoguaianone A–D (1–4)

The *n*-hexane–ethyl acetate (7:3) fraction of the column chromatography of the ethyl acetate solubles was further fractionated repeatedly using silica-gel and reverse-phase column to give four sesquiterpenoids, nardoguaianone A (**1**), B (**2**), C (**3**) and D (**4**).

Structure of nardoguaianone A (1)

Nardoguaianone A (**1**), $[\alpha]_D^{25}=+19.6$ (*c* 0.429, MeOH), gave a molecular ion peak at *m/z* 266.1557 (*m/z* 266.1518 calculated for $C_{15}H_{22}O_4$) in its HREI-MS. Complete proton decoupled ^{13}C NMR and DEPT spectra showed the presence of four methyl groups, three methylene carbons, two methine carbons, one oxymethine carbon (δ_C 73.7), two oxygen attached quaternary carbons (δ_C 77.4 and 82.9), tetrasubstituted double bond carbons (δ_C 141.9 and 181.4) and one carbonyl carbon (δ_C 205.2), and they were very similar to those of nardoperoxide (**5**) reported previously.[4] Signals of the ^1H NMR spectrum observed between δ_H 1.75 and 2.05 were rather complicated compared to that of nardoperoxide (**5**) (δ_H 1.75 (1H, dd, J=14.0, 8.2 Hz; H-9β), 1.94 (1H, dd, J=14.0, 8.4 Hz; H-8α) and 2.07 (1H, ddd, J=14.0, 10.2, 8.4 Hz; H-9α)). ^1H–^1H and ^1H–^{13}C COSY spectra proposed partial structures as shown in Fig. 1, and long-range C–H coupling demonstrated by COLOC spectrum made them link to afford a planar structure for nardoguaianone A (**1**). The structure was the same as that of nardoperoxide (**5**) and isonardoperoxide (**6**), and they were different in their stereochemistry discussed below.

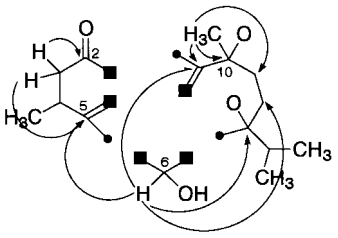

Figure 1. Partial structures of nardoguaianone A (**1**) and its correlations observed on the COLOC spectrum. The arrows indicate the correlations.

Structure of nardoguaianone B (2)

The ^1H and ^{13}C NMR spectra as well as UV and mass spectra displayed by nardoguaianone B (**2**) resembled those of nardoguaianone A (**1**). The planar structure of **2** was substantiated by 2D NMR spectra to be the same as that of compound **1**, but physical properties of this compound were different from those of nardoperoxide (**5**) and isonardoperoxide (**6**). Accordingly, compounds **1**, **2**, **5** and **6** were suggested to be diastereomers.

Stereochemistry of nardoguaianone A (1) and B (2)

The H-8 methylene signals of **1** (δ_H 1.78 and 1.96) and **2** (δ_H 2.03–2.09, 2H) in ^1H NMR spectra were observed in lower field than those of **5** (δ_H 1.00 and 1.94) and **6** (δ_H 1.06 and 1.96). This finding must be due to the anisotropic effect of the endoperoxide bridge and hydroxyl group at C-6. In other words, the two hydrogen atoms at C-8 of **1** and **2** are close to the oxygen atoms of the hydroxyl group and endoperoxide, whereas only one is near one of the oxygen atoms in the case of **5** and **6**. From these results and a model study of this molecule, the endoperoxide of **1** and **2** is suggested to be placed opposite the hydroxyl groups, as shown in Fig. 2. Moreover, the chemical shifts of H-6 signals of **1** (δ_H 4.95) and **2** (δ_H 4.59) which were in lower field than those of **5** (δ_H 4.18) and **6** (δ_H 4.35) supported that H-6 of **1** and **2** were directed to the same side as the endoperoxide. On the other hand, NOEs of H-6–H-14 (CH_3) of **1** and H-4–H-6 of **2** revealed that the methyl group at C-4 and the hydroxyl group at the C-6 group are directed to the same and opposite side, respectively.

Absolute configurations of **1** and **2** were investigated using the modified Mosher's method[7] with MTPA-esters of **2** and the exciton chirality method[8] with 6-*O*-benzoate of **2**. Unfortunately, a resonance distribution pattern of the $\Delta\delta$ values of (*S*)- and (*R*)-MTPA esters were not proportional to the distance from the MTPA moiety, and CD curve of the benzoate gave ambiguous couplets that lead the absolute

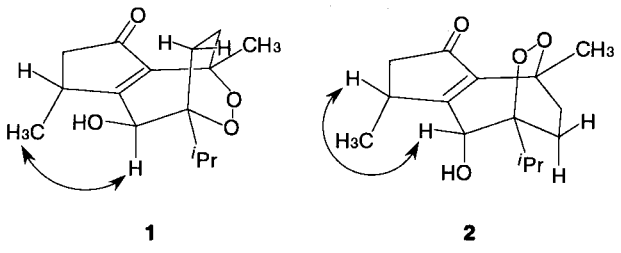

Figure 2. NOEs of nardoguaianone A (**1**) and B (**2**).

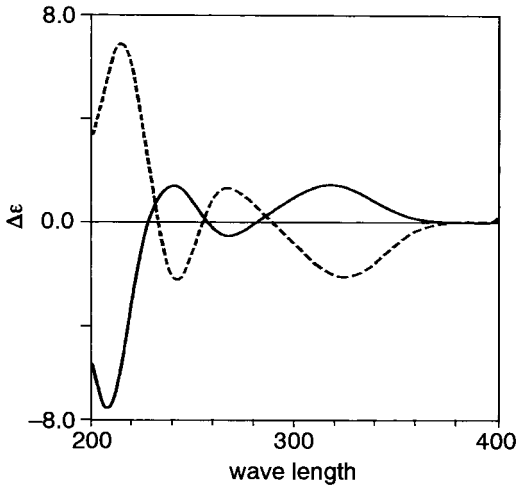

Figure 3. CD spectra of nardoguaianone A (**1**) and B (**2**). (—) Nardoguaianone A (**1**), (- - -) nardoguaianone B (**2**).

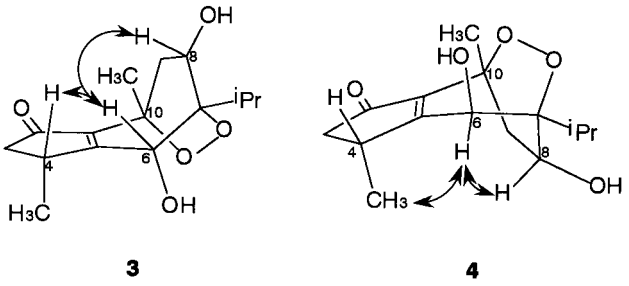

Figure 4. NOEs of nardoguaianone C (**3**) and D (**4**).

configuration. These results may be due to the interaction between the ester groups and the neighboring isopropyl group that interferes to form the ideal conformation to be adapted to the above methods. In consideration of biogenesis the guaiane-type compounds from *N. chinensis*, the absolute configuration of C-4 position of **1** and **2** are estimated to be *R*. Accordingly, together with the antipodal relationship of the CD spectrum of **1** and **2** (Fig. 3), the absolute configurations of **1** and **2** would be 4*R*, 6*S*, 7*R* and 10*R* and 4*R*, 6*R*, 7*S* and 10*S*, respectively, though they were not determined by the spectroscopic methods.

Structure of nardoguaianone C (3) and D (4)

The molecular formula $C_{15}H_{22}O_5$ of nardoguaianone C (**3**) and D (**4**) was different from those of nardoperoxide (**5**) and isonardoperoxide (**6**) by only one oxygen. Compounds **3** and **4** demonstrated the corresponding oxymethine signals (**3**: δ_H 3.52, δ_C 66.6; **4**: δ_H 3.54, δ_C 66.3) to one additional oxygen atom, and other spectral patterns closely resembled those of **5** and **6** in their NMR spectra, respectively. Interpretation of the 2D spectra of **3** and **4** revealed that both of the compounds had structures corresponding to 8-hydroxylated congeners of **5** and **6**. From the information gained from the NOESY spectra of these compounds (Fig. 4), the two hydroxyl groups at C-6 and C-8 and the endoperoxide were shown to be oriented to the same direction. Furthermore, the relative configuration of the methyl group at the C-4 position of each compound was determined as shown in Fig. 5 in the same manner as nardoperoxide (**5**) with the chemical shifts of H-4 and H-14 along with NOEs. The CD spectra of **3** and **4** were almost the same as those of **5** and **6**, respectively (Fig. 5). As compounds **5** and **6** displayed antipodal CD spectra, the absolute configurations of nardoguaianone C (**3**) and D (**4**) were thus confirmed to be 4*R*, 6*S*, 7*S*, 8*R* and 10*S*, and 4*R*, 6*R*, 7*R*, 8*S* and 10*R*, respectively.

Antinociceptive activities of compounds from *N. chinensis*

Antinociceptive activities of some of the major constituents from *N. chinensis*, which were obtained in sufficient amount to carry out a biological test, were investigated. These compounds were isolated not only from the *n*-hexane–ethyl acetate (7:3) fraction, which showed antinociceptive activity, but also other fractions of the methanol extract of *N. chinensis*. Table 1 exhibited the activities of the compounds and indomethacin as a positive control. Among these compounds, nardoguaianone A (**1**) and D (**4**) shortened the licking time at the second phase of the pain induced by 2% formalin. Considering that nardoguaianone A (**1**) and D (**4**) could not reproduce the activity of the

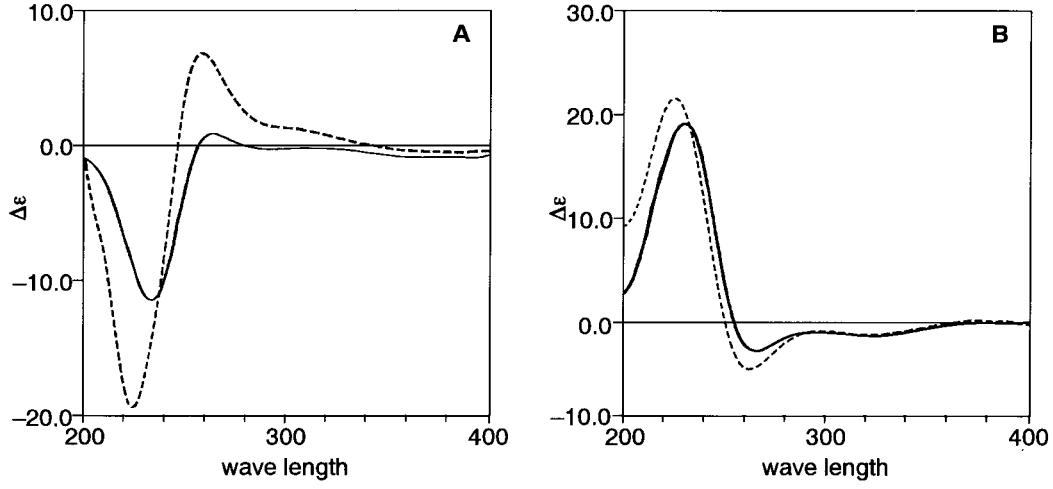

Figure 5. CD spectra of nardoguaianone C (**3**), D (**4**), nardoperoxide (**5**) and isonardoperoxide (**6**). (A) (—) **3**, (- - -) **5**; (B) (—) **4**, (- - -) **6**.

Table 1. Antinociceptive activities of the constituents of *N. chinensis* (*$P<0.05$. **$P<0.01$ different from control groups (Dunnett's test))

Compounds	Dose (mg/kg)	*n*	Licking and biting time (s)	
			The first phase	The second phase
Methanol extract	124	10	107.1±8.3	106.9±14.1*
EtOAc solubles	88	10	103.9±9.4	88.0±16.6**
Nardoguaianone A (**1**)	30	10	128.8±6.4	95.2±14.2*
Nardoguaianone C (**3**)	30	5	111.9±8.2	143.5±51.6
Nardoguaianone D (**4**)	30	10	112.9±8.3	95.7±11.6*
Nardoperoxide (**5**)	30	10	113.8±7.7	175.0±26.0
Isonardoperoxide (**6**)	30	5	109.6±11.8	135.6±38.9
(+)-Pinoresinol	30	3	100.0±14.1	150.8±20.2
Nardosinone diol	30	4	94.8±6.3	216.7±36.2
Nardosinone (**8**)	50	5	104.7±17.8	195.0±55.5
Indomethacin[a]	40	6	76.8±10.9	55.2±11.9**
Control		10	112.7±8.2	147.0±4.9

[a] Serve as a positive control.

n-hexane–ethyl acetate (7:3) fraction of the methanol extract of *N. chinensis*, the antinociceptive potency of *N. chinensis* may arise from the synergistic action of several constituents.

Antimalarial activities of nardoguaianones

In our previous letter, the antimalarial activities of some compounds isolated from *N. chinensis* were described, and the endoperoxide moiety of the molecules was assumed to relate to the activity.[4] To ascertain this hypothesis, the antimalarial activity of nardoguaianone A–D (**1**–**4**) was investigated. As a result, it was seen that nardoguaianones showed similar activities to those of nardoperoxide (**5**) and isonardoperoxide (**6**), and the evidence supported the necessity of endoperoxide to exhibit the antimalarial activity (see Table 2).

Experimental

General

UV and IR spectra were recorded on Hitachi U-3200 and JASCO FT/IR-5399 spectrometers, respectively. Optical rotations and CD spectra were measured with a JASCO DIP-370 polarimeter and a JASCO J-720 circular dichroism spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on JEOL LAMBDA-600 (^{1}H: 600 MHz and ^{13}C: 150 MHz), JEOL JNM GSX-500 (^{1}H: 500 MHz and ^{13}C: 125 MHz) and Varian Gemini 2000 (^{1}H: 300 MHz and ^{13}C: 75 MHz) spectrometers. All NMR spectra were recorded in $CDCl_3$. Chemical shifts for ^{1}H and ^{13}C NMR are given in parts per million (δ) relative to tetramethylsilane (δ_H 0.00) and $CDCl_3$ (δ_C 77.1) as internal standards, respectively. LR and HR EI-MS were obtained with JEOL JMS DX-303 and AX-500 mass spectrometers. Analytical TLC was performed on silica gel 60 F254 (Merck). Column chromatography was carried out on silica gel 60 (70–230 mesh, Merck) and Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto). (*R*)-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride ((*R*)-MTPA-Cl) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride ((*S*)-MTPA-Cl) were purchased from Aldrich.

Animals

Male Std-ddY mice (Japan SLC Co., Hamamatsu, Japan) weighing 20–22 g were used. They were housed in plastic cages with free access to food and water prior to the

Table 2. Antimalarial activities of the constituents of *N. chinensis*

Compounds	EC_{50} (M)		Selectivity[c]
	P. falciparum[a]	FM3A cells[b]	
Nardoguaianone A (**1**)	4.1×10^{-6}	$>1.9\times10^{-5}$ (98%)[d]	>5
Nardoguaianone B (**2**)	5.3×10^{-6}	$>1.8\times10^{-5}$ (99%)[d]	>3
Nardoguaianone C (**3**)	9.0×10^{-6}	$>1.0\times10^{-4}$ (90%)[d]	>11
Nardoguaianone D (**4**)	4.1×10^{-6}	$>1.0\times10^{-4}$ (97%)[d]	>24
Nardoperoxide (**5**)	1.5×10^{-6}	3.4×10^{-5}	23
Isonardoperoxide (**6**)	6.0×10^{-7}	$>4.5\times10^{-5}$ (80%)[d]	>75
Nardoxide (**7**)	$>4.0\times10^{-5}$ (86%)[d]	$>4.0\times10^{-5}$ (97%)[d]	>1
Nardosinone (**8**)	4.5×10^{-6}	$>6.5\times10^{-5}$	>14
Chloroquine	1.8×10^{-8}	3.2×10^{-5}	1778

[c] Selectivity=EC_{50} against FM3A cells/EC_{50} against *P. falciparum*.
[a] *Plasmodium falciparum* malaria parasite.
[b] Mouse mammary cells.
[d] Growth percent at the concentration indicated.

Table 3. ^{1}H NMR data of nardoguaianone A–D (**1**–**4**)

Position	1	2	3	4
3α	2.09 (dd, 18.3, 1.8)	2.09 (dd, 18.3, 1.8)	2.15 (dd, 18.4, 1.8)	2.11 (dd, 18.7, 1.8)
3β	2.68 (dd, 18.3, 6.7)	2.75 (dd, 18.3, 6.9)	2.71 (dd, 18.4, 6.4)	2.79 (dd, 18.7, 6.6)
4	3.17 (ddq, 6.7, 1.8, 7.3)	2.94 (ddq, 6.9, 1.8, 7.2)	2.79 (ddq, 6.4, 1.8, 6.9)	3.15 (ddq, 6.6, 1.8, 7.3)
6	4.95 (s)	4.59 (s)	4.49 (d, 11.5)	4.55 (br.s)
8α	1.96 (m)	2.03–2.09 (2H, m)		3.54 (t, 8.1)
8β	1.78 (m)		3.52 (t, 8.1)	
9α	1.86 (m)	1.87–1.93 (2H, m)	1.95 (dd, 14.2, 8.1)	2.42 (dd, 13.6, 8.1)
9β	2.00 (m)		2.39 (dd, 14.2, 8.1)	1.95 (dd, 13.6, 8.1)
11	1.95 (sept, 6.7)	1.91 (sept, 6.9)	2.41 (sept 7.3)	2.42 (sept, 7.0)
12 (CH_3)	1.10 (d, 6.7)	1.06 (d, 6.9)	1.21 (d, 7.3)	1.21 (d, 7.0)
13 (CH_3)	1.06 (d, 6.7)	1.05 (d, 6.9)	1.12 (d, 7.3)	1.14 (d, 7.0)
14 (CH_3)	1.28 (d, 7.3)	1.37 (d, 7.2)	1.38 (d, 6.9)	1.22 (d, 7.3)
15 (CH_3)	1.59 (s)	1.64 (s)	1.65 (s)	1.64 (s)

experiments. They were maintained in climate- and light-controlled rooms (22±2°C, 55±5%, 12/12 h dark/light cycle with lights on at 8:30). Each mouse was placed in the cage for testing at least 30 min before the formalin test to adapt to the new environment.

Formalin test

The formalin test was performed by the modified method reported in the literature.[5,6] 20 μL of 2% formalin in saline was injected subcutaneously into the dorsal hind paw of the mouse at 30 min after administration of sample intraperitoneally. Each mouse was then put back into the chamber and the observation period started, and the amount of time that the animal spent licking or biting the injected paw was recorded for 30 min. The summation of time for pain response during each 5 min block was measured. Samples and indomethacin as positive control were suspended in 4% Gum Arabic (Nacalai Tesque, Inc., Kyoto, Japan). Mice injected 4% Gum Arabic only were served as control.

Statistical analysis

The data were examined by analysis of variance and Dunnett's procedure. Level of significance was set to 5% ($P<0.05$). Results are given as mean ±S.E.M.

Antimalarial activity

Antimalarial activity of the compounds were measured by the method described in Ref. 9.

Extraction and isolation

Dried rhizome (3 kg) of *N. chinensis*, purchased from Tochimoto-tenkaido, Osaka, was extracted three times with methanol at room temperature to give the extract (357 g). The methanol extract was partitioned with ethyl acetate and water to yield ethyl acetate solubles (230 g). Ethyl acetate solubles were chromatographed over silica gel, and the column was eluted with *n*-hexane–ethyl acetate mixtures by increasing polarity (10:0 to 0:10). The resulted *n*-hexane–ethyl acetate (7:3) eluate (28.9 g) was further fractionated with repeated chromatography using silica gel with a mixed solvent of *n*-hexane–acetone, followed by *n*-hexane–isopropanol as eluants. Fractionation of the *n*-hexane–isopropanol (93:7) eluate (818 mg) using silica gel column (*n*-hexane–isopropanol and chloroform–methanol) and ODS columns (H_2O–acetone) afforded nardoguaianone A (**1**) (40 mg). Moreover, nardoguaianone B (**2**) (14 mg) was obtained from the *n*-hexane–isopropanol (9:1) eluate (212 mg) by fractionation using silica gel column (chloroform–methanol) followed by preparative HPLC equipped with ODS column (30% acetonitrile–H_2O).

Nardoguaianone C (**3**) (7 mg) and nardoguaianone D (**4**) (25 mg) were obtained from *n*-hexane–ethyl acetate (6:4) eluate (21.8 g) by fractionation with silica gel (*n*-hexane–ethyl acetate, *n*-hexane–acetone, *n*-hexane–isopropanol and chloroform–methanol as eluant) and a preparative HPLC using ODS column (25% acetonitrile–H_2O).

Nardoguaianone A (1). $[\alpha]_D^{25}=+19.6$ (*c* 0.429, MeOH); a colorless amorphous solid; UV λ_{max} (MeOH) (nm (log ϵ)) 236 (3.97); CD λ (MeOH) nm ($\Delta\epsilon$) 317.4 (+1.54), 266.8 (−0.55), 239.8 (+1.48), 207.4 (−7.47); IR ν_{max} ($CHCl_3$) 1698 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data are shown in Tables 3 and 4; HREI-MS: *m/z* 266.1557 $[M]^+$ (266.1518 calculated for $C_{15}H_{22}O_4$).

Nardoguaianone B (2). $[\alpha]_D^{25}=+53.7$ (*c* 0.867, MeOH); a colorless amorphous solid; UV λ_{max} (MeOH) (nm (log ϵ)) 233 (3.93); CD λ (MeOH) nm ($\Delta\epsilon$) 324.2 (−2.20), 266.8

Table 4. ^{13}C NMR data of nardoguaianone A–D (**1**–**4**) (abbreviations of multiplicity: q, primary carbon; t, secondary carbon; d, tertiary carbon; s, quaternary carbon)

Position	1	2	3	4
1	141.9 (s)	140.9 (s)	141.8 (s)	143.5 (s)
2	205.2 (s)	205.5 (s)	206.0 (s)	204.3 (s)
3	44.7 (t)	45.9 (t)	45.8 (t)	45.4 (t)
4	32.6 (d)	36.2 (d)	36.3 (d)	32.4 (d)
5	181.4 (s)	178.9 (s)	177.0 (s)	178.3 (s)
6	73.7 (d)	75.4 (d)	70.7 (d)	67.6 (d)
7	82.9 (s)	83.8 (s)	86.9 (s)	87.9 (s)
8	21.3 (t)	20.1 (t)	66.6 (d)	66.3 (d)
9	31.1 (t)	33.9 (t)	39.8 (t)	40.0 (t)
10	77.4 (s)	77.1 (s)	78.9 (s)	78.7 (s)
11	35.2 (d)	36.3 (d)	30.0 (d)	30.2 (d)
12	16.9 (q)	17.4 (q)	19.4 (q)	18.9 (q)
13	16.7 (q)	16.7 (q)	18.2 (q)	18.2 (q)
14	19.2 (q)	20.4 (q)	19.0 (q)	18.6 (q)
15	22.7 (q)	23.0 (q)	22.3 (q)	22.5 (q)

(+1.38), 241.8 (−2.30), 214.0 (+7.14); IR ν_{max} ($CHCl_3$) 1701 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data are shown in Tables 3 and 4; HREI-MS *m/z* 266.1488 $[M]^+$ (266.1518 calculated for $C_{15}H_{22}O_4$).

Nardoguaianone D (4). $[\alpha]_D^{25}=+14.1$ (*c* 0.768, MeOH); a yellow amorphous solid; UV λ_{max} (MeOH) (nm (log ϵ)) 235 (3.87); CD λ (MeOH) nm ($\Delta\epsilon$) 264.8 (−0.86), 229.0 (+6.66); IR ν_{max} ($CHCl_3$) 1701 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data are shown in Tables 3 and 4; HREI-MS: *m/z* 283.1544 $[M]^+$ (283.1546 calculated for $C_{15}H_{23}O_5$).

Nardoguaianone C (3). $[\alpha]_D^{25}=+18.4$ (*c* 0.577, MeOH); a yellow amorphous solid; UV λ_{max} (MeOH) (nm (log ϵ)) 236 (3.90); CD λ (MeOH) nm ($\Delta\epsilon$) 263.4 (+1.39), 232.2 (−7.96); IR ν_{max} ($CHCl_3$) 1701 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data are shown in Tables 3 and 4; HREI-MS: *m/z* 283.1525 $[M]^+$ (283.1546 calculated for $C_{15}H_{23}O_5$).

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