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ORIGINAL PAPER

Anti-inflammatory and analgesic components from "hierba santa," a traditional medicine in Peru

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Abstract "Hierba santa," a Peruvian herbal medicine, is used to alleviate many symptoms, including headache, hemorrhoids, fever, and rheumatism. Several *Cestrum* species are said to be the origin of hierba santa. Three lots of hierba santa: *Cestrum auriculatum* (herb 1 and herb 2) and *C. hediundinum* (herb 3), which were purchased from Peruvian markets at Cuzco (Andes area) and Equitos (Amazon area), respectively, were examined for their pharmacological activities and active components. Herbs 1–3 showed anti-inflammatory and analgesic activities in the in vivo writhing inhibition test in mouse and inhibited prostaglandin E_1-, E_2-, or ACh-induced contractions of guinea pig ileum in the Magnus method. Activity-based separation of each extract yielded cestrumines A and B, cestrusides A and B, a mixture of (+)- and (−)-pinoresinol glucosides, nicotiflorin, rutin, sinapoyl glucose, ursolic acid, β-sitosteryl glucoside, and 2-*sec*-butyl-4,6-dihydroxyphenyl-β-D-glucopyranoside. Among them, cestrumine A and cestrusides A and B are new compounds. All three lots of hierba santa do not contain exactly the same active components.

Keywords Hierba santa · *Cestrum* · Anti-inflammation · Analgesic · β-Carboline · Spirostanol

This work is dedicated to the late Mr. Tetsuo Shiota, one of the authors.

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Introduction

As part of our long-term research on traditional medicine, we investigated "hierba santa," a Peruvian herbal medicine. Hierba santa or "holy weed" is used to alleviate many kinds of symptoms in Peru. It is also used in magical rituals to cure diseases and to protect against bad luck. The leaf decoction is taken orally to treat fever, typhoid fever, rheumatism, cough, bronchitis, colic of the stomach, high blood pressure, and diabetes [1–3]. The decoction is also applied externally to treat headache and hemorrhoids [1, 2], and the crushed leaves are used to disinfect wounds [4]. It is said that this herb is incensed to cure colds or magically to send away bad shadows [3].

Such plants as *Cestrum auriculatum*, *C. hediundinum*, and *C. coriaceum* are listed as the origin of hierba santa in herbal medicine books [5–7]. The same plants are also known by another local name: "hierba hedionda." One herbal medicine book states that hierba santa and hierba hedionda are the same [5]. According to Professor

A. Tupayachi Herrera of Universidad Nacional de San Antonio Abad del Cuzco, *C. coriaceum* is commonly used as hierba santa and *C. auriculatum* as hierba hedionda at Cuzco in Andes. A herb sold as hierba santa at a market in Equitos, a city in Amazon, was identified as *C. hediundinum* by Mr. J. Ruiz (Herbario Ethnobotánico Amazónico). Several plants of the genus *Cestrum* (Solanaceae) grow in Peru and they are expected to have similar pharmacological activities, such as anti-inflammatory and analgesic activities. Hierba santa, therefore, seems to have a few *Cestrum* plants as its origin.

We purchased three lots of hierba santa from local markets at Cuzco (Andes area) and Equitos (Amazon area) and identified them as *C. auriculatum* (herb 1 and herb 2) and *C. hediundinum* (herb 3), respectively. We present herein the active components in hierba santa as well as their anti-inflammatory and analgesic activities.

Results and discussion

Activity of extracts

Herb 1, herb 2, and herb 3 were extracted with methanol at room temperature. After evaporation in vacuo, methanol extracts (ext. 1, 2, and 3) were obtained and their analgesic activities in vivo were evaluated using the acetic-acid-induced writhing inhibition test in mice. The three extracts showed 44% ($P < 0.05$, 2.0 g/kg), 49% ($P < 0.01$, 3.0 g/kg), and 32% ($P < 0.01$, 1.0 g/kg) inhibition, respectively.

Ext. 1–3 were also evaluated in vitro for their ability to inhibit prostaglandin (PG)- and acetylcholine (ACh)-induced contractions of guinea pig ileum using the Magnus method. Ext. 3 inhibited PGE_1-, PGE_2- or ACh-induced contraction at a concentration of 3×10^{-4} g/ml. Ext. 1 inhibited PGE_1- or ACh-induced contraction but not PGE_2-induced contraction at 3×10^{-4} g/ml. Ext. 2 inhibited only ACh-induced contraction at 1×10^{-3} g/ml.

All three extracts inhibited neither substance-P-induced contraction of guinea pig ileum nor norepinephrine-induced contraction of rat aorta at the concentration of 3×10^{-4} g/ml.

The results showed that the three lots of hierba santa, herb 1, herb 2, and herb 3, have significant analgesic activity (probably anti-inflammatory) in vivo, but their in vitro inhibitory activities against PG- and ACh-induced contractions were not the same.

Activity-based separation of ext. 3

The separation of ext. 3 was carried out by monitoring pharmacological activity, such as ability to inhibit acetic-acid-induced writhing in mouse by oral administration, and ability to inhibit guinea pig ileum contractions induced by PGE_1, PGE_2 or ACh.

Ext. 3 was chromatographed on DIAION HP-20 and the methanol eluate yielded precipitate **1**. The detailed activities of the isolated compounds are discussed later. The mother liquor, fr. 1-A-sol, inhibited writhing in mice (40%, $P < 0.01$) at 1 g/kg and inhibited contraction induced by PGs or ACh at 1×10^{-4} g/ml. Fr. 1-A-sol was also evaluated for its ability to inhibit electrical-stimulation-induced contractions of guinea pig ilea. The inhibitory effect was observed at a concentration of 1×10^{-3} g/ml and was not reversed by naloxone, an opioid antagonist.

Fr. 1-A-sol was further separated by partition with chloroform, *n*-butanol, and water, in that order. From the chloroform fraction, compound **2** was obtained as an insoluble solid by the addition of methanol. The mother liquor of **2**, which showed the ability to inhibit writhing (26%, $P < 0.05$, 200 mg/kg) and to inhibit PGE_1- or ACh-induced contraction at concentrations of 1×10^{-4} and 3×10^{-5} g/ml, respectively, was separated on a silica gel flash column by gradient elution with chloroform–methanol–water. Two of the fractions, the 10:1:0.1 and 7:1:0.1 fractions, were independently purified by Sephadex LH-20 column chromatography to give **3** together with **2**.

The *n*-butanol fraction of fr. 1-A-sol was dissolved in methanol to yield **1** as an insoluble precipitate. The mother liquor was further separated because it was able to inhibit writhing (33%, $P < 0.05$) in mice at a dose of 300 mg/kg and to inhibit PG- or ACh-induced contractions of guinea pig ileum at a concentration of 3×10^{-5} g/ml. Separation on a DIAION HP-20 column with methanol–water as eluent gave fr. 4-A–fr. 4-D. After silica gel flash column chromatography of fr. 4-D with chloroform–methanol–water gradient, the fractions were repeatedly separated to afford **4**, **6**, and **7**, together with **1** and **3**. Fr. 4-B and fr. 4-C were independently separated by LH-20 (methanol) and/or octadecyl silica gel (ODS) HPLC (methanol–water). From them, **8**, and **4** and **5** were obtained, respectively.

Compound **1** was obtained as pale yellow needles, m.p. 271–273°C, and its molecular formula, $C_{20}H_{20}O_8N_2$, was established from *m/z* 417.1276 $[M+H]^+$ by HR-FAB-MS. Although **1** was negative in the Dragendorff test, it was positive in the Ehrlich test, suggesting that **1** is an indole alkaloid. The ^{13}C-NMR spectral data of **1** revealed signals of a carbonyl group at δ 201.0; an ester carbonyl at δ 163.4; 11 aromatic carbons at δ 113.5, 120.3, 121.1, 121.7, 122.2, 129.4, 131.3, 135.0, 135.1, 135.6, and 142.3; a sugar moiety at δ 60.6, 69.5, 72.7, 76.5, 78.0, and 95.3; and a methyl group at δ 25.6. The ^{1}H-NMR spectra showed signals due to an *ortho*-substituted aromatic ring at δ 7.38 (1H, td, $J = 8.0$, 0.9 Hz; 8-H), 7.65 (1H, ddd, $J = 8.3$, 8.0, 1.1 Hz; 9-H), 7.87 (1H, d, $J = 8.3$ Hz; 10-H), and 8.46

(1H, d, J = 8.1 Hz; 7-H), and an isolated proton at δ 9.23 (1H, s; 4-H). The signal at δ 12.34 (1H, s; 12-H) was assigned to an imino proton as the compound was predicted to have no phenolic OH based on the negative result of the ferric chloride test. In the HMBC spectrum, cross peaks were observed between δ 12.34 and δ 131.3 (C-5), 120.3 (C-6), 142.3 (C-11), and 135.1 (C-13). HMBC correlations were also observed between δ 9.23 (4-H) and δ 120.3 (C-6), 135.1 (C-13), and 163.4 (C-16), and between δ 2.85 (3H, s; 15-H_3) and δ 201.0 (C-14) and 135.6 (C-1). From these data, 1,3-substituted β-carboline was estimated. The glucopyranoside unit was elucidated from the ^{1}H-NMR signals at δ 3.23 (1H, td, J = 8.7, 5.3 Hz; 4′-H), 3.33–3.39 (2H, m; 3′-H, 5′-H), 3.43 (1H, td, J = 8.3, 5.4 Hz; 2′-H), 3.51 (1H, dt, J = 12.0, 5.9 Hz; 6′-H), 3.71 (1H, ddd, J = 12.0, 5.9, 2.1 Hz; 6′-H), 4.65 (1H, t, J = 5.9 Hz; 6′-OH), 5.08 (1H, d, J = 5.6 Hz; 4′-OH), 5.19 (1H, d, J = 4.9 Hz; 3′-OH), 5.45 (1H, d, J = 5.1 Hz; 2′-OH), and 5.74 (1H, d, J = 8.0 Hz; 1′-H), together with ^{13}C-NMR data. The coupling constant of the anomeric proton at δ 5.74 (J = 8.0 Hz) indicated a β-configuration. The position of the glucosyl moiety at C-16 was determined from the HMBC correlation between δ 5.74 (1′-H) and δ 163.4 (C-16).

Compound **1** was acid-hydrolyzed to yield D-glucose and an aglycone, which was identical with **2**. The structure of the aglycone, 1-acetyl-β-carboline-3-carboxylic acid, was already reported in *Vestia lycioides* (Solanaceae) [8]. After the aglycone was esterified with dicyclohexylcarbodiimide, dimethyl aminopyridine, and methanol, the methyl ester was identified as 1-acetyl-3-methoxycarbonyl-β-carboline, which was isolated from *V. lycioides* [9], by comparison with published data [9, 10]. Therefore, compounds **1** and **2**, named cestrumines A and B, were determined as β-D-glucopyranosyl-1-acetyl-β-carboline-3-carboxylate and the desglucoside, respectively (Fig. 1).

Compound **6** is a white powder, $[\alpha]_D^{20}$ −53°, and its molecular formula, $C_{39}H_{62}O_{15}$, was determined from m/z 793.3989 $[M+Na]^+$ by HR-FAB-MS. The ^{1}H-NMR spectrum showed signals for two tertiary methyl groups at δ 0.76 (3H, s; 18-H_3) and δ 0.91 (3H, s; 19-H_3), a secondary methyl at δ 0.99 (3H, d, J = 6.4 Hz; 21-H_3), and an exomethylene at δ 4.79 and 4.82 (each 1H, br.s; 27-H_2). In the ^{13}C-NMR spectrum, the signal for the spiroketal quaternary carbon was observed at δ 110.8 (C-22) and those for one terminal double bond, at δ 108.9 (C-27) and 145.2 (C-25). Signals indicating the presence of a glucose moiety [δ 101.1 (C-1′), δ 77.1 (C-2′), δ 76.1 (C-3′), δ 71.0 (C-4′), δ 76.7 (C-5′), and δ 62.6 (C-6′)] and rhamnose [δ 102.4 (C-1″), δ 72.2 (C-2″), δ 72.4 (C-3″), δ 73.9 (C-4″), δ 69.7 (C-5″), and δ 17.9 (C-6″)] also appeared in the ^{13}C-NMR spectrum. These data suggested that **6** is a steroidal saponin having Δ25(27) spirostene with two hexose units.

The ^{13}C-NMR data of **6** were similar to those of 1β, 2α-dihydroxy-5α-spirost-25(27)-en-3β-yl-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside [11] from *Cestrum sendtenerianum*, except for the signals assigned to protons in the C-ring. The existence of a hydroxyl group in C-ring of **6** was estimated and the position of C-12 assigned to δ_C 80.9

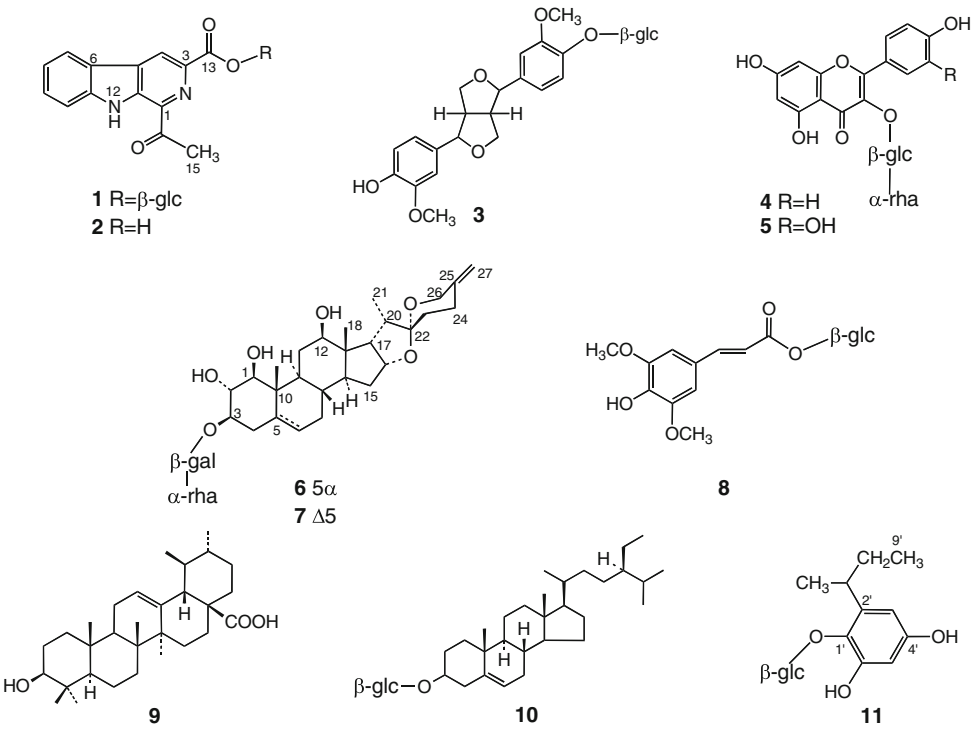

Fig. 1 Isolated compounds from three lots of hierba santa. Compounds **1**–**8** were isolated from herb 3, **9** and **10** from herb 2, and **11** from herb 1, which contained also **2** and **9**

and δ_H 3.22 (1H, br.dd, $J = 11.4$, 4.4 Hz) was determined from the HMBC correlations between δ_H 0.76 (3H, s; 18-H_3) and δ_C 46.7 (C-13), 55.9 (C-14), 63.6 (C-17), and 80.9 (C-12).

The sugar unit was also determined by enzymatic hydrolysis of **6**. Hydrolysis of **6** with hesperidinase (including α-L-rhamnosidase) gave rhamnose and a monoglycoside, the latter of which was subsequently hydrolyzed with β-D-galactosidase to give galactose and the aglycone of **6**.

In the HMBC experiment of **6**, anomeric protons at δ 5.15 (1H, d, $J = 1.5$ Hz) in L-rhamnose and at δ 4.42 (1H, d, $J = 7.6$ Hz) in D-galactose showed correlations to δ 77.1 (C-2′) and δ 81.1 (C-3), respectively. Considering the coupling constants of both anomeric protons, the 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl unit was estimated.

Configuration analysis revealed that the hydroxyl groups at C-1 and C-2 and the glycosyl unit at C-3 were trans-equatorial, because of the coupling constants of the corresponding protons at δ 3.13 (1H, d, $J = 9.2$ Hz; 1-H) and δ 3.38 (1H, t-like, $J = 9.2$ Hz; 2-H). Rotating frame Overhauser effect spectroscopy (ROESY) experiment was carried out in methanol-d_4 and pyridine-d_5. 2-H/19-H_3 and 1-H/9-H correlations supported the stereochemistry of A- and B-rings. In the C-ring, the methine proton at C-12 showed NOE with 17-H, and the methyl group (18-H_3) at C-13, with 11-H_β and 20-H in the E-ring. Additional correlation was observed between 16-H and 26-H_{ax} in the F-ring. From these data and the comparison with the data of related Δ25 (27) spirostenes [11], the structure was determined as 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl]-spirosta-25(27)-ene-1β,2α,3β,12β-tetrol.

All other NMR data, including 2D-NMR data, such as COSY, homonuclear Hartmann-Hahn spectroscopy (HOHAHA), and HMBC, supported the proposed structure (Fig. 1). Compound **6** is a new compound named cestruside A.

Compound **7** was obtained as a white powder, $[\alpha]_D^{20}$ −68°. Its molecular formula was determined to be $C_{39}H_{60}O_{15}$ from *m/z* 791.3815 $[M+Na]^+$ by HR-FAB-MS. The ^{1}H- and ^{13}C-NMR data were similar to those of **6**, except for the signals assigned to C-4–7 (Table 2). The signal for an olefinic proton was observed at δ 5.61 (1H, br.d, $J = 5.5$ Hz; 6-H), and those for methylene protons at δ 2.41–2.43 (2H, m; 4-H_2) and δ 1.38–1.44 and 1.99–2.04 (each 1H, m; 7-H_2) were deshielded. The ^{13}C-NMR spectrum also indicated two olefinic carbons at δ 126.3 (C-6) and 138.5 (C-5). The chemical formula of new compound **7**, named cestruside B, was estimated to be 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl]-spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol, which was also supported by comparison with published data of the Δ5 derivative [12].

The ^{1}H- and ^{13}C-NMR data of **3** were identical with those of pinoresinol glucoside [13]. Compound **3**, however, had a small $[\alpha]_D$. HPLC analysis on a chiral column demonstrated that **3** was a 9:1 mixture of (+) and (−) stereoisomers.

Compounds **4** and **5** were identified as nicotiflorin and rutin, respectively, by direct comparison with authentic samples. Compound **8** was identified as sinapoyl glucose by comparing with published data [14].

Separation of ext. 2

Ext. 2 (77 g) was partitioned with ethyl acetate and water, and the ethyl acetate fraction (22 g) was subjected to DIAION HP-20 column chromatography. The methanol eluate yielded precipitate **9** (310 mg). The activity of **9** is discussed later. The methanol-soluble part was further separated on a silica gel column by gradient elution with *n*-hexane and acetone. By precipitation from the fractions with methanol, **10** (180 mg) was obtained together with additional **9** (2.4 g).

Compounds **9** and **10** were identified as ursolic acid and β-sitosteryl glucoside, respectively, by direct comparison of ^{1}H-NMR and TLC with authentic compounds.

Separation of ext. 1

The extract was partitioned successively with *n*-hexane, ethyl acetate, *n*-butanol, and water. The *n*-hexane and water fractions inhibited (60%, $P < 0.01$ and 43%, $P < 0.05$, respectively) acetic-acid-induced writhing in mouse at oral dose of 2 g/kg. The ethyl acetate fraction could not be evaluated for its activity because it did not give a suitable sample for the preparation at the same dose. The hypothermic effect on mice was additionally evaluated, as hierba santa was expected to exert a sedative effect. *n*-Hexane and ethyl acetate fractions administered by i.p. injection at 300 mg/kg showed hypothermic effect on mice. The *n*-hexane and ethyl acetate fractions were independently separated as follows.

The *n*-hexane fraction was chromatographed on a silica gel column by gradient elution with *n*-hexane–ethyl acetate. Fractions that showed hypothermic effect on mice were separated repeatedly by HPLC or medium-pressured liquid chromatography (MPLC) to give palmitic acid and stearic acid.

The ethyl acetate fraction (1.92 g) included a component which showed a major spot on TLC and gave a positive reaction in the ferric chloride test. The fraction was chromatographed on Sephadex LH-20 column with methanol, and then on silica gel containing 10% water with chloroform–methanol as eluent. Crude **11** (218 mg) was

obtained, which was further purified by ODS flash column chromatography with methanol–water (1:3).

Compound **11**, a white amorphous powder, had a molecular weight of 344 as determined by FAB-MS. NMR spectral analysis, including 2D-NMR analysis, gave 2-*sec*-butyl-4,6-dihydroxyphenyl-β-D-glucopyranoside, whose structure was identical with that of cesternoside A isolated from *Cestrum nocturnum* [15]. As only published data of the acetylated compound were available, **11** was acetylated and the hexaacetate of **11** was identified as cesternoside A hexaacetate [13].

It was also found that ext. 1 contained cestrumine B (**2**) and ursolic acid (**9**), which were identified by comparison with authentic samples on TLC.

The activities of the isolated compounds are summarized later.

In vivo activities of isolated compounds

The anti-inflammatory and analgesic activities of **1**, **2**, **3**, **9**, and **11** administered orally were evaluated using the acetic-acid-induced writhing inhibition test in mice. In the case of β-carbolines, cestrumine A (**1**) inhibited writhing significantly at a dose of 70 mg/kg (36%, $P < 0.01$), although it did not show inhibition at a lower dose (30 mg/kg). Cestrumine B (**2**), the aglycone of **1**, showed significant inhibition at low doses of 25 mg/kg (45%, $P < 0.01$) and 50 mg/kg (54%, $P < 0.01$). Lignan **3** also showed analgesic activity at doses of 30 mg/kg (40%, $P < 0.01$) and 70 mg/kg (32%, $P < 0.01$), but the activity was not dose dependent.

Ursolic acid (**9**) has been reported to exhibit 31% (150 mg/kg, *p.o.*) and 34% (50 mg/kg, *i.p.*) writhing inhibition [16]. In our test, oral doses of 100 and 200 mg/kg did not show significant activity. Considering the poor absorption of **9**, the dosage form for the sample preparation was examined. Finally, crystals of **9** were dissolved in ethanol and the solution evaporated to leave a powder. The powder with 5% Tween 80 gave a suitable sample for administration. Writhing was inhibited when a dose of 75 mg/kg was administered (41%, $P < 0.01$), whereas doses as high as 150 mg/kg did not show any inhibitory activity. Writhing was inhibited by β-sitosteryl glucoside (**10**) at a dose of 100 mg/kg [17].

sec-Butylphenol-type compound **11** also exhibited analgesic activity at doses of 30 mg/kg (47%, $P < 0.01$) and 100 mg/kg (32%, $P < 0.01$), but the activity was not dose dependent.

The hypothermic effect was evaluated by intraperitoneally injecting mice with the compounds isolated from ext. 1. Compound **11** showed hypothermic effect at a dose of 100 mg/kg (−0.9°C, 0.5 h, $P < 0.05$). In the case of isolated fatty acids, the hypothermic effect was observed in palmitic acid at a dose of 50 mg/kg (−1.2°C, 0.5 h, $P < 0.01$), but not in stearic acid even at a high dose of 200 mg/kg.

In vitro activities of isolated compounds

Compounds **1**–**6**, **9**, and **11** were evaluated for their ability to inhibit PGE_1- or PGE_2-induced contractions of guinea pig ileum in the Magnus method. Compound **2** inhibited both PGE_1- and PGE_2-induced contractions at concentration of 30 μM, although its glucoside **1** did not show any inhibitory activity. Compounds **3** at 30 μM and **6** at 30 and 100 μM inhibited PGE_1- and PGE_2-induced contractions, respectively. In the case of ACh-induced contractions, **1**–**6**, **9**, and **11** did not show any inhibitory activity at concentration of 100 μM.

The inhibitory activities of **1**–**5** on norepinephrine-induced contractions of rat aorta were evaluated, and only **4** showed the activity at a concentration of 100 μM. The aglycone of **3**, (+)-pinoresinol, however, was found to strongly inhibit norepinephrine-induced rat aorta contractions at the same concentration used in our previous study [18].

Hierba santa is used in Peru to alleviate many symptoms, including inflammation and pain. Some *Cestrum* species, such as *C. auriculatum*, *C. hediundinum*, and *C. coriaceum*, are known to be the origin of this herbal medicine. However, these plants are also known by the other local name, hierba hedionda. To clarify the pharmacological activity of hierba santa, a scientific study of its efficacy was conducted. Two lots of *C. auriculatum* (herb 1 and herb 2) and one lot of *C. hediundinum* (herb 3) were examined for their anti-inflammatory and analgesic activities, and for the active components responsible for the activities.

All the extracts showed analgesic activity in the writhing inhibition test in mice. However, the active components in the extracts were not the same. In herb 3, β-carbolines, cestrumines A (**1**) and B (**2**), and lignan **3** seemed to contribute to the activity as they inhibited writhing in mice and/or PGE_1- and PGE_2-induced contractions of guinea pig ileum in the Magnus method. Spirostene glycosides, cestrusides A (**6**) and B (**7**) may be the active components as well, because **6** inhibited PG-induced contraction. Ursolic acid (**9**), the major component of herb 2 (approximately 6.4% of the extract), might be the active component in this herb because it showed writhing inhibition. Many biological effects of ursolic acid have been reported, including antihyperlipidemic and hepatoprotective effects [19–22]. β-Sitosteryl glucoside (**10**) may somewhat contribute to the efficacy of herb 2. In herb 1, isobutylphenol glycoside (**11**) showed the writhing inhibition. Herb 1 contained two other analgesic compounds, **2** and **9**, which were also the active components of herb 3 and herb 2, respectively.

Therefore, all three lots of hierba santa showed anti-inflammatory and analgesic activities, although they do not contain exactly the same active components.

Experimental

General experimental procedures

Melting points were determined on a Yanagimoto melting point apparatus. Optical rotation was recorded on a DIP 140 digital polarimeter (JASCO); ORD, on a J-20 polarimeter (JASCO); UV, on a U-3400 (Hitachi); and IR, on an FT-IR230 (JASCO). ^{1}H- and ^{13}C-NMR spectra were measured with JEOL JNM GSX-A400 and JEOL JNM GSX-A500 spectrometers with tetramethylsilane or a deuterated solvent as internal standard. Mass spectra were measured with Hitachi M-60 and JEOL JMX-HX 110A. HPLC was run on an SSC Flow System 3100 with a UV detector (SSC 3000). Column chromatographies were performed on DIAION HP-20 (Mitsubishi Chemical Corporation), Wakogel C-200 (Wako Pure Chemical Industries), and Chromatorex ODS (Fuji-Davison Chemical). Prepacked columns for HPLC, Develosil ODS-UG-5 (10 $\phi \times$ 250 mm; Nomura Chemical) and Pegasil ODS (20 $\phi \times$ 250 mm; Senshu Scientific), were also used for purification.

PGE_1 and PGE_2 (Cayman Chemical Company) and SC-51089 (BIOMOL Research Labs.) were dissolved in dimethylsulfoxide and then diluted with saline (Otsuka Pharmaceutical) or water. Acetylcholine chloride and atropine sulfate (Nacalai Tesque) dissolved in water were used. All other chemicals used in the experiments were of analytical grade.

Plant material

Three lots of hierba santa, herb 1, herb 2, and herb 3, were purchased in Peru. The air-dried aerial part of *Cestrum auriculatum* L. Heritier was obtained through the late Mr. T. Shiota, Institute de Agri-Cultura Andina y Amazonia, in 1989 (herb 1; LNP18904-01) and 1990 (herb 2; LNP19012-01) by his own identification, and was also identified by Dr. M. Satake. Fresh herbs of *C. hediundinum* Dunal were purchased in 1996 (herb 3; LNP19612-01) and were identified by Mr. J. Ruiz, curator of Herbario Ethnobotánico Amazónico. Voucher specimens were deposited in the Laboratory of Pharmacognosy, Josai International University, and the herbarium of Research Center of Medicinal Plant Resources, National Institute of Biomedical Innovation.

Isolation

The fresh aerial part of *C. hediundinum* (herb 3, 90.2 g) was extracted three times with methanol at room temperature. After evaporation of the solvent in vacuo, 10.6 g of methanol extract was obtained. The extract orally administered at doses of 1 g/kg (32%, $P < 0.01$) and 3 g/kg (36%, $P < 0.01$) demonstrated analgesic activity in the acetic-acid-induced writhing inhibition test in mice. The extract also inhibited ACh-, PGE_1- or PGE_2-induced contraction of guinea pig ileum at concentration of 3×10^{-4} g/ml. The isolation was carried out by monitoring fractions according to their ability to inhibit writhing in mice and to inhibit ACh-, PGE_1- or PGE_2-induced contractions of guinea pig ileum.

The extract dissolved in methanol was applied to DIAION HP-20 to remove chlorophyll fractions. The methanol eluate gave precipitate **1** (4 mg). The remaining methanol-soluble fraction (3.6 g) inhibited writhing at a dose of 1 g/kg (40%, $P < 0.01$) as well as ACh-, PGE_1- or PGE_2-induced contractions at 1×10^{-4} g/ml. The fraction was partitioned successively with chloroform, *n*-butanol, and water. Both chloroform (490 mg) and *n*-butanol (920 mg) fractions gave precipitates, **2** (16 mg) and **1** (18 mg), respectively.

The mother liquors, fr. 2-A-sol and fr. 2-B-sol, also inhibited writhing in mice (26%, $P < 0.05$, 200 mg/kg and 33%, $P < 0.05$, 300 mg/kg, respectively). Both inhibited PGE_1-, PGE_2- or ACh-induced contractions of guinea pig ileum at concentration of 3×10^{-5} g/ml as well.

Fr. 2-A-sol (470 mg) was further subjected to silica gel flash column chromatography with gradient elution of chloroform–methanol–water to yield four fractions fr. 3-A–fr. 3-D. By LH-20 purification (methanol), fr. 3-B (the 10:1:0.1 eluate, 19 mg) and fr. 3-C (the 7:1:0.1 eluate, 22 mg) yielded **3** (4 mg) and **2** (1 mg), respectively.

Fr. 2-B-sol (900 mg) was subjected to DIAION HP-20 chromatography to obtain fr. 4-A (115 mg) with water as eluent, and fr. 4-B (211 mg), 4-C (36 mg), and 4-D (396 mg) with methanol–water (7:3, 1:1, and 3:7, respectively). Only the test for ACh-induced contraction of guinea pig ileum was conducted and the inhibitory effect was observed in fr. 4-C and fr. 4-D at concentration of 1×10^{-5} g/ml.

From fr. 4-C, **4** (1 mg) and **5** (1 mg) were obtained by purification on LH-20 with methanol and then on ODS-HPLC with methanol–water (1:1). Fr. 4-D was separated by silica gel flash column chromatography with gradient elution of chloroform–methanol–water. The 4:1:0.1 eluate (19 mg) afforded precipitate **1** (2 mg), whose mother liquor yielded **3** (2 mg) by ODS-HPLC with methanol–water (4:6). The 3:1:0.1 eluate (168 mg) was chromatographed repeatedly on an ODS flash column with methanol–water

(4:6–6:4) and then on an ODS-HPLC column with methanol–water (4:6 or 7:3) to give **4** (6 mg), **6** (35 mg), and **7** (3 mg). Compound **8** (6 mg) was obtained from fr. 4-B by ODS-HPLC with methanol–water (4:6) as eluent.

The dried aerial part of *C. auriculatum* (herb 2, 787 g) was extracted with methanol at room temperature, and evaporation of the solvent in vacuo afforded the extract (82.0 g). The extract orally administered showed analgesic activity in the acetic-acid-induced writhing inhibition test in mice at doses of 1 g/kg (49%, $P < 0.01$) and 3 g/kg (44%, $P < 0.01$). The extract also inhibited ACh-induced contraction of guinea pig ileum at concentration of 1×10^{-3} g/ml, while it did not inhibit PGE_1- or PGE_2-induced contraction at the same concentration.

The extract was partitioned with ethyl acetate and water. The ethyl acetate fraction (22.2 g) was subjected to DIAION HP-20 chromatography with methanol to remove chlorophylls. The methanol eluate (15.5 g) yielded precipitate **9** (310 mg), which was further purified by recrystallization from ethanol. The remaining fraction (15.2 g) was separated by silica gel column chromatography with gradient elution of *n*-hexane-acetone. From the *n*-hexane-acetone (3:1) eluate, **9** (2.44 g) was additionally obtained by precipitation and by ODS column chromatography with methanol–water (1:6). Compound **10** (180 mg) was obtained as a white precipitate from the 2:1 eluate by the addition of methanol.

The dried aerial part of *C. auriculatum* (herb 1, 730 g) was extracted with methanol at room temperature and then evaporated in vacuo to obtain 80.4 g of the methanol extract. The extract orally administered showed analgesic activity (44%, $P < 0.01$) in the acetic-acid-induced writhing inhibition test in mice at dose of 2 g/kg. The extract also induced sedation and blepharoptosis in mouse at dose of 3 g/kg, *p.o*. Rectal temperature was decreased by i.p. injection of 300 mg/kg in mice (-1.5°C, 0.5 h, $P < 0.01$). The extract also inhibited methamphetamine-induced locomotor activity in mice at dose of 300 mg/kg, i.p. ($P < 0.01$), as well as ACh- or PGE_1-induced contraction of guinea pig ileum at concentration of 3×10^{-4} g/ml, while it had no effect on PGE_2-induced contraction at the same concentration. The extract was partitioned successively with *n*-hexane, ethyl acetate, *n*-butanol, and water to obtain fractions weighing 27.2 g, 1.92 g, 9.91 g, and 24.1 g, respectively. The *n*-hexane and water fractions showed 60% ($P < 0.01$) and 43% ($P < 0.05$) writhing inhibition, respectively, at oral dose of 2 g/kg. The ethyl acetate fraction could not be tested because it did not give a suitable sample for administration at the similar doses. The *n*-hexane and ethyl acetate fractions at dose of 300 mg/kg (i.p.) had a hypothermic effect in mice, such as -1.0°C (0.5 h, $P < 0.05$) and -1.0°C (1.0 h, $P < 0.01$), respectively.

The ethyl acetate fraction included a major component which showed a positive reaction in the ferric chloride test on TLC. The corresponding compound was isolated from the fraction (1.5 g) by column chromatography using Sephadex LH-20 with methanol and silica gel with gradient elution of chloroform–methanol. Crude **11** (218 mg) was obtained from the fraction eluted with chloroform–methanol (20:1). Further purification was accomplished by ODS flash column chromatography with methanol–water (1:3).

The *n*-hexane fraction was separated according to its effect on mouse body temperature. The fraction (18.9 g) was subjected to silica gel chromatography with gradient elution of *n*-hexane–ethyl acetate. The 15:1 eluate (960 mg) that exhibited the activity was repeatedly separated by MPLC (silica gel, ODS) and HPLC (silica gel) to yield palmitic acid (51 mg) and stearic acid (7 mg).

*Cestrumine A (**1**)* (*β*-D-glucosyl-1-acetyl-*β*-carboline-3-carboxylate): pale yellow needles. M.p.: 271.2–272.7°C (DMSO). HR-FAB-MS (NBA) *m/z*: 417.1276 ($[M+H]^+$; calcd. for $C_{20}H_{21}O_8N_2$: 417.1298). UV λ_{max} (MeOH) nm (log ε): 219 (4.39), 239 (4.02), 287 (4.58), 370 (3.73). IR (KBr) cm^{-1}: 3484, 3397, 1735, 1664. ^{1}H-NMR (DMSO-d_6) δ: 2.85 (3H, s; 15-H_3), 3.23 (1H, td, $J = 8.7$, 5.3 Hz; 4′-H), 3.33–3.39 (2H, m; 3′-H, 5′-H), 3.43 (1H, td, $J = 8.3$, 5.4 Hz; 2′-H), 3.51 (1H, dt, $J = 12.0$, 5.9 Hz; 6′-H), 3.71 (1H, ddd, $J = 12.0$, 5.9, 2.1 Hz; 6′-H), 4.65 (1H, t, $J = 5.9$ Hz; 6′-OH), 5.08 (1H, d, $J = 5.6$ Hz; 4′-OH), 5.19 (1H, d, $J = 4.9$ Hz; 3′-OH), 5.45 (1H, d, $J = 5.1$ Hz; 2′-OH), 5.74 (1H, d, $J = 8.0$ Hz; 1′-OH), 7.38 (1H, td, $J = 8.0$, 0.9 Hz; 8-H), 7.65 (1H, ddd, $J = 8.3$, 8.0, 1.1 Hz; 9-H), 7.87 (1H, d, $J = 8.3$ Hz; 10-H), 8.46 (1H, d, $J = 8.1$ Hz; 7-H), 9.23 (1H, s; 4-H), 12.34 (1H, s; 12-H). ^{13}C-NMR (DMSO-d_6) δ: 25.6 (C-15), 60.6 (C-6′), 69.5 (C-4′), 72.7 (C-2′), 76.5 (C-3′), 78.0 (C-5′), 95.3 (C-1′), 113.5 (C-10), 120.3 (C-6), 121.1 (C-8), 121.7 (C-4), 122.2 (C-7), 129.4 (C-9), 131.3 (C-5), 135.0 (C-3), 135.1 (C-13), 135.6 (C-1), 142.3 (C-11), 163.4 (C-16), 201.0 (C-14).

*Acid hydrolysis of **1***: A solution of **1** (1 mg) in 1 N HCl (1 ml) was stirred for 16 h at room temperature and then warmed at 50°C for 1 h. The reaction solution was extracted with chloroform and the extract was applied to a silica gel column to obtain the aglycone (0.4 mg), which was identified as **2** by TLC and ^{1}H-NMR. The remaining aqueous part was subjected to Sephadex LH-20 column chromatography. The obtained sugar was identified by comparing ^{1}H-NMR spectra with those of authentic D-glucose.

*Methyl esterification of aglycone of **1***: To a solution of the aglycone of **1** (3 mg) in tetrahydrofuran (500 μl) were added dicyclohexylcarbodiimide (10 mg), dimethyl aminopyridine (2 mg), and methanol (10 μl). The mixture was stirred for 2 h at room temperature. After purification by silica gel column chromatography, the methyl ester (1 mg) was obtained.

Table 1 NMR spectral data of **6** (methanol-d_4 and pyridine-d_5), monoglycoside of **6** (methanol-d_4), and aglycone of **6** (pyridine-d_5)

Position		**6** (CD_3OD)					**6** (C_5D_5 N)				Monoglycoside of **6** (CD_3OD)			Aglycone of **6** (C_5D_5 N)			
		d_H		*J* (Hz)	d_C	HMBC	d_H		*J* (Hz)	d_C	d_H		*J* (Hz)	d_H		*J* (Hz)	d_C
1		3.13	d	9.2	82.4	2,3,5,9,19	3.52	br.d	7.6	81.7				3.54	br.d	7.6	81.8
2		3.38	dd	9.2, 9.2	77.2	1,3	3.98–4.00	m		76.4							79.0
3		3.55–3.63	m		81.1		3.98–4.00	m		81.8							73.3
4		1.43–1.50	m		33.0	5	1.84–1.94	m		33.4							37.2
		1.66–1.70	m			5	2.01–2.04	m									
5		1.03–1.10	m		42.7		0.99–1.05	m		41.9							42.6
6		1.39–1.48	m		29.2		1.30–1.33	m		28.5							28.6
		1.39–1.48	m				1.30–1.33	m									
7		0.83–0.91	m		33.5		0.81–0.85	m		32.1							32.3
		1.66–1.70	m				1.59–1.63	m									
8		1.41–1.49	m		35.5	10	1.52–1.60	m		34.7							34.7
9		0.94–1.01	m		55.5		1.02–1.14	m		54.5							54.7
10		–			42.9		–			41.7							42.3
11		1.41–1.49	m		34.6	8,9,12,13	1.89–1.97	m		34.8							34.8
		2.49	dt	14.0, 4.3			3.23	dt-like	13.7, 3.7					3.23	dt-like	13.7, 4.2	
12		3.22	br.dd	11.4, 4.4	80.9	13,17,18	3.57–3.61	m		79.7				3.60	dt-like	11.0, 4.7	79.8
13		–			46.7		–			46.2							46.2
14		1.03–1.10	m		55.9		1.08–1.15	m		55.0							55.1
15		1.34–1.39	m		32.5	14,16	1.56–1.65	m		32.1							32.1
		2.00	ddd	11.9, 7.3, 5.8		13,16,17	2.09–2.13	m									
16		4.38–4.43	m		82.3		4.58–4.63	m		81.5	4.41	q-like	6.8	4.62	q-like	7.3	81.5
17		1.86–1.92	m		63.6		2.19	dd	7.6, 7.0	63.2							63.2
18		0.76	s		11.2	12,13,14,17	1.13	s		11.3	0.89	s		1.13	s		11.8
19		0.91	s		8.6	1,9,10	1.20	s		8.8	1.28	s		1.14	s		8.9
20		1.86–1.92	m		43.6	13,17,22	2.19–2.26	m		43.0							43.0
21		0.99	d	6.4	14.0	17,22	1.40	d	6.7	14.4	0.99	d	6.6	1.39	d	6.7	14.4
22		–			110.8		–			109.7							109.7
23		1.66–1.70	m		34.0	22	1.79–1.83	m		33.4							33.4
		1.75	td	13.1, 4.9		22,25	1.83–1.88	m									
24		2.24	br.d	13.1	29.5		2.24–2.27	m		29.1							29.1
		2.54	td	13.1, 4.9			2.76	td-like	12.5, 5.5					2.74	td-like	13.0, 5.7	
25		–			145.3		–			144.7							144.7
26	eq	3.81	br.d	11.6	65.8	22,24,25,27	4.06	br.d	12.2	65.0	3.80	d	11.2	4.04	d	12.5	65.0
	ax	4.25	br.d	12.2		24,25,27	4.52	br.d	12.2		4.26	d	11.7	4.54	d	11.9	
27	a	4.76	br.s		108.9		4.82	br.s		108.6	4.76	br.s		4.81	br.s		108.6

Table 1 continued

Position		**6** (CD_3OD)					**6** (C_5D_5 N)				Monoglycoside of **6** (CD_3OD)				Aglycone of **6** (C_5D_5 N)			
		d_H		*J* (Hz)	d_C	HMBC	d_H		*J* (Hz)	d_C	d_H		*J* (Hz)	d_C	d_H		*J* (Hz)	d_C
	b	4.73	br.s				4.79	br.s			4.73	br.s			4.77	br.s		
1′		4.42	d	7.6	101.1	3	5.02	d	7.9	101.4	4.30	d	7.3					
2′		3.59	dd	9.5, 7.6	77.1	1′,3′	4.61–4.65	m		76.1								
3′		3.59	dd	9.5, 3.1	76.1	2′,4′	4.24–4.28	m		76.5								
4′		3.74	br.d	4.0	71.0	3′	4.39–4.45	m		70.9								
5′		3.50	br.dd	7.8, 4.4	76.7	4′	4.12	t-like	6	77.0								
6′	a	3.64	dd	11.6, 4.0	62.6	4′	4.36–4.45	m		62.3	3.66	dd	11.4, 4.6					
	b	3.76	dd	11.6, 7.6		4′,5′	4.36	dd	10.5, 5.1									
1″		5.15	d	1.5	102.4	2′,5″	6.27	br.s		102.1								
2″		3.9	dd	3.4, 1.5	72.2	3″,4″	4.79	br.s		72.5								
3″		3.64	dd	9.6, 3.2	72.4	4″	4.60–4.64	m		72.8								
4″		3.38	dd	9.6, 9.6	73.9	5″,6″	4.24–4.28	m		74.2								
5″		4.07	dq	9.6, 6.4	69.7	4″,6″	4.79–4.84	m		69.4								
6″		1.23	d	6.4	17.9	4″	1.53	d	5.5	18.4								

*Methyl ester of aglycone of **1** (methyl ester of **2**)*: greenish pale brown powder. EI-MS *m/z* (%): 268 (100) [M^+], 236 (30), 208 (65), 194 (67), 182 (48), 166 (54), 154 (23). ^{1}H-NMR data were identical with published data [9].

*Cestrumine B (**2**)* (1-acetyl-*β*-carboline-3-carboxylic acid): yellowish powder. EI-MS *m/z* (%): 254 (36) [M^+], 207 (100), 194 (26), 182 (27). ^{1}H-NMR data were identical with published data [23].

*Pinoresinol glucoside (**3**)*: pale yellow amorphous solid. $[\alpha]_D^{17}$ +0.6° (*c* 0.1, MeOH) {ref. (+)-pinoresinol glucoside, $[\alpha]_D^{22}$ +8.0° (*c* 0.1, MeOH) [13]; (−)-pinoresinol glucoside, $[\alpha]_D^{24}$ −82.3° (*c* 0.89, MeOH) [24]}. HR-FAB-MS (NBA) *m/z*: 543.1834 ([M+Na]$^+$; calcd. for $C_{26}H_{32}O_{11}Na$: 543.1842). CD (8.3×10^{-4} M, MeOH) nm ($\Delta\varepsilon$): 289 (−0.2), 273 (−1.5), 249 (+0.2), 229 (−5.4). {ref. (+)-pinoresinol glucoside; 288 (+1.6), 270 (−2.5), 246 (+0.5), 233 (−17) [13]}, {(−)-pinoresinol glucoside; 301(−0.2), 280.8(−22.7), 250.6(−2.1), 227 (−45.4), 212 (−99.7) [24]}.

*Enantioselective HPLC of **3***: Compound **3** was analyzed on a chiral column [Chiral CD-Ph (Shiseido)] with acetonitrile-water (4:1) as eluent at a flow rate of 0.4 ml/min, and detected by measuring UV at 270 nm. In this condition, the retention times (rt) of authentic (+)-pinoresinol glucoside and (−)-pinoresinol glucoside were 34 and 37 min, respectively. HPLC of **3** gave two peaks at rt 34 min and rt 37 min, whose ratio was ca. 9:1, and these were identified as (+)- and (−)-pinoresinol glucoside, respectively.

Cestruside A (6) (3-*O*-[α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl]-spirosta-25(27)-ene-1*β*,2*α*,3*β*,12*β*-tetrol): white powder. ORD (*c* 0.064, MeOH) $[\alpha]^{21}$ nm: −53° (589), −80° (500), −138° (400), −304° (300), −562.5° (250). HR-FAB-MS (NBA) *m/z*: 793.3989 ([M+Na]$^+$; calcd. for $C_{39}H_{62}O_{15}Na$: 793.3986). IR *v* (KBr) max cm^{-1}: 3420, 2930, 1458, 1375, 1230, 1051, 941, 922, 878, 818. ^{1}H-NMR (CD_3OD), ^{13}C-NMR (CD_3OD), ^{1}H-NMR (pyridine-d_5), and ^{13}C-NMR (pyridine-d_5) data are shown in Table 1.

*Hydrolysis of **6***: To **6** (3 mg) in water (200 μl) was added hesperidinase (2 mg) and the reaction mixture was stirred for 2 days at 40°C. The reaction mixture was extracted with ethyl acetate and the extract was purified by preparative TLC (silica gel, chloroform–methanol–water 3:1:0.1). The monoglycoside (1 mg) was obtained.

*Monoglycoside of **6***: white powder. HR-FAB-MS (NBA) *m/z*: 647.3378 ([M+Na]$^+$; calcd. for $C_{33}H_{52}O_{11}Na$: 647.3407). ^{1}H-NMR (CD_3OD) data are shown in Table 1.

*Hydrolysis of monoglycoside of **6***: The monoglycoside of **6** (1 mg) was hydrolyzed with *β*-D-galactosidase (1 mg) in water (200 μl) for 3 days at 40°C. Extraction of the reaction mixture with ethyl acetate, followed by purification on

Table 2 NMR spectral data of **7** (methanol-d_4 and pyridine-d_5)

Position		**7** (CD_3OD)				**7** (C_5D_5N)			
		d_H		*J* (Hz)	d_C	d_H		*J* (Hz)	d_C
1		3.09	d	9.2	83.0	3.56	d	9.2	82.2
2		3.48	dd	9.2, 9.2	76.2	4.09	dd	9.2, 8.5	75.6
3		3.40–3.48	m		81.0	3.92–3.97	m		81.5
4		2.41–2.43	m		37.6	2.88	dd	13.4, 5.8	37.6
		2.41–2.43	m			2.93	br.t	13.4	
5		–			138.5				138.1
6		5.61	br.d	5.5	126.3	5.52	br.d	5.5	125.2
7		1.38–1.44	m		32.7	1.45–1.52	m		32.1
		1.99–2.04	m			1.83–1.92	m		
8		1.35–1.41	m		35.8	1.54–1.59	m		31.6
9		1.05–1.14	m		51.8	1.35–1.40	m		50.8
10		–			46.1				43.2
11		1.50–1.56	m		34.1	3.27	dt-like	13.4, 4.0	34.2
		2.53	dt-like	13.7, 4.3		1.97	br.dd	13.4, 12.2	
12		3.28	br.dd	11.9, 4.9	80.5	3.65	dt-like	10.7, 4.0	79.4
13		–			46.6				45.9
14		1.24–1.30	m		56.1	1.14–1.18	m		55.2
15		1.48–1.52	m		32.5	2.07–2.12	m		32.1
		1.96–2.02	m			1.66	m		
16		4.38–4.46	m		82.3	4.61–4.66	td-like	12.7, 6.7	82.0
17		1.87–1.92	m		63.5	2.19	dd	8.2, 6.4	63.1
18		0.78	s		11.1	1.14	s		11.2
19		1.09	s		14.9	1.37	s		14.8
20		1.87–1.92	m		43.6	2.21–2.27	m		43.0
21		1.00	d	6.7	14.0	1.39	d	6.7	14.3
22		–			110.9				109.7
23		1.65–1.71	m		34.1	1.80–1.86	m		33.4
		1.75	td	13.1, 5.2		1.80–1.86	m		
24		2.24	ddd	13.4, 4.6, 1.8	29.5	2.24–2.27	m		29.1
		2.53–2.55	m			2.72–2.79	m		
25		–			145.3				144.7
26	eq	3.81	br.d	11.9	65.8	4.05	d	11.9	65.0
	ax	4.26	br.d	11.9		4.51	d	11.9	
27	a	4.76	br.s		108.9	4.81	br.s		108.6
	b	4.73	br.s			4.78	br.s		
1′		4.41	d	7.6	101.3	5.00	d	7.9	101.8
2′		3.69	dd	9.7, 7.6	76.9	4.61–4.66	m		75.6
3′		3.60	dd	9.8, 3.4	76.2	4.24–4.28	m		76.9
4′		3.74–3.75	m		71.0	4.43–4.46	m		70.9
5′		3.50	br.dd	7.6, 4.6	76.7	4.06–4.11	m		76.6
6′	a	3.65	dd	11.6, 4.6	62.7	4.38–4.42	m		62.2
	b	3.76	dd	11.6, 7.6		4.32–4.35	m		
1″		5.18	d	1.5	102.2	6.30	s		101.9
2″		3.89–3.91	m		72.2	4.78–4.82	m		72.6
3″		3.63–3.67	m		72.4	4.58–4.62	m		72.8
4″		3.38	t-like	9.5	74.0	4.24–4.28	m		74.2
5″		4.09	dq	9.8, 6.4	69.8	4.81–4.87	m		69.7
6″		1.24	d	6.4	17.9	1.52	d		18.4

preparative TLC (silica gel, chloroform–methanol–water 3:1:0.1), yielded the aglycone (0.6 mg).

Aglycone of ***6***: white powder. $[\alpha]_{589}^{22}$ −30° (*c* 0.07, chloroform). HR-FAB-MS (NBA) *m/z*: 463.3039 ($[M+H]^+$; calcd. for $C_{27}H_{43}O_6$: 463.3060). IR *v* (KBr) max cm^{-1}: 3400, 2924, 2853, 1658, 1461, 1378, 1229, 1048, 1019, 923, 880. ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (pyridine-d_5) data are shown in Table 1.

Cestruside B (7) (3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl]-spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol): white powder. ORD (*c* 0.063, MeOH) $[\alpha]^{20}$ nm: −68° (589), −84° (500), −154° (400), −292° (327), −320° (300), −611° (250). HR-FAB-MS (NBA) *m/z*: 791.3815 ($[M+Na]^+$; calcd. for $C_{39}H_{60}O_{15}Na$: 791.3830). IR *v* (KBr) max cm^{-1}: 3446, 2926, 1651, 1365, 1057, 674. ^{1}H-NMR (CD_3OD), ^{13}C-NMR (CD_3OD), ^{1}H-NMR (pyridine-d_5), and ^{13}C-NMR (pyridine-d_5) data are shown in Table 2.

2-sec-*butyl-4,6-dihydroxyphenyl-β-D-glucopyranoside* ***(11)***: amorphous powder. ORD (*c* 0.107, MeOH) $[\alpha]^{21}$ nm: −28° (589), −40° (500), −79° (400), −327° (300), −748° (287), 0° (275), +0.6° (263), 0° (255), −1028° (235). FAB-MS *m/z* (%): 345 (50) $[M+1]^+$, 182 (100). CD (9.6×10^{-4} M, MeOH) nm (Δε): 277 (−0.96), 244 (−0.05), 227 (−1.98), 218 (−1.82), 205 (−6.97). ^{1}H-NMR (DMSO-d_6) δ: 0.69 (3H, t, $J = 7.3$ Hz; 9′-H_3), 1.05 (3H, d, $J = 6.8$ Hz; 10′-H_3), 1.32–1.47 (2H, m; 8′-H_2), 3.10–3.13 (1H, m; 5-H), 3.19–3.26 (1H, m; 3-H), 3.20–3.24 (1H, m; 4-H), 3.21–3.28 (1H, m; 2-H), 3.32–3.41 (1H, m; 7′-H), 3.50 (1H, dt, $J = 11.8$, 5.6 Hz; 6-H), 3.65 (1H, ddd, $J = 11.8$, 4.4, 2.2 Hz; 6-H), 4.24 (1H, d, $J = 7.6$ Hz; 1-H), 4.36 (1H, dd, $J = 5.6$, 4.4 Hz; 6-OH), 4.93 (1H, d, $J = 4.2$ Hz; 3-OH), 5.13 (1H, brs; 4-OH), 6.02 (1H, d, $J = 2.7$ Hz; 3′-H), 6.06 (1H, d, $J = 2.7$ Hz; 5′-H), 6.54 (1H, brs; 2-OH), 8.88 (1H, brs; 6′-OH), 8.92 (1H, s; 4′-OH). ^{13}C-NMR (DMSO-d_6) δ: 12.0 (C-9′), 21.8 (C-10′), 30.2 (C-8′), 32.1 (C-7′), 60.7 (C-6), 69.6 (C-4), 73.9 (C-2), 76.3 (C-3), 77.3 (C-5), 100.8 (C-5′), 102.8 (C-3′), 106.5 (C-1), 136.5 (C-1′), 141.7 (C-2′), 149.6 (C-6′), 154.6 (C-4′).

Acetylation of ***11***: Compound **11** (17 mg) was stirred with acetic anhydride (1 ml) and pyridine (1 ml) for 24 h. After water (1 ml) was added, the reaction mixture was extracted with ethyl acetate and then purified by silica gel column chromatography with benzene-acetone. The hexaacetate of **11** (24 mg) was obtained.

Hexaacetate of ***11***: white needles. M.p.: 61–62°C (ethanol). $[\alpha]_D^{24}$ −16° (*c* 2.07, acetone). {ref. $[\alpha]_D^{24}$ −27.7° (*c* 1.2, MeOH) [15]). EI-MS *m/z* (%): 554 (1), 331 (15) 182 (14), 169 (89), 109 (64), 43 (100). ^{1}H-NMR data were identical with published ones [15].

Pharmacological experiments

Male ddy mice (22–35 g, 5–7 weeks old), and Hartley male guinea pigs (330–450 g, 4–6 weeks old) were purchased from Japan SLC. The animals were conditioned for at least 1 week in a 12 h light/dark room with controlled temperature and humidity, and water and food were provided ad libitum. Animal tests were carried out in Chiba University and all procedures were in accordance with the experimental animal welfare guidelines of Chiba University.

The acetic-acid-induced writhing inhibition test, the test for hypothermic effect on normal body temperature, and the methamphetamine-induced locomotor activity test in mice were performed as described previously [25–27]. Samples were dissolved or suspended in water containing 5% Tween 80. Aminopyrine (50 mg/kg) was used as positive control of the writhing inhibition test.

The effects of the isolated compounds on ACh- or PG-induced contraction of guinea pig ileum in the Magnus method were evaluated as previously reported [28]. Resting tension of 1 g was initially applied to each ca. 1 cm ileum preparation. Then, the ileum was equilibrated in Tyrode solution (137.9 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 5.6 mM glucose) that was bubbled with 5% CO_2–O_2 at 28°C. PGE_1, PGE_2, or ACh was added at concentrations of 0.3 μM, 0.1 μM, and 1 μM, respectively. When muscle contractions stabilized, a sample dissolved or suspended in 5% DMSO-water solution was added. A force displacement transducer (TB-611T, NIHON KOHDEN, Japan) coupled to an amplifier (AP-601G, NIHON KOHDEN, Japan) was used to measure isometric contractions, and data were recorded on a chart recorder (TI-102, TOKAI IRIKA, Japan). Inhibitory activity was evaluated after two applications of samples, and more than 20% inhibition was considered positive. SC-51089, a PGE_2 antagonist (EP1 receptor), was used as positive control at a concentration of 3 μM. The effects on norepinephrine-induced contractions of rat aorta in the Magnus method were evaluated as previously reported [18].

Statistics

After data were analyzed for outlier by the Smirnov-Grubbs test, statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by the Dunnett's test, and was defined as $P < 0.05$ and $P < 0.01$.

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