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NOTE

Novel urushiols with human immunodeficiency virus type 1 reverse transcriptase inhibitory activity from the leaves of *Rhus verniciflua*

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Abstract Two novel urushiols, **1** and **2**, and two known urushiols, **3** and **4**, were isolated from the leaves of *Rhus verniciflua* and were examined for their human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) inhibitory activity. The novel urushiols were found to be 1,2-dihydroxyphenyl-3-[7′(*E*),9′(*Z*),11′(*Z*)-pentadecatrienyl]-14′-ol (**1**) and 1,2-dihydroxyphenyl-3-[8′(*Z*),10′(*E*),12′(*E*)-pentadecatrienyl]-14′-ol (**2**) by spectroscopic analyses. The absolute configuration at C-14′ in **1** and **2** was determined to be a racemic mixture of (*R*) and (*S*) isomers by ozonolysis. Compound **2** (IC_{50}: 12.6 μM) showed the highest HIV-1 RT inhibitory activity among the four urushiols, being 2.5-fold more potent than the positive control, adriamycin (IC_{50}: 31.9 μM). Although the known urushiols were isolated from the sap and leaves of *R. verniciflua*, **1** was exclusively present in the leaves, and higher amounts of **2** were found in the leaves than in the sap. Present findings indicate that the leaves of *R. verniciflua* represent a new biological resource from which novel and known urushiols may be prepared, and the possible use of novel urushiols as bioactive products.

Keywords *Rhus verniciflua* · Anacardiaceae · Leaves · Urushiol · HIV-1 reverse transcriptase inhibitory activity

Introduction

Rhus verniciflua, a member of the Anacardiaceae family, is commonly known as the lacquer tree [1]. It is used as a food additive and as traditional herbal medicine to treat cancer and stomach diseases [2]. The bark, branch and stem of *R. verniciflua* have traditionally been eaten as an additive in chicken and duck soups, especially in Korea [3]. In previous studies, urushiol [4, 5], butein [6], sulfuretin [7], fustin, fisetin and gallic acid [8] were isolated from the sap, bark and heartwood of *R. verniciflua*. Biological evaluation of the constituents has shown that these components have antioxidant [5, 8], antimicrobial [8], anti-inflammatory [7], and cytotoxic [4] properties and can inhibit nitric oxide (NO) production [6]. To date, only one study used the leaves as the study material and revealed the protective effect of dopaminergic neuronal cells using a 70 % methanol extract of the leaves [9]. However, research on the utilization of *R. verniciflua* leaves as a biological resource has not yet been performed.

The present study attempted to isolate novel compounds from *R. verniciflua* leaves and to test human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) inhibitory activity in order to evaluate the leaves as a biological resource.

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Results and discussion

Compound **1** was pale yellow oil. The FAB-MS of **1** showed a dehydrated ion $[MH–H_2O]^+$ at *m/z* 313 in a positive mode, and a deprotonated-molecular ion $[M–H]^-$ at *m/z* 329 in a negative mode. This result allowed us to establish its molecular weight as 330. The molecular formula, $C_{21}H_{30}O_3$ of **1** was deduced from the elemental analysis data (anal. calcd for $C_{21}H_{30}O_3$: C, 76.33; H, 9.15; O, 14.52. Found: C, 76.37; H, 9.16). The ^{1}H NMR spectrum showed a broad singlet of three protons on a benzene ring at δ 6.69, and signals from an unsaturated and hydroxylated alkyl group. These signals are characteristic of 1,2-dihydroxy-3-alkyl-benzene, strongly suggesting that **1** is an urushiol-type compound. Existence of the HMBC correlation between H-1′ (δ 2.60), and C-2 (δ 142.1) and C-4 (δ 119.9) confirmed that C-1′ of the alkyl group bonded at C-3 of a benzene ring. The molecule formula and ^{1}H and ^{13}C NMR spectra indicated that the alkyl group contained 15 carbons with three double bonds and one hydroxy group. The position of the double bonds in the alkyl group was determined by the H–H COSY, HSQC and HMBC spectra. In the HSQC spectrum, the methyl proton (H-15′) signal at δ 1.23 correlated with the ^{13}C signal at δ 22.9, showing that the ^{13}C signal was assigned to C-15′. The H-15′ signal is coupled with the proton signal at δ 3.89 assignable to protons attached to a carbon having a hydroxy group. Furthermore, the proton signal at δ 3.89 coupled with the proton signal at δ 2.36 assignable to protons adjacent to a double bond. This signal relationship indicated that the hydroxy group bonded to C-14′, and that a double bond was located at C-11′–C-12′. The cross peak between H-14′ signal and ^{13}C signal at δ 127.2 observed in the HMBC spectrum assigned the ^{13}C signal to C-12′. The proton signal at δ 5.51 assigned to H-12′ from the HSQC spectrum showed a cross peak with the ^{13}C signal δ 121.8, in the HMBC spectrum, so the ^{13}C signal was assigned to C-10′. This indicated that the second double bond was located at C-10′−C-9′. The H-10′ signal at δ 6.13 that showed a cross peak with C-10′ in the HSQC spectrum had a cross peak with the ^{13}C signal at δ 125.5, indicating that the ^{13}C signal was C-8′, and that the third double bond was located at C-7′−C-8′. In the HMBC spectrum, C-8′ also showed a cross peak with the proton signal at δ 2.12 assignable to H-6′ adjacent to a double bond at C-7′. The coupling constants between H-7′ and H-8′ (J = 15.0 Hz), H-9′ and H-10′ (J = 11.0 Hz), and H-11′ and H-12′ (J = 11.0 Hz) indicated 7′*E*, 9′*Z* and 11′*Z* geometry, respectively. The chemical structure of **1** was therefore elucidated to be 1,2-dihydroxyphenyl-3-[7′(*E*),9′(*Z*),11′(*Z*)-pentadecatrienyl]-14′-ol (Fig. 1).

Compound **2** had the same molecular formula, $C_{21}H_{30}O_3$ as **1**, shown by elementary analysis and FAB-MS. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1** (Table 1) indicating that **2** was an urusiol-type compound that had three double bonds and one hydroxy group in the alkyl group. The position of the hydroxy group was confirmed by the HSQC spectrum to be C-14′. In the HMBC spectrum, the methyl proton (H-15′) signal at δ 1.30 correlated with the ^{13}C signal at δ 136.4 corresponding to an olefinic carbon, indicating that a double bond was located at C-12′−C-13′. The positions of two other double bonds at C-8′−C-9′ and C-10′−C-11′ were confirmed by correlation between H-12′ (δ 6.27) and C-10′ (δ 128.8), and H-10′ (δ 6.49) and C-8′ (δ 133.5), respectively, in the HMBC spectrum. The geometry of C-8′−C-9′, C-10′−C-11′, and C-12′−C-13′ double bonds were assigned as *Z*, *E*,

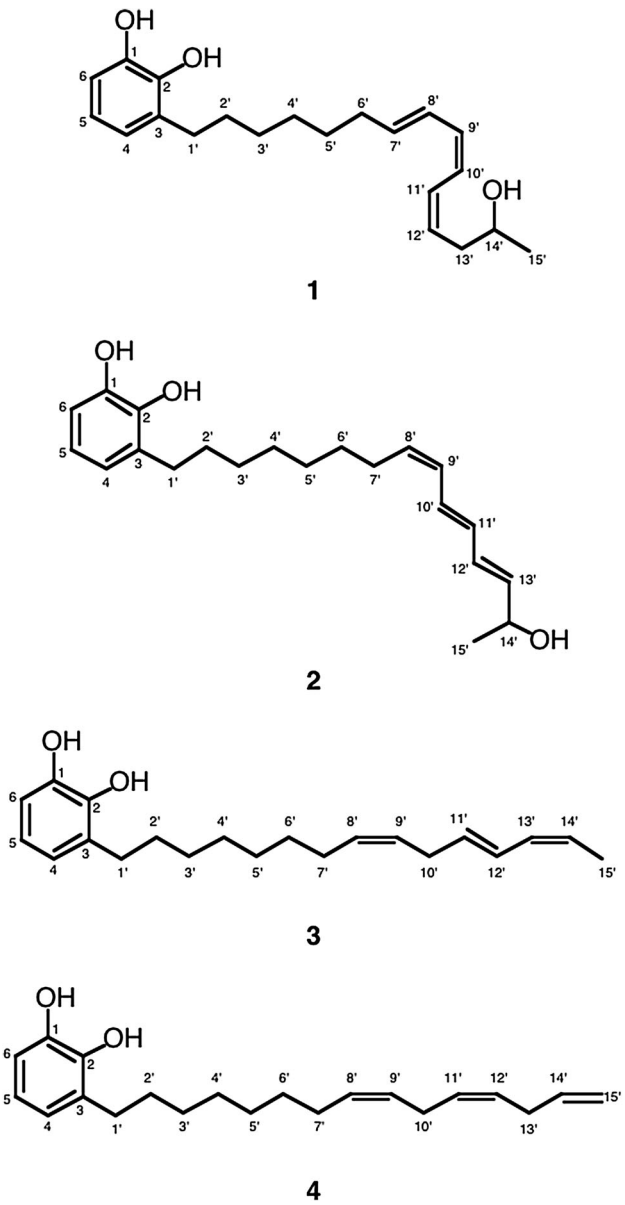

Fig. 1 Structures of isolated compounds **1–4**

Table 1 ^{1}H and ^{13}C NMR data for compounds 1 and 2 in $CDC1_3$[1]

Position	1		2	
	^{1}H	^{13}C	^{1}H	^{13}C
1		143.2		143.2
2		142.1		142.1
3		129.3		129.4
4	6.69 bs	121.9	6.69 bs	122.0[c]
5	6.69 bs	119.9[a]	6.69 bs	120.0[c]
6	6.69 bs	112.9[a]	6.69 bs	113.0[c]
1′	2.60 t ($J_{1',2'}$ = 7.7 Hz)	29.7	2.59 t ($J_{1',2'}$ = 7.7 Hz)	29.8
2′	1.60 m	29.7	1.60 m	29.7
3′	1.35 m	29.3[b]	1.36 m	29.5[d]
4′	1.35 m	29.1[b]	1.36 m	29.4[d]
5′	1.42 m	29.0[b]	1.36 m	29.2[d]
6′	2.12 dt ($J_{5',6'}$ = 13.7, $J_{6',7'}$ = 7.0 Hz)	32.9	1.36 m	29.0[d]
7′	5.75 dt ($J_{6',7'}$ = 7.0, $J_{7',8'}$ = 15.0 Hz)	137.2	2.18 dt ($J_{6',7'}$ = $J_{7',8'}$ = 7.0 Hz)	27.8
8′	6.49 dd ($J_{7',8'}$ = 15.0, $J_{8',9'}$ = 11.0 Hz)	125.5	5.47 dt ($J_{7',8'}$ = 7.0, $J_{8',9'}$ = 11.0 Hz)	133.5
9′	6.01 dd ($J_{8',9'}$ = $J_{9',10'}$ = 11.0 Hz)	130.6	6.01 dd ($J_{8',9'}$ = $J_{9',10'}$ = 11.0 Hz)	128.4
10′	6.13 dd ($J_{9',10'}$ = $J_{10',11'}$ = 11.0 Hz)	121.8	6.49 dd ($J_{9',10'}$ = 11.0, $J_{10',11'}$ = 15.0 Hz)	128.8
11′	6.61 dd ($J_{10',11'}$ = $J_{11',12'}$ = 11.0 Hz)	126.7	6.16 dd ($J_{10',11'}$ = 15.0, $J_{11',12'}$ = 11.0 Hz)	131.4
12′	5.51 dd ($J_{11',12'}$ = 11.0, $J_{12',13'}$ = 8.0 Hz)	127.2	6.27 dd ($J_{11',12'}$ = 11.0, $J_{12',13'}$ = 15.0 Hz)	130.2
13′	2.36 m	37.2	5.73 dd ($J_{12',13'}$ = 15.0, $J_{13',14'}$ = 6.4 Hz)	136.4
14′	3.89 tq ($J_{13',14'}$ = $J_{14',15'}$ = 6.2 Hz)	67.9	4.38 dq ($J_{13',14'}$ = $J_{14',15'}$ = 6.4 Hz)	68.8
15′	1.23 d	22.9	1.30 d	23.3

[1] Superscripts [a], [b], [c] and [d] on chemical shift date indicate that the assignments could be interchanged within the respective groups

and *E* on the basis of ^{1}H-^{1}H coupling constants of 11.0, 15.0 and 15.0, respectively. Compound **2** was therefore identified as 1,2-dihydroxyphenyl-3-[8′(*Z*),10′(*E*),12′(*E*)-pentadecatrienyl]-14′-ol (Fig. 1). Compounds **1** and **2** were both determined as novel urushiols which possess a hydroxy group at C-14′.

The optical rotations of **1** and **2** were nearly zero, suggesting that their specific rotations were very small, or that both **1** and **2** were racemic. Elucidation of the absolute configuration at C-14′ of **1** was attempted by the Mosher method [10], but protection of the phenolic hydroxy group and esterification of the hydroxy group at C-14′ by MTPA-chloride was unsuccessful. We finally elucidated it by converting the four carbon units from C-12′ to C-15′ to 3-hydroxybutyric acid by ozonolysis followed by oxidation with H_2O_2. 3-Hydroxybutyric acid from **1** was analyzed with HPLC using a chiral column which can separate (*R*) and (*S*)-3-hydroxybutyric acids. The result showed that 3-hydroxybutyric acid from **1** was completely racemic (*R*: *S* = 49.9: 50.0, ± 0.5), indicating that **1** was a racemic mixture of (*R*) and (*S*) isomers. Compound **2** was oxidized in the same manner as **1** to give lactic acid containing the C-13′ to C-15′ units. HPLC analysis with a chiral column indicated that lactic acid from **2** was racemic (*R*: *S* = 50.3: 49.7, ± 1.4), indicating that **2** was a racemic mixture of (*R*) and (*S*) isomers as well as **1**. This result suggested that the hydroxy group at C-14′ of **1** and **2** was not stereoselectively introduced by an enzyme-like monooxygenase. The hydroxy group at C-14′ might be introduced by auto-oxidation although the mechanism is unknown. We could not separate the optical isomers of **1** and **2** to measure the biological activity.

The other two compounds were determined to be 3-[8′(*Z*),11′(*E*),13′(*Z*)-pentadecatrienyl] catechol (**3**) and 3-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl] catechol (**4**) by comparing spectral data with the literature [11, 12] (Fig. 1). Spectroscopic data revealed that compounds **3** and **4** from the leaves were identical to the structures reported for major urushiols found in the sap.

HIV-1 RT inhibitory activity of compounds **1–4** were investigated. Adriamycin, which is known to be a DNA synthesis inhibitor, served as a positive control for both HIV-1 RT inhibitory activity and a cytotoxicity assay [13, 14]. Among the test compounds, **2** showed the highest HIV-1 RT inhibitory activity, with an IC_{50} value of 12.6 μM, and its activity level was 2.5-fold higher than that of adriamycin (IC_{50}, 31.9 μM) and 4.4- to 4.5-fold greater than **3** and **4** (Table 2). Although **1** and **2** are geometric isomers, they showed more than a 6-fold difference in activity level.

Table 2 HIV-1 RT inhibitory activity and cytotoxicity in PC-3 and MRC-5 cells of isolated compounds and in adriamycin (positive control)

Compounds	Calculated IC_{50} values (μM) ± SEM[a]		
	HIV-1 RT inhibitory activity	Cytotoxicity	
		PC-3	MRC-5
1,2-dihydroxyphenyl-3-[7′(*E*),9′(*Z*),11′(*Z*)-pentadecatrienyl]-14′-ol **(1)**	78.10 ± 3.52	61.78 ± 2.81	49.58 ± 8.38
l,2-dihydroxyphenyl-3-[8′(*Z*),10′(*E*),12′(*E*)-pentadecatrienyl]-14′-ol **(2)**	12.58 ± 3.16	86.31 ± 2.72	48.70 ± 4.78
3-[8′(*Z*),11′(*E*),13′(*Z*)-pentadecatrienyl] catechol **(3)**	56.84 ± 3.38	4.56 ± 0.57	7.58 ± 1.03
3-[8′(*Z*),11′(*E*),14′-pentadecatrienyl] catechol **(4)**	55.36 ± 2.36	4.53 ± 0.47	5.83 ± 0.76
Adriamycin	31.86 ± 0.81	0.45 ± 0.07	0.39 ± 0.03

[a] HIV-1 RT inhibitory activity and cytotoxicity in PC-3 and MRC-5 cells are the average ($n = 5$) of calculated IC_{50} values

Compounds **1** and **2** showed low cytotoxicity not only against the noncancerous cell line (MRC-5), but also against the cancer cell line (PC-3). The cytotoxicity of **1** and **2** against MRC-5 was much lower than **3** and **4** and adriamycin (Table 2).

To evaluate the utility of the leaves as a biological resource, HPLC analysis was performed using the leaves and sap extract, after which the amounts of **1–4** in the extract were calculated. The amounts of **1** and **2** per 100 g dry weight of leaf extracts were 12.7 mg and 126.0 mg, respectively, and the amount of **2** in the sap extract was 22.3 mg; compound **1** was not detected in the sap extract. By comparison, the amounts of **3** and **4** per 100 g dry weight of leaf and sap extracts were 20.08 and 8.54 g, and 31.63 and 9.71 g, respectively. Quantitative analysis of compounds in the leaves and sap extracts showed that the leaves can be utilized as a new biological resource for the preparation of urushiols.

Materials and methods

General experimental procedures

NMR spectra were recorded on a JEOL ECA500, JNM400A and Avance III 400 spectrometer (Bruker). Low-resolution mass spectra were obtained using a JEOL JMS-SX102A instrument. HPLC was performed using an LC-6A system (Shimadzu). Pegasil-ODS SP-100 (Sensyu Scientific), Develosil ODS-5 (Nomura Chemical), CHIRALPAK IC (Daicel), YMC-Pack ODS-AQ (YMC) and Sumichiral OA-6100 (Sumika Chemical Analysis Service) were used for HPLC columns. Column chromatography was performed using silica gel 60 N (100–210 μm; Kanto Chemical). TLC was performed using precoated silica gel 60 F_{254} plates (Merck).

Plant material

The leaves of *R. verniciflua* were collected from Guizhou, China in June 2002, and identified by Prof. Zhu Shougian, College of Forestry of Guizhou University. A voucher specimen (No.R20020620) was deposited at the Food Material Center for Research and Development, Kibun Foods Inc., Tokyo, Japan. The sap of *R. verniciflua* was collected from Chengkou, Chongqing, China in July 2012 and was purchased from Hariyo Shikko, Tokyo, Japan.

Extraction and isolation

The dried and ground leaves of *R. verniciflua* (0.5 kg) were extracted with Et_2O (5 L) while stirring at room temperature for 14 h. The solvent was concentrated in vacuo to yield 28 g. The crude extract was subjected to silica gel column chromatography (500 g) with *n*-hexane–EtOAc (2:3) to afford ten fractions (**Fr. 1–10**). **Fr. 7–9** (361 mg) were applied to silica gel column chromatography (20 g) and eluted with *n*-hexane–EtOAc (4:1) to give five fractions (**fr. 6–10**) (96.7 mg). The above-mentioned extraction and isolation steps were repeated five times, and **fr. 6–10** (484 mg) were obtained from the dried leaves (2.5 kg). Fractions (**fr. 6–10**) were purified by preparative HPLC [Pegasil-ODS SP-100 (20 × 250 mm), CH_3CN–H_2O (48:52) containing 0.05 % acetic acid] to yield compounds **1** (20 mg) and **2** (46 mg).

Part of **Fr. 5** (1.6 g) was separated by silica gel column chromatography (50 g) with *n*-hexane–EtOAc (10:1) and subsequently by preparative HPLC [Develosil ODS-5 (10 × 250 mm), CH_3CN–H_2O (85:15) containing 0.05 % acetic acid and CHIRALPAK IC (4.6 × 250 mm), *n*-hexane–CH_2Cl_2 (90:10)] to yield compounds **3** (41.6 mg) and **4** (37.2 mg).

HPLC analysis

Analyses of compounds **1** and **2** were performed using Pegasil-ODS SP-100 (4.6 × 250 mm) with CH_3CN–H_2O (48:52) containing 0.05 % acetic acid, and analyses of **3** and **4** were conducted using CHIRALPAK IC (4.6 × 250 mm) with *n*-hexane–CH_2Cl_2 (90:10), at a flow

rate of 2.0 ml/min with detection at 272 nm. The amounts of compounds **1–4** in the extracts were calculated from standard curves derived from isolated compounds.

HIV-1 RT inhibitory activity assay

HIV-1 RT (recombinant, Bio Academia) was adjusted to 4 mU/μl with a solution of 50 mM Tris–HCl buffer (pH 8.0), 100 mM KCl, 50 % glycerol, and 10 mM dithiothreitol. The reaction mixture (50 μl) containing 50 mM Tris–HCl (pH 8.0), 100 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2.0 μg/ml (ca. 6 μM) polyA $(dT)_{15}$ as a template primer, 3 μM dTTP, 0.33 μM [*methyl*-^{3}H] dTTP (37 MBq/ml, Moravek Biochemicals), 40 mU of RT, and 10 μl of compound dissolved in DMSO (final concentration, 10 %) was incubated at 37 °C for 20 min. The reaction was terminated by the addition of 20 μl of 0.1 M EDTA. The resulting mixture (50 μl) was applied to a Whatman DE81 filter and washed four times with 5 % Na_2HPO_4, and twice with distilled water. The filter was then dried and immersed in 5 ml of scintillation fluid. The amount of polymer fraction was determined by measuring the radioactivity on the filter according to the incorporation of [^{3}H]-labelled substrate into the polynucleotide products.

Cytotoxicity assay

PC-3 (human prostate cancer) and MRC-5 (noncancerous human foetal lung fibroblast) cells were obtained from the JCRB cell bank, Japan. PC-3 cells were cultured in RPMI-1640 (Gibco) with 10 % FBS (Gibco), and MRC-5 cells were maintained in DMEM (Gibco) with 10 % FBS and 0.1 mM non-essential amino acids at 37 °C, 5 % CO_2. Approximately 1,500–4,000 cells were seeded into 96-well plates and incubated for 16 h. Then test compounds were added. After an incubation period of 72 h, 10 μl of WST-1 solution was added to each well, followed by further incubation at 37 °C for 3 h. The dissolved formazan product was quantified spectrophotometrically at 450 nm/655 nm.

Absolute configuration of **1** and **2**

Compounds **1** (10 mg) and **2** (15 mg) were individually dissolved in 3 ml of acetic acid, and ozone gas from an ozone generator (Nippon Ozone) was passed through the solution for 15 h at room temperature. Next, 0.4 ml of 30 % hydrogen peroxide was added to the solution, and the reaction mixture was stirred for 8 h (**1**) or 70 h (**2**) at room temperature [15]. The solution was concentrated and purified by preparative HPLC [YMC-Pack ODS-AQ (6.0 × 100 mm), 0.1 % acetic acid/100 % water, 0.7 ml/min with detection at 210 nm]. The materials were collected to give 3-hydroxybutyric acid (0.4 mg) from **1** and lactic acid (0.4 mg) from **2**. Optical resolution of 3-hydroxybutyric acid and lactic acid were used (Sumichiral OA-6100; 4.6 × 150 mm) with 1 mM $CuSO_4$ at a flow rate of 1.0 ml/min with detection at 254 nm. The retention times were (*R*)-3-hydroxybutyric acid, t_R9.6 min; (*S*)-3-hydroxybutyric acid, t_R8.2 min; (*R*)-lactic acid, t_R4.5 min; and (*S*)-lactic acid, t_R7.5 min.

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