CARBOXYLIC ACIDS FROM *Phyllanthus urinaria*

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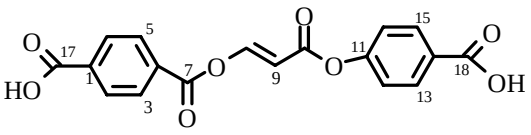
Five compounds, terephthalic acid mono-[2-(4-carboxy-phenoxycarbonyl)-vinyl] ester (1), (E)-3-(5′-hydroperoxy-2,2′-dihydroxy[1,1′-biphenyl]-4-yl)-2-propenoic acid (2), 3,4,5-trihydroxybenzoic acid (3), succinic acid (or butanedioic acid) (4), and 2,3,4,5,6-pentahydroxybenzoic acid (5), were isolated from Phyllanthus urinaria. *The structures of these compounds were elucidated by means of spectral techniques including IR, MS, and 1D/2D NMR. 1 and 2 are new compounds.*

Key words: *Phyllanthus urinaria*, terephthalic acid mono-[2-(4-carboxy-phenoxycarbonyl)-vinyl] ester, (E)-3-(5′-hydroperoxy-2,2′-dihydroxy[1,1′-biphenyl]-4-yl)-2-propenoic acid, 3,4,5-trihydroxybenzoic acid, succinic acid, 2,3,4,5,6-pentahydroxybenzoic acid.

Phyllanthus urinaria Linn. is a small plant, which is widespread in China. This plant is reputed in folklore to be a remedy for jaundice [1], hepatitis B, neprolithiasis, and painful disorders [2–4]. Investigation of its bioactivity showed an anticancer effect [5]. The ethyl acetate fraction of *P. urinaria* showed anti-proliferative activities. The ethyl acetate fraction and one compound obtained from it is capable of inhibiting telomerase activity and also could inhibit *bcl2* and activate *caspase* 3 and *caspase* 8 [6]. Some investigations of the chemical composition of this plant have been reported [7–9]. In this paper we report the isolation and structural identification of two new compounds (**1** and **2**) and three known compounds from *P. urinaria*, which grow in Guangxi, south of China. In our presently work on this plant, five carboxylic acids were isolated from the butanol fraction. The structures of these compounds were elucidated by means of spectral techniques including IR, ESIMS, NMR (1D and 2D). Two of them, terephthalic acid mono-[2-(4-carboxy-phenoxycarbonyl)-vinyl] ester, (E)-3-(5′-hydroperoxy-2,2′-dihydroxy[1,1′-biphenyl]-4-yl)-2-propenoic acid, are new compounds, and 2,3,4,5,6-pentahydroxybenzoic acid was first obtained from *P. urinaria.*



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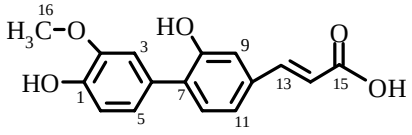

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ESIMS gave the molecular weight of the compound as 354. The proton-proton coupling constants [(δ 7.50, J = 15.86) and (δ 6.29, J = 15.93)], and a cross-peak in the HMQC of these two protons show that these two protons are linked to each carbon of the C=C fragment, and they adopt the *trans*-orientation. The amount of protons and their coupling constants and chemical shifts imply that there are phenyl groups in this compound, and these phenyl groups should be symmetrical structures, or the phenyl group has the para-substituted orientation. The proton signals at δ 7.80 and δ 6.83 appear in the same symmetrical phenyl group and these two protons are mutually coupled, and the proton signals at δ 7.52 and δ 6.80 appear in another phenyl group and they are also mutually coupled. The chemical shifts of the carbons show that C=O groups of **4** appear in this compound, and these C=O groups are not ketones, nor aldehydes. So these C=O groups should be OC=O ester groups or carboxylic acid groups.

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TABLE 1. NMR Data of Compound **1** (DMSO-d_6)

Atom C	^{1}H (Multiplicity)	^{13}C	HMBC (^{13}C)
10	-	168.18	-
17	-	167.41	-
7	-	161.81	-
18	-	159.81	-
8	7.50 (d, 1H, J = 15.86)	144.37	5, 11, 10
1	-	132.50	-
13, 15	7.52 (d, 2H, J = 8.53)	131.75	11, 18
2, 6	7.80 (d, 2H, J = 8.65)	130.30	7, 17, 9
11	-	125.50	-
6	-	121.61	-
3, 5	6.83 (d, 2H, J = 8.67)	115.97	6, 10
9	6.29 (d, 1H, J = 15.93)	115.58	11, 10
12, 16	6.80 (d, 2H, J = 8.57)	115.39	11, 18
14	-	115.07	-

Multiplicity was provided by DEPT (90 and 135) spectra.
HMQC provided links of protons and carbons.

The IR spectrum of the compound shows an absorption band between 2550–3320cm^{-1}, and strong absorption bands at 1728, 1715, 1694, 1683. Then compound **1** was deduced to be a carboxylic acid. We may conclude that the compound is constituted by two phenyls, four OC=O, and one C=C. Compared with the molecular weight of this compound, the molecular formula was obtained as $C_{18}H_{10}O_8$. The degree of unsaturation U = 13 shows that besides the two phenyl groups there are no ring structures in this compound. HMBC shows that the proton signals at δ 7.50 and 6.29 correlated to the carbon signal at δ 168.18 means that the C=O (δ 168.18) is connected to the C=C to compose an acrylic fragment C=C–C=O. The proton signal of NMR at δ 7.80 correlated to the carbon signal at δ 168.18 shows that the C=C–C=O is connected to the phenyl group that contains the proton signal at δ 7.80 by an oxygen bridge to yield phenyl acrylate. The HMBC of the proton signals at δ 7.80 and 6.83 correlated to C=O (δ 167.41 and 161.83) shows that these two C=O are connected to the phenyl group that these two protons are on. This means that this phenyl group is a *p*-phenyl dicarboxylic fragment.

The HMBC of protons (signals at δ 7.52, and 6.80) indicates only one carboxyl group (δ 159.81) linked to the phenyl ring, which consists of carbons (δ 125.50, 131.75, 115.39, 115.07). The chemical shifts of carbons of this phenyl group also imply a carbon atom (δ 131.75) linked to an oxygen atom.

NMR data show that there are no ketone nor aldehyde structures in this compound, which indicates that all C=O in this molecule is OC=O. Because of reactivity, anhydride fragments would not appear in compound **1**. These facts lead to the conclusion that the two phenyl groups are connected to each other by one acrylic group and form an ester fragment. Using IUPAC rules, this compound was given the name terephthalic acid mono-[2-(4-carboxy -phenoxycarbonyl)-vinyl] ester. Assignments of all NMR signals are showed in Table 1.

When sprayed with $FeCl_3$-methanol solution on a silica gel TLC plate, compound **2** appeared as a yellow brown spot. This fact shows that the phenol group is a fragment of the compound. A strong wide absorption band at 3350–2550 cm^{-1} in the IR spectra of compound **2** shows that the COOH group is a fragment of this it. ESI-MS gave a molecular weight of 286. The coupling constant of protons δ 7.50, J = 15.89 and δ 6.22, J = 15.88 in NMR indicate that these two protons adopt the *trans*-orientation in the carbon-carbon double bond. The chemical shift of carbons in ^{13}C-NMR indicate that 15 carbons are unsaturated, among which one is OC=O; two carbons compose a fragment of the CH=CH group in which two protons adopt the *trans*-orientation. The other 12 unsaturated carbons compose two phenyl rings. The chemical shift data of carbons also show three carbons in the two phenyl groups connected with oxygen atoms. DEPT shows only one methyl group connected to one oxygen atom. HMBC and HMQC show that six carbon signals at δ 149.70, 114.22, 112.01, 148.64, 125.58, 116.81 compose one phenyl group ring A, and the other six carbon signals at δ 123.44, 150.82, 112.12, 128.61, 124.25, 116.30 compose another phenyl group ring B.

TABLE 2. NMR Data of Compound **2** (DMSO-d_6)

Atom C	^{1}H (Multiplicity)	^{13}C	HMBC (^{13}C)
15	-	171.25	-
8	-	150.82	-
2	-	149.70	-
1	-	148.64	-
13	7.50 (d, 1H, J = 15.89)	147.15	9, 11, 15
10	-	128.61	-
5 (6)	7.46 (d, 1H, J = 6.47)	125.58	1, 4, 3, 11
11	6.97 (m, 1H, J = 6.58, 1.42)	124.25	9, 10, 13, 8
4	-	123.44	-
6 (5)	6.72 (m, 1H, J = 6.41, 1.53)	116.81	4, 2, 1
12	6.73 (d, 1H, J = 6.62)	116.30	10, 7
14	6.22 (1H, J = 15.88)	116.15	10, 15
3	7.44 (1H, d, J = 1.58)	114.22	4, 2, 8
9	7.08 (1H, s, J = 1.45)	112.12	10, 11, 13, 8
7	-	112.01	-
16	3.79 (3H, s)	56.77	1

Multiplicity was provided by DEPT (90 and 135) spectra.
HMQC provided links of protons and carbons.

Data of ^{1}H–^{1}H COSY show that every phenyl ring possesses three protons, and the coupling constant shows two of each three protons couple each other in every phenyl ring. The proton not correlated to any other protons is in the *meta*-position of one of the two coupling protons. The coupling constant of the proton signal at δ 7.08 (J = 1.45) and 6.97 (J = 1.42) shows that their correlative carbon signal at 112.12 and 124.25 were at the *meta*-position in ring B. HMBC of proton signals at δ 7.50 and δ 6.22 led to the conclusion that the C=O is connected to the CH=CH fragment, and the fragment CH=CH–CO is connected to the carbon (δ 128.61) in ring B. The HMBC signal at δ 7.08 in ring B shows that the proton is at the *ortho*-position of the carbon (δ 152.82) where the oxygen atom is connected. The HMBC of protons (δ 6.73, 6.97, 7.08) confirms that the carbon (δ 128.61) is at the *ortho*-position (δ 115.58, 125.50). The position of the carbon connected with ring A was determined to be the *ortho*-position of the carbon connected to the hydroxyl group.

The coupling constants of the proton signal at δ 7.44 (J = 1.58) and 6.72 (J = 1.53) reveal that the carbon signal at δ 114.22 is at the *meta*-position of the carbon signal at 116.81 in ring A. The chemical shifts of carbons on ring A reveal that two of them δ 149.70, 148.64 are connected to oxygen atoms. The carbon signal at δ 123.44 is connected to ring B by a carbon-carbon bond. The HMBC of the proton signal at 7.44 and 7.46 in ring A reveals that the carbon signal at 123.44 is at the *ortho*-position of the carbon signal at 114.22 and 125.58. The carbons that are connected to oxygen atoms are at the *ortho*-position mutually in ring A. The HMBC of the proton signal at 6.72 shows that the carbon signal at 116.81 is at the *ortho*-position of the carbon signal at δ 148.64, and the proton signal at 7.44 shows that the carbon signal at δ 114.22 is at the *ortho*-position of the carbon signal at 149.70. Meanwhile HMBC shows that the methoxyl group is connected to the carbon signal at δ 149.70 in ring A. We conclude that the carbons (signal at δ 150.82 in ring B, signal at δ 148.64 in ring A) are connected to hydroxyl groups. Ring A and ring B are connected mutually by a carbon-carbon bond directly at the positions of the carbons signals at δ 112.01 in ring A and signals at δ 123.44 in ring B. On the basis of all the evidence mentioned above, the structure of compound **2** was established. The assignment of all NMR signals is shown in Table 2.

Compound **2** was given the name (E)-3-(5′-hydroperoxy-2,2′-dihydroxy[1,1′-biphenyl]-4-yl)-2-propenoic acid.

Compound **3** shows acidity in aqueous solution. The spectral data (IR, NMR, and MS) coincides with a known compound, 3,4,5-trihydroxybenzoic acid. So compound **3** was identified as 3,4,5-trihydroxybenzoic acid [10]. Compound **4** was identified as 2,3,4,5,6-pentahydroxybenzoic acid [11], and compound **5**, as succinic acid, or butanedioic acid [12, 13], on the basic of their spectral data and physical properties.

EXPERIMENTAL

General Methods. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) were recorded on a Bruker AM-500 spectrometer. The sample was dissolved in CD_3SOCD_3 for NMR studies. Chemical shifts are reported in ppm, and coupling constants in Hz. The ESIMS spectrum was recorded on a Bruker Esquire 3000 *plus* ESIMS spectrometer. IR spectra were recorded on a Nicolet Protage 460 FT-IR spectrometer.

Plant Material. The plant material was collected from Nanning, Guangxi province in August 2002 and was identified as *Phyllanthus urinaria* L. A voucher specimen was kept in the College of Life Sciences of Guangxi University.

Isolation and Purification. The air-dried *P. urinaria* (7 kg) was extracted with ethanol under constant mechanical stirring. This procedure was repeated until no further extract mud was obtained. The combined extracts were filtered while warm through glass wool and then concentrated. The residue (480 g) was extracted successively with petroleum ether, ethyl acetate, and butanol. The extracts of petroleum ether, ethyl acetate, and butanol were centrifuged and then the extracts were concentrated to dryness to obtain 8 g residue from petroleum ether extract, 25 g residue from ethyl acetate extract, and 36 g residue from butanol extract. Chromatography of the 36-g residue from butanol extract was carried out on a silica gel (40–60 um) column by using a solvent composed of ethyl acetate and methanol (v/v) in various ratios. Different polarities of the eluent systems were applied after a 25×500 ml eluent was used. The fraction obtained from an eluent composed of 100% ethyl acetate gave 11 g of residue F1. The fraction obtained at a ratio of ethyl acetate–methanol = 20:1 gave 850 mg of solid residue F2. The fraction obtained at a ratio of ethyl acetate–methanol =10:1 gave 120 mg of residue F3 and the fraction of ethyl acetate–methanol = 5:1 gave 2.5 g of residue F4.

The separation of residue F2 was carried out on a silica gel H column (packed with activated silica gel H under vacuum). The F2 was dissolved in methanol and then mixed with silica gel H. Then the solvent was carefully removed from the mixture. The F2 which was mixed with silica gel H was put on the top of the silica gel H column under vacuum [14]) and was eluated with eluent consisting of 10 parts of chloroform, 3 parts of methanol, and 1 part of formic acid under 1 atm pressure. After this process was repeated for three times, 5 mg compound **1** and 8 mg compound **2** were obtained**.** Residue F3 was subjected to the same procedure used in F2 and was eluated with an eluent consisting of 10 parts of chloroform, 5 parts of methanol, and 1.5 parts of formic acid. After this procedure, 10 mg compound **3** and 5 mg of **4** were obtained. The F4 was crystallized repeatedly in methanol to give 400 mg of **5**.

Compound 1. Flaxen powder, mp 164–166°C. ESIMS provided gave a molecular weigh of 356. NMR data included ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HMQC, and HMBC, as shown in Table 1.

IR (KBr, ν_{max}, cm^{-1}): 3320–2500, 1728, 1715, 1694, 1683, 1629, 1582, 1495, 1285.

Compound 2. Brown powder, mp 142–145°C. ESIMS gave the molecular weight as 286. NMR data included ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HMQC, and HMBC, as shown in Table 2.

IR (KBr, ν_{max}, cm^{-1}): 3350–2550, 1714, 1615, 1598, 1484, 1271.

Compound 3. Brown powder, mp 252°C. Reaction of this compound with $FeCl_3$–methanol solution on a TLC plate showed a blue-black spot. ESIMS showed the molecular weight as 170. ^{13}C-NMR (DMSO-d_6) showed five types of carbon (108.87, 120.65, 138.16, 145.53, 167.63) in this compound. ^{1}H-NMR showed one H (6.92, s) in this compound, and DEPT showed only one CH appearing in its molecule.

Compound 4. Brown powder, degraded when heated. Reaction of this compound with $FeCl_3$–methanol solution on a TLC plate gave a blue-yellow spot. ESIMS gave a molecular weight as 202.

^{13}C-NMR (δ): 101.346 (C1), 112.966 (C3 C5), 130.616 (C4), 137.40 (C2 C6), 161.431 (C7).

^{1}H-NMR and DEPT showed that there were no CH, CH_2, and CH_3 in this compound.

Compound 5. Needle crystals, mp 182°C. ESIMS showed the molecular weight as 118.

^{1}H-NMR (δ): H (2.56, s). ^{13}C-NMR (δ): C (20.50) and C (166.84). DEPT showed that the carbon (20.50) was a methylene group CH_2.

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