TWO NEW POTENT INHIBITORS OF XANTHINE OXIDASE FROM LEAVES OF PERILLA FRUTESCENS BRITTON VAR. ACUTA KUDO

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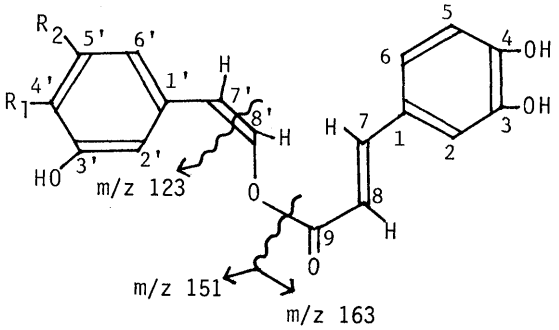
A known and a new caffeic ester (**1** and **2**), new inhibitors of xanthine oxidase (XO), were isolated from leaves of Perilla frutescens var. acuta and their structures have been established as (Z,E)-2-(3,4-dihydroxyphenyl)ethenyl ester (**1**) and (Z,E)-2-(3,5-dihydroxyphenyl)ethenyl ester (**2**) of 3-(3,4-dihydroxyphenyl)-2-propenoic acid, respectively, based on detailed spectral studies, including 2D COSY, long range COSY, difference NOE, etc. Both caffeic esters strongly inhibited XO in vitro and especially, the inhibition by **1** was as potent as that by allopurinol. The inhibition mode of **1** was also shown to be non-competitive.

KEYWORDS Perilla frutescens var. acuta; Labiatae; leaf; xanthine oxidase inhibitor; caffeic ester

Xanthine oxidase (XO; EC 1.2.3.2) is an enzyme of purine nucleotide degradation, mainly in humans. It catalyzes the oxidation of hypoxanthine to xanthine, and xanthine to uric acid. The inhibition of XO by allopurinol became the basis for the control of hyperuricemia in human gout[1] and thus, new XO inhibitors other than allopurinol are also expected to be useful as a remedy of human gout. We studied the XO inhibitory activities of 143 kinds (in their EtOH extracts) of common edible plants (77 vegetables, 42 spices, and 24 fruits). The XO activity was assayed with xanthine as a substrate in vitro and monitored spectrophotometrically at 290 nm according to the method by Noro et al.[2,3] with a partial modification. In the present preliminary screening tests, it appeared that the EtOH extract of leaves of Perilla frutescens Britton var. acuta Kudo (Shiso in Japanese; Labiatae) potently inhibited XO. Further column and HPLC separation of the extract with the guidance of the XO inhibitory activity led us to the isolation of two new potent XO inhibitory principles, **1** and **2** (0.028 and 0.007% from the extr.), having a known caffeic ester structure (**1**) and a new one (**2**). The structural elucidation and the XO inhibitory activities of both esters are dealt with here.

Both esters (**1**) (yellow fine crystals of mp 183-185°C (decomp.) (from aq.MeOH), IR (KBr) cm^{-1}: 3400, 1690, 1625, 1605, 1270, 1150) and (**2**) (yellow needles of mp 188-190°C (from aq.MeOH), IR (KBr) cm^{-1}: 3380, 1720, 1625, 1600, 1280, 1140) had the same molecular formula, $C_{17}H_{14}O_6$ (calcd: 314.0791, found: 314.0794 for both **1** and **2**) and gave common characteristic fragments (m/z 123, 151, and 163) (see structural formulas). Based on detailed and precise ^1H- and ^{13}C-NMR assignments (Table I) based on ^1H-^1H, ^{13}C-^1H and long range ^{13}C-^1H COSY (Table II) and difference NOE experiments,[4] the full structures for the caffeic esters were defined as (Z,E)-2-(3,4-dihydroxyphenyl)-ethenyl ester (**1**) and (Z,E)-2-(3,5-dihydroxyphenyl)-

(phenyl B) (phenyl A)



1 R_1 = OH, R_2 = H

2 R_1 = H, R_2 = OH

ethenyl ester (**2**) of 3-(3,4-dihydroxyphenyl)-2-propenoic acid, respectively. Some important points in the structural elucidations and the signal assignments are briefly discussed below. The geometries (Z,E) of two kinds of double bonds, Δ7' and Δ7 in both **1** and **2** were determined based on the ^{1}H-NHR J-values due to the respective double bonds and further confirmed by the difference NOE experimental proofs.[4] The presence and location of the ester bond in both **1** and **2** were revealed by the IR and mass spectral data (vide ante) and further, the cross peak between H-8' and C-9 in the long range ^{13}C-^{1}H COSY spectrum of **1** (Table II) provides corroborating evidence for the located position of the ester bond in **1**. Unfortunately, in **2** the corresponding cross peak was not observed, probably due to the low concentration of the measured solution. However, concerning the location of the ester bond in **2**, we reached the same conclusion as in **1**. Ester **2** is a new, naturally-occurring product but ester **1** has been isolated from leaves of Plectranthus caninus Roth[5] (Labiatae) and as the callus component of Lavandula angustifolia Mill[6] (Labiatae). However, the full and precise ^{1}H- and ^{13}C-NMR assignments for **1** are first reported here.

Table I. ^{1}H- and ^{13}C-NMR Spectral Data for **1** and **2** (δ, ppm from TMS in DMSO-d_6)[a]

	^{1}H-NMR (400 MHz)		^{13}C-NMR (100.5 MHz)		
	1	**2**		**1**	**2**
H-2	7.18 (d, 2.0)	7.11 (d, 2.2)	C-1	125.318 (s)	125.288 (s)
H-5	6.83 (d, 8.0)	6.79 (d, 8.0)	C-2	114.996 (d)	115.011 (d)
H-6	7.11 (dd, 8.0, 2.0)	7.08 (dd, 8.0, 2.2)	C-3	145.597 (s)	145.521 (s)
H-7	7.69 (d, 16.0)	7.64 (d, 16.0)	C-4	148.952 (s)	148.846 (s)
H-8	6.49 (d, 16.0)	6.36 (d, 16.0)	C-5	115.710 (d)	115.695 (d)
H-2'	7.24 (d, 2.0)	6.69 (brs)	C-6	122.009 (d)	121.812 (d)
H-4'	-----	6.82 (brs)	C-7	147.328 (d)	146.994 (d)
H-5'	6.75 (d, 8.3)	-----	C-8	112.249 (d)	112.158 (d)
H-6'	6.91 (dd, 8.3, 2.0)	6.69 (brs)	C-9	163.478 (s)	163.888 (s)
H-7'	5.67 (d, 7.5)	6.38 (d, 12.5)	C-1'	125.364 (s)	124.999 (s)
H-8'	7.20 (d, 7.5)	7.70 (d, 12.5)	C-2'	116.287 (d)	115.755 (d)[b]
4 x OH	9.13 (brs)	9.08 (brs)	C-3'	144.899 (s)	145.339 (s)[c]
	(3,4,3',4')	(3,4,3',5')	C-4'	144.899 (s)	112.932 (d)
			C-5'	115.497 (d)	145.066 (s)[c]
			C-6'	120.946 (d)	117.835 (d)[b]
			C-7'	111.824 (d)	115.300 (d)
			C-8'	131.617 (d)	134.046 (d)

a) ^{1}H and ^{13}C Assignments and multiplicities were decided with the aid of ^{1}H-^{1}H, ^{13}C-^{1}H, and long range ^{13}C-^{1}H COSY data and difference NOE experiments. Multiplicities and coupling constants in ^{1}H-NMR and multiplicities in ^{13}C-NMR are in parentheses. b,c) These assignments may be interchanged with each other.

Table II. Long Range ^{13}C-^{1}H COSY Data of **1** and **2**[a]

1	**2**
H-2 ---C-6, C-4	H-2 ---C-6, C-4, C-3, C-7
H-5 ---C-1, C-3	H-5 ---C-1, C-3
H-6 ---C-4, C-2	H-6 ---C-4, C-2
H-7 ---C-9, C-2, C-6, C-8	H-7 ---C-9, C-2, C-6
H-8 ---C-1, C-9	H-8 ---C-1, C-9
H-2'---C-6', C-4'[b], C-7'	H-2'---C-1' C-7'
H-5'---C-1', C-3'[c]	(H-6')
H-6'---C-4'[b], C-2', C-7'	H-4'---C-2'[d], C-5', C-3', C-6'[d]
H-7'---C-8', C-2', C-6'	H-8'---C-7'
H-8'---C-7', C-1', C-9	

a) Between the proton and each of the carbons on the same horizontal line, the C-H cross peaks were observed, respectively, and the carbons on the same horizontal line are arranged in the order of the high peak intensities. b,c) The C-3' and 4' showed the same chemical shift but in the one marked b) C-4' must be preferable to the other, and contrary to this, in c) the C-3' is better than the other. d) May be interchanged with each other.

Table III. XO Inhibitory Activities of **1**, **2**, Flavonoids, and Allopurinol

Compounds	IC_{50} (μg/ml)
1	0.021
2	0.124
Luteolin[a]	0.11
Quercetin[a]	>0.40
Allopurinol[b]	0.021

a) cf. lit. 2c,7). b) cf. lit. 8)

On the detailed HPLC examination, the existence of the XO inhibitory ester (**1**) in the original EtOH extract was confirmed, so ester **1** (also **2**) must be a native component of leaves of P. frutescens var. acuta.

In Table III, the XO inhibitory activities of **1** and **2** are compared with three references known for XO inhibitory activity. Both caffeic esters, especially **1**, were highly active against XO. Recently, a number of natural XO inhibitors such as flavonoids,[2c,7] anthraquinones,[9] phenylpropanoids,[2b,10] xanthones,[11] and tannins[12] have been isolated from the plant kingdom. The XO inhibition by **1** was as potent as that by allopurinol which has been used as a remedy for human gout.[1c,8] The inhibition mode of **1** was also studied based on kinetic analysis, the Lineweaver-Burk plots[13] of the reactions in the presence and absence of the ester **1** in an XO reaction mixture and was found to be non-competitive. In the present study, we have demonstrated that the caffeic esters **1** and **2**, especially **1** are among the most potent XO inhibitors so far determined. Thus, these caffeic esters should be useful in the medical treatment for hyperuricemia in human gout.

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3) The assay of XO activity and the estimation of XO inhibitory activity in the present paper are as follows: An assay mixture of 1.0 ml of test solution (aq.EtOH), 2.9 ml of 1/15 M phosphate buffer (pH 7.5), and 0.1 ml of 1/15 M phosphate buffer (pH 7.5) solution containing the enzyme, XO (0.23 units/ml) was preincubated at 37°C for 10 min. To this mixture, 1.0 ml of 0.15 mM aq. solution of the substrate, xanthine was added and the resulting mixture was incubated at 37°C for 30 min and without delay, the absorbance of the mixture at 290 nm was measured spectrophotometrically. As a blank, 0.1 ml of 1/15 M phosphate buffer without XO was used in the place of 0.1 ml of the buffer containing XO. The XO inhibition (%) was calculated as shown in ref. 2c.
4) The respective irradiation of H-6 in **1** and H-2 in **2** enhanced H-7 (0.3% in **1** and 2.7% in **2**) and H-8 (0.5% in **1** and 7.2% in **2**) in both **1** and **2**, demonstrating that the Δ7 double bond is of E geometry, and this Δ7 is connected with the phenyl A ring in both compounds (see the structural figures). In addition, the higher intensity of H-8 than H-7 led us to the assignment of H-7 and H-8 in both compounds. While, when H-6' in **1** and H_2-2'(6') in **2** were respectively irradiated, H-7' was only enhanced in both **1** (3.14%) and **2** (3.50%) but H-8' was not enhanced in both esters, suggesting that Δ7' is a Z form bonded to the phenyl B in both esters (see the structural figures). These facts also effected the assignment for H-7' in both compounds.
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