A Mutagenic New Iridoid in the Water Extract of Catalpae Fructus

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A mutagenic principle in the water extract from Catalpae Fructus (originated from *Catalpa ovata* G. DON) (Bignoniaceae) was isolated and characterized as a new iridoid named catalpin. The iridoid exhibited mutagenic activity towards *Salmonella typhimurium* strain TA100 in the presence and absence of rat liver homogenate (S9) mix in Ames' test.

Keywords mutagenicity; iridoid; Catalpae Fructus; *Catalpa ovata*; Bignoniaceae; relative stereochemistry

In the previous screening study of crude drugs for mutagenicity, the water extract of Catalpae Fructus, the fruits of *Catalpa ovata* G. DON (Bignoniaceae) used as a diuretic in Japan, was weakly mutagenic in Ames'test with *Salmonella typhimurium* strain TA100 in the presence and absence of rat liver homogenate (S9 mix).[1] We attempted the isolation of the mutagenic principle from the drug, and isolated a new iridoid compound.

The water extract of Catalpae Fructus was separated into the ethyl acetate-soluble part and an aqueous layer (see Experimental). Employing the mutagenicity test for monitoring, the ethyl acetate part was further fractionated by a combination of preparative thin-layer chromatography (TLC) and Sephadex LH-20 column chromatography to give a new iridoid, named catalpin (**1**).

Catalpin (**1**) was obtained as colorless needles, mp 93—94 °C, $C_{16}H_{18}O_7$. The infrared (IR) spectrum of (**1**) showed hydroxyl (3340, 3450, 3510 cm^{-1}), ester carbonyl (1685 cm^{-1}), benzene ring (1615, 845 cm^{-1}) and ester (1295, 1120 cm^{-1}) absorptions. The presence of a *p*-hydroxybenzoate moiety was indicated by λ_{max}^{MeOH} 258 nm in the ultraviolet (UV) spectrum.

In the proton (^1H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra (Table I), resonances due to a sugar moiety were not observed, suggesting that **1** was not a glycoside; instead, the resonances due to a *p*-hydroxybenzoate moiety were observed. Inspection of the ^{13}C-NMR spectrum of (**1**) led us to suppose that the compound (**1**) might be a C_9-type iridoid compound. In the ^1H-NMR spectrum, however, olefinic proton signals due to a dihydropyran ring, which is characteristic of usual iridoids such as catalposide (**2**), were absent, and instead, a proton signal at δ5.38 (dd) ascribable to a proton on the acetal carbon at C-3, which coupled with the H-4 methylene protons at δ1.55 (ddd) and δ1.92 (ddd), was observed. Sequences starting from H-3 to H-4, H-5 (δ2.69, dddd), H-6 (δ5.26, ddd) and H-7 (δ2.54, dd and δ2.01, ddd) and from H-5 to H-9 (δ2.48, dd) and H-1 (δ5.51, d) were firmly established by the two-dimensional (2D) NMR technique and the corresponding carbons, C-3 (δ90.07, d), C-4 (δ30.10, t), C-5 (δ41.02, d), C-6 (δ76.64, d), C-7 (δ45.92, t), C-9 (δ51.53, d) and C-1 (δ101.31, d), were assigned from the ^1H–^{13}C correlation spectroscopy (COSY) spectrum. A quaternary carbon (δ85.16, s) bearing an oxygen atom was assigned as C-8 in the iridoid framework. Thus, the presence of a partial structure (A) was suggested.

In the ^1H-NMR of (**1**) (in acetone-d_6), besides the *p*-substituted phenolic hydroxyl group (δ9.15), the presence of a tertiary hydroxyl group (δ4.45, s) and a secondary hydroxyl group (δ5.35, d, J=5.1 Hz) was suggested. These hydroxyl signals disappeared on addition of D_2O and the H-3 signal changed to double doublet, indicating that the secondary hydroxyl group is linked to the C-3 acetal carbon. The tertiary hydroxyl group must be linked to the quaternary carbon at C-8. The chemical shift of H-6 suggested the presence of the *p*-hydroxybenzoate group at C-6.

In the ^1H-NMR spectrum of **1** (in methanol-d_4), the signals at δ3.76 and 3.98 seem to be due to C-10 methylene protons from their coupling constants and splitting patterns. In addition to geminal coupling (J=10.8 Hz) between the C-10 methylene protons, long-range coupling (J=1.5 Hz) between H-7β (δ2.01) and H-10β (δ3.76), as observed in the case of dihydrocatalpol hexaacetate, cistanin and iridoids isolated from Rehmanniae Radix,[2] is present. These data suggested that the C-10 methylene links to the C-1 oxygen to form a five-membered ether ring.[2]

The relative stereochemistry in **1** was studied by examination of the ^1H nuclear Overhauser effect (NOE) difference spectra (Fig. 1). NOE enhancements were observed between H-5 and H-9, and H-9 and H-1, but not between H-5 and H-6. These data confirmed the relative stereochemistry between H-5 and H-9 (*cis*), H-5 and H-6 (*trans*) and H-9 and H-1 (*cis*). NOE enhancements were also observed between H-6 and H-3 as well as H-4α and H-3, suggesting that H-6 is located near H-3. Thus, the relative stereochemistry of **1** was determined.

A close structural relationship was seen between **1** and

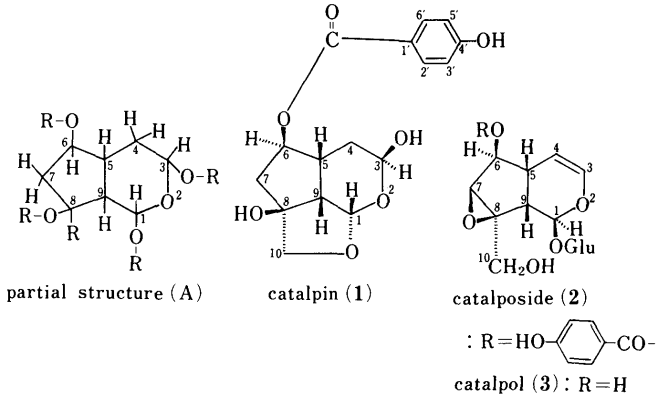

Chart 1

Table I. ^{1}H-NMR and ^{13}C-NMR Data of Catalpin (1)

Carbon or proton	^{13}C-NMR[a] m	δ	^{1}H-NMR[b]	m	δ	J (Hz)
1	d	101.31		d	5.51	5.4
3	d	90.07		dd	5.38	3.6, 7.5
4	t	30.10	H-4α	ddd	1.92	3.6, 3.6, 11.5
			H-4β	ddd	1.55	3.6, 7.5, 11.5
5	d	41.02		dddd	2.69	3.6, 3.6, 6.5, 11.5
6	d	76.64		ddd	5.26	6.5, 7.8, 8.2
7	t	45.92	H-7α	dd	2.54	7.8, 11.0
			H-7β	ddd	2.01	1.5, 8.2, 11.0
8	s	85.16				
9	d	51.53		dd	2.48	5.4, 11.5
10	t	79.28	H-10α	d	3.90	10.8
			H-10β	dd	3.76	1.5, 10.8
1′	s	122.94				
2′,6′	d	133.62		d	7.88	8.8
3′,5′	d	116.52		d	6.82	8.8
4′	s	163.18				
C=O	s	166.96				

a) δ (ppm) in acetone-d_6. *b*) δ (ppm) in CD_3OD.

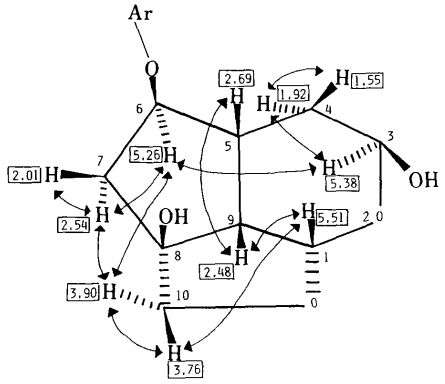

Fig. 1. NOE Results of Catalpin (1) (δ ppm in CD_3OD)

catalposide (2), the absolute configuration of which has been established.[3] Although the absolute stereochemistry of 1 has not been studied due to its poor yield from the crude durg, the same absolute configuration at C_5 and C_9 of 1 as in other iridoids such as catalposide (2) isolated from the same source[4] was suggested, as depicted in the chart and Fig. 1.

Catalpin (1) is a new tricyclic iridoid compound,[5] and such compounds of synthetic origin have been reported to have antifeedant activity against spruce budworm.[6]

Catalpin (1) showed weak but reproducible mutagenic activities towards *Salmonella typhimurium* TA100 in the presence and absence of S9 mix, but did not show mutagenicity towards *Salmonella typhimurium* TA98 in the presence or absence of S9 mix. The dose-response curves of the mutagenicity of catalpin (1) are shown in Fig. 2. From the results of Ames'test, the specific mutagenic activities were calculated to be 900 revertants per milligram with S9 mix and 820 revertants per milligram without S9 mix towards TA100 at the dose of 100 μg per plate. The mutagenicity of catalpin (1) without S9 mix was not affected by the addition of S9 mix. The negligible effect of S9 mix in the case of direct mutagens acting on TA100 has been reported.[7] The mutagenic potency of catalpin (1) is comparable to that of anethol.[8]

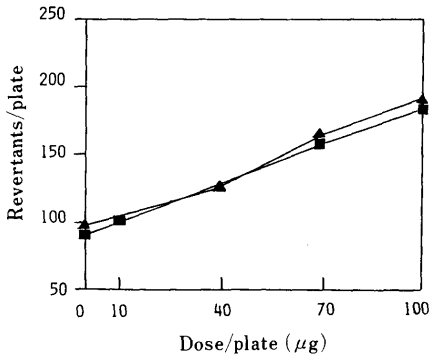

Fig. 2. Dose-Response Curves of Catalpin (1)

TA100. ▲—▲, +S9; ■—■, −S9. Each point is the average of 3 plates. Spontaneous revertants have not been subtracted.

Table II. Yield and Mutagenicity[a] of Samples

Sample	Yield (g)[b]	Specific mutagenic activity (His$^+$/mg) −S9	+S9
Water extract	150.0	62	20
Ethyl acetate extract	3.0	204	28
Aq. layer of ethyl acetate extraction	143.0	19	12
Catalpin (1)	0.03	820	900
Catalposide (2)	—	(—)[c]	(—)
Catalpol (3)	—	(—)	(—)

a) *Salmonella typhimurium* TA100 was used. *b*) From 2000 g of Catalpae Fructus. *c*) (—): not mutagenic.

Since catalpin (1) is structurally similar to catalposide (2) and catalpol (3), the major iridoid compounds from *Catalpa* species, the mutagenicity of catalposide (2) and catalpol (3) was examined. In our experiments, no mutagenic activity towards *Salmonella typhimurium* strains TA98 and TA100 was observed at doses of 10 to 60 μg of catalposide (2) per plate and 10 μg to 1 mg of catalpol (3) per plate in the presence and absence of S9 mix.

One gram of extract of Catalpae Fructus induced 6.2×10^4 revertants of TA100 without S9 mix while one gram of ethyl acetate extract of Catalpae Fructus induced 2.0×10^5 revertants of TA100 without S9 mix. The mutagenic potency of the water extract of Catalpae Fructus is comparable to that of the extract of coffee.[9] Yields and mutagenicity of samples are summarized in Table II.

Some biological activities of iridoid compounds have been reported[10] but this is the first report on the mutagenicity of iridoid compounds.

Experimental

Melting points were determined on a Yazawa BY-2 apparatus and are uncorrected. Shimadzu IR 435 and Hitachi model 330 spectrometers were used for measurements of IR and UV spectra, respectively. The chemical ionization (CI) mass spectrum (MS) was measured on a Shimadzu LKB-9000 GC-MS high-resolution spectrometer and high-resolution MS was measured on a JEOL JMS-SX102 apparatus equipped with a JMA-DA6000 data system. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-GX 400 instrument and chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal reference and coupling constants (J) in Hz (s, singlet; d, doublet; dd, double doublet; ddd, doublet of double doublets; dddd, doublet of doublets of double doublets). Optical rotatory dispersion (ORD) spectrum was measured on a model J-20 apparatus (Japan Spectroscopic Co., Ltd.).

Materials Commercial Catalpae Fructus was purchased from Kino-

kuniya Kanyakkyoku Ltd., (Japan). Catalposide was a gift from Prof. T. Okuda, Okayama University. Catalpol was obtained from Wako Pure Chemical Industries, Ltd.

Mutagenicity Assay Mutagenicity was tested by the use of Ames' method with the modification of preincubation[11] using *Salmonella typhimurium* TA98 and TA100 in the presence or absence of S9 mix. S9 was prepared from the liver of Sprague-Dawley rats pretreated with phenobarbital and β-naphthoflavone. Benzo(a)pyrene was used as a positive control for TA98 and TA100 with S9 mix and furylfuramide (trade name AF-2) for TA98 and TA100 without S9 mix. Spontaneous revertants amounted to 26 (TA98, −S9), 44 (TA98, +S9), 90 (TA100, −S9), 100 (TA100, +S9). Furylfuramide (0.2 μg) yielded 437 His^+ revertants per plate from TA98 without S9 mix and it (0.02 μg) yielded 530 His^+ revertants per plate from TA100 without S9 mix. Benzo(a)pyrene (5 μg) yielded 230 His^+ revertants per plate from TA98 and 654 His^+ revertants per plate from TA100 with S9 mix. The numbers of revertants in tests are averages of 3 plates.

Extraction and Fractionation Dried materials (2 kg) of Catalpae Fructus were extracted with water (12 l) at room temperature for 2 weeks. The water extract (150 g) was extracted with EtOAc (12 l). The EtOAc layer was dried ($MgSO_4$) and concentrated *in vacuo* to give an amorphous residue (3 g) (fraction-1). Fraction-1 was separated by preparative TLC ($CHCl_3$: MeOH : H_2O = 65 : 35 : 10, lower layer). The eluate from the band on the TLC plate showing UV absorption (Rf = 0.6) exhibited mutagenicity in Ames'test. The fraction was collected and further purified on a Sephadex LH-20 column with MeOH to give catalpin (**1**) (30 mg), colorless needles from $CHCl_3$.

Catalpin (1) mp 93—94 °C. ORD (c = 0.0085, methanol) $[\alpha]^{20}$ (nm): +79° (264), +39° (252), +35° (216). UV λ_{max}^{MeOH} nm: 258. IR ν_{max}^{KBr} cm^{-1}: 3510, 3450, 3340, 1685, 1615, 1295, 845. CI-MS (reactant gas, isobutane) m/z (%): 323 ($(M+H)^+$) (11.0), 322 ($(M)^+$) (1.2), 305 ($((M+H)-18)^+$) (36.0), 139 (100.0), 121 (C_6H_4OHCO) (96.2). High-resolution MS (negative fast atom bombardment (FAB) MS) m/z: Calcd for $C_{16}H_{17}O_7$ ($(M-H)^-$), 321.0974, Found, 321.1006. ^{1}H-NMR and ^{13}C-NMR (in methanol-d_4 or acetone-d_6) δ: Table I.

Acknowledgements The authors with to thank Prof. T. Okuda, Okayama University, for providing a sample of catalposide, Mr. J. Kokatsu, Taisho Pharm. Co., Ltd., for the CI-MS analyses and staff of JEOL Ltd. for measurements of high-resolution MS.

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