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Inhibitors of Xanthine Oxidase from *Athyrium mesosorum*[1)]

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Xanthine oxidase (XO) inhibitor was isolated from the aerial parts of *Athylium mesosorum* MAKINO (Aspidiaceae) and was identified as norathyriol (1,3,6,7-tetrahydroxyxanthone) (**1**). The type of inhibition by **1** with respect to xanthine as a substrate was uncompetitive. The inhibitory activities of related xanthone derivatives towards XO were assayed.

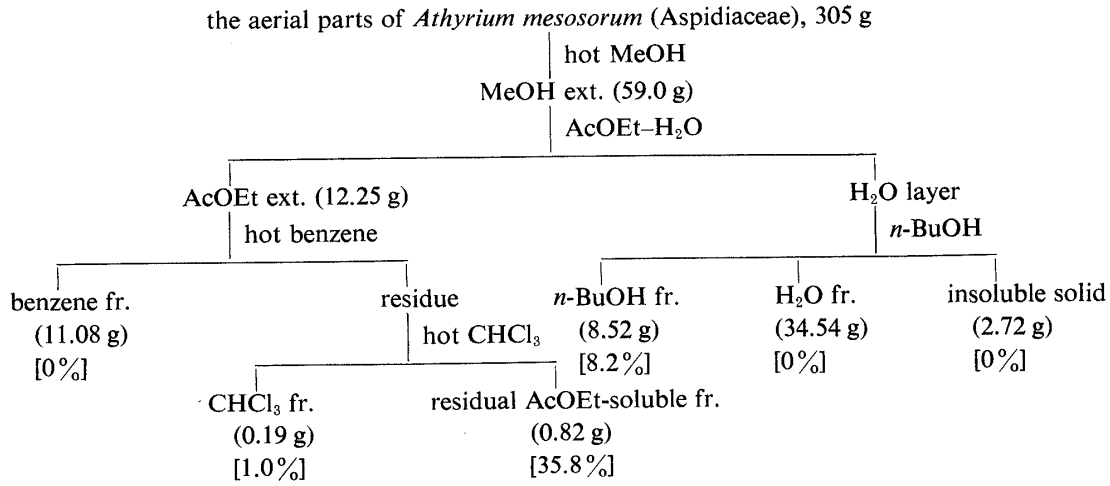
Keywords——xanthine oxidase; inhibitor; uncompetitive inhibition; xanthine; *Athyrium mesosorum*; aspidiaceae; norathyriol; xanthone

The inhibitors of xanthine oxidase (XO, EC 1.2.3.2) may be candidate drugs for the treatment of gout.[2)] In the previous paper,[1)] luteolin and apigenin, which are components of *Daphne genkwa* SIEB. *et* ZUCC. (Thymelaeaceae), were reported as inhibitors of XO. In this paper, we report the isolation of a strong *in vitro* XO inhibitor from the aerial parts of *Athyrium mesosorum* MAKINO (Aspidiaceae, Japanese name "nuriwarabi"), as well as the inhibitory activities of related xanthone derivatives against XO.

Results and Discussion

The activity of XO *in vitro* towards xanthine as a substrate was assayed spectrophotometrically at 290 nm by the method described in the previous paper.[1)] The aerial parts of *Athyrium mesosorum* were extracted with hot methanol under reflux and the methanol extract was fractionated with ethyl acetate, *n*-butyl alcohol and water. The ethyl acetate extract was washed with benzene and chloroform as shown in Chart 1. The inhibitory activities against XO and the yields of each extract and fraction are also shown in Chart 1. The most active ethyl acetate-soluble fraction was chromatographed repeatedly on a silica gel column with a benzene–ethyl acetate–methanol or a chloroform–methanol gradient system as the developer, and the fractions were monitored by thin layer chromatography (TLC, silica gel), and also by measurement of inhibitory activity against XO. The most active fraction from the column was subjected to preparative TLC on silica gel to give the most active compound (**1**), $C_{13}H_8O_6$, which was identified as norathyriol (1,3,6,7-tetrahydroxyxanthone)[3)] on the bases of melting point, spectral comparisons and elemental analysis. The concentration of **1** in the assay mixture required to give 50% inhibition (IC_{50}) was 9.2×10^{-7} M. Kinetic studies were done on the effect of **1** on the oxidation of xanthine by XO under the assay conditions. The results are shown as Lineweaver–Burk plots[4)] in Fig. 1. The type of inhibition by **1** was uncompetitive.

The inhibitory activities towards XO of **1**, mangiferin(2-*C*-glucosyl-1,3,6,7-tetrahydroxyxanthone, **2**),[3)] athyriol(3-methoxy-1,6,7-trihydroxyxanthone, **3**),[3)] isoathyriol(6-methoxy-1,3,7-trihydroxyxanthone, **4**)[3)] and some related synthetic xanthones (**5**—**14**)[5-11)] are shown in Table I. Compounds **1**—**4** are constituents of *A. mesosorum*. In this study, **1** was the strongest inhibitor. Various naturally occurring inhibitors of XO, flavonoids,[1)] hy-



( ), yields
[ ], percent inhibition, 10 μg of test sample per 1 ml of reaction solution

Chart 1

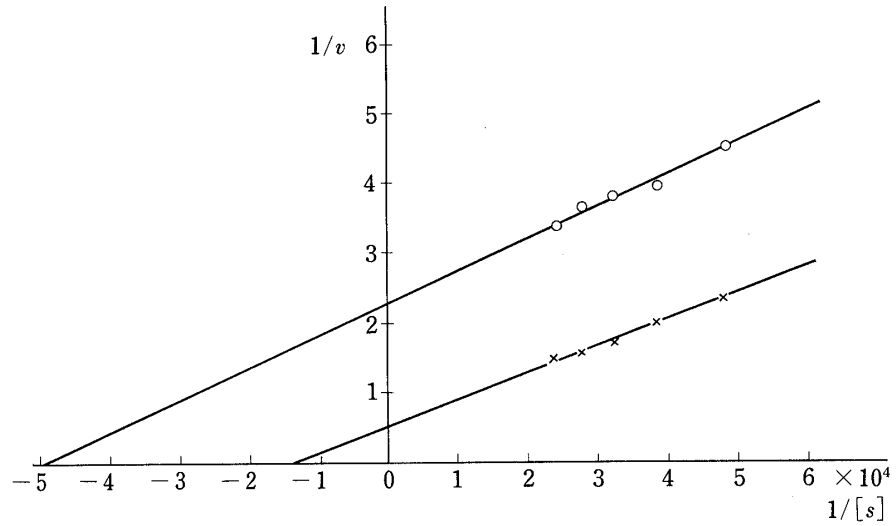


Fig. 1. Inhibitory Effects of Norathyliol (**1**) on XO

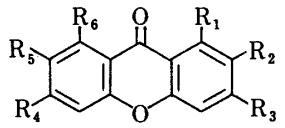
Lineweaver–Burk plots in the absence (0 M, ×—×) and in the presence of **1** (1.92×10^{-6} M, ○—○) with xanthine as the substrate.
v: μM substrate metabolized/mg enzyme/min. s: substrate.

droxychalcones,[12] coumarins,[12] 2,8-dihydroxyadenine,[13] 5-formyluracil,[14] β-carbolines,[15] and so on, are known. In this paper, we show that xanthones are another category of strong XO inhibitors.

Experimental

The following instruments were used to obtain physical data. Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on a JASCO IRA-202 infrared spectrophotometer. The ultraviolet (UV) spectra were recorded on a Shimadzu UV-360 recording spectrophotometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectra and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a JEOL LNM-FX 90Q FT NMR spectrometer (90 MHz) with tetramethylsilane as an internal standard (δ value) (s, singlet; d, doublet; t, triplet; q, quartet; br, broad). Mass spectra (MS) were recorded on a JEOL JMS-O1SG-2 mass spectrometer. Silica gel 60 GF_{254} (Merck) was used for TLC and detection was achieved by illumination with an ultraviolet lamp, by spraying 3% $FeCl_3$ ethanol solution or by spraying 20%

TABLE I. XO-Inhibitory Activities of Xanthones



Compound	R_1	R_2	R_3	R_4	R_5	R_6	Inhibition (%)	
							10 μg/ml	1 μg/ml
1	OH	H	OH	OH	OH	H	85.0	77.3
2	OH	Gluc.	OH	OH	OH	H	0	0
3	OH	H	OMe	OH	OH	H	32.2	15.8
4	OH	H	OH	OMe	OH	H	29.1	21.1
5	OH	H	OH	OH	H	OH	24.3	19.2
6	OH	H	OH	H	H	OH	15.1	0
7	OH	H	OH	H	OH	H	34.2	27.7
8	OH	H	OH	OH	H	H	58.1	21.4
9	H	H	OH	OH	H	H	16.7	10.5
10	OH	H	H	OH	H	H	10.5	0
11	OH	H	OH	H	H	H	22.4	11.3
12	H	H	OH	H	H	H	13.7	4.3
13	OH	H	H	H	H	H	1.9	1.2
14	H	H	H	H	H	H	23.0	8.7

Gluc = glucose.

aq. H_2SO_4 followed by heating. For preparative TLC, Silica gel 60 PF_{254} (Merck) was used. For column chromatography, Silica gel 60 (Merck) was used. The spectrophotometric measurements were carried out with a Hitachi model 101 spectrophotometer.

Enzyme and Chemicals——Xanthine oxidase (EC 1.2.3.2) from cow's milk was obtained from Boehringer Mannheim Co., Ltd. Xanthine was obtained from ICN Pharmaceutical Inc. Tween 80 was obtained from Wako Pure Chemical Industries, Ltd. Sodium phosphate dibasic 12 hydrate and potassium phosphate monobasic were obtained from Kanto Chemical Co., Inc. The buffer was Hasting–Sendroy's 1/15 M potassium phosphate–sodium phosphate buffer, pH 7.5. The substrate solution, 0.15 mM xanthine in water, was prepared immediately before use. Enzyme solution containing about 0.07 unit per ml in 1/15 M phosphate buffer, pH 7.5, was prepared immediately before use.

Test Solution——The test solution was prepared as described previously.[1]

Assay of Xanthine Oxidase Activity——For the assay of XO activity, the method described previously[1] was employed.

Estimation of Xanthine Oxidase Inhibitory Activity——The same method as described previously[1] was used for the estimation of XO inhibitory activity.

Extraction and Separation——The dried aerial parts (305 g) of *Athyrium mesosorum* (collected at Yaezawa in Shizuoka city in September, 1981) were extracted three times with 5 l of MeOH under reflux for 3 h, and the extract was concentrated to give 59.0 g of MeOH extract under reduced pressure. The MeOH extract was suspended in water, and extracted with AcOEt to give the AcOEt extract. The water layer was extracted with *n*-BuOH to give *n*-BuOH extract, and the residual water layer was filtered to give the insoluble solid and water-soluble extract. The AcOEt extract was washed with hot benzene to give the benzene-soluble fraction, and the residue was washed with hot $CHCl_3$ to give the $CHCl_3$-soluble fraction and the residual AcOEt-soluble fraction. These extracts and fractions were assayed for inhibitory activity against XO. The yields and inhibitory activities are shown in Chart 1. The most active AcOEt fraction was chromatographed repeatedly on a silica gel column with a benzene–AcOEt–MeOH or $CHCl_3$–MeOH gradient system as a developer, and the fractions were monitored by TLC on silica gel and by measurements of inhibitory activity against XO. The most active fraction from the column was subjected to preparative TLC on silica gel using $CHCl_3$: MeOH : AcOH = 22 : 2 : 1 (v/v) as a developer and the desired fraction (Rf = 0.25) was recrystallized from acetone to give the most active compound (**1**).

Norathyriol (1,3,6,7-Tetrahydroxyxanthone, 1)[3,10]——Yellow-brown needles from acetone. IR (KBr) cm^{-1}: 3200, 1645, 1610, 1565, 1485, 1445, 1300, 1180, 1130, 1080, 1010, 830, 805, 790. UV λ_{max}^{EtOH} nm (log ε): 255 (4.46), 312 (4.23), 365 (4.16). MS *m/z* (rel. int %): 260 (M^+, 100), 230 (10), 203 (12), 69 (25), 51 (15), 28 (15), 16 (18). ^{1}H-NMR (DMSO-d_6) δ: 5.42 (br, OH), 6.18 (1H, d, J = 1.7 Hz, C_2–H), 6.34 (1H, d, J = 1.7 Hz, C_4–H), 6.85 (1H, s, C_5–H), 7.39

Table II. Physical Data for Xanthones

Compound	Form of crystals (solvent)	mp (°C) (lit., °C)	Formula	Analysis (%) Calcd (Found)		Ref.
				C	H	
1	Yellow-brown needles (acetone)	>320 (>320)	$C_{13}H_8O_6$	60.01 (59.90	3.10 3.18)	3, 10
2	Yellow-brown needles (aq. MeOH)	263 (263)	$C_{19}H_{18}O_{11}$	54.01 (53.95	4.30 4.41)	3, 10
3	Yellow-brown needles (aq. EtOH)	300 (300)	$C_{14}H_{10}O_6$	61.32 (61.06	3.68 3.70)	3
4	Yellow needles (aq. EtOH)	325 (325)	$C_{14}H_{10}O_6$	61.32 (61.15	3.68 3.83)	3
5	Yellow needles (benzene–acetone)	350 (350)	$C_{13}H_8O_6$	60.01 (59.98	3.10 3.05)	5
6	Yellow needles (aq. EtOH)	268 (265)	$C_{13}H_8O_5$	63.94 (63.92	3.30 3.38)	6
7	Red-brown needles (EtOH)	320 (316—318)	$C_{13}H_8O_5$	63.94 (63.75	3.30 3.35)	7
8	Orange needles (aq. EtOH)	322 (323—324)	$C_{13}H_8O_5$	63.94 (63.75	3.30 3.40)	7, 11
9	Red-orange needles (aq. EtOH)	347 (>330)	$C_{13}H_8O_4$	68.42 (68.31	3.53 3.48)	7, 11
10	Yellow needles (benzene–acetone)	250 (246—247)	$C_{13}H_8O_4$	68.42 (68.34	3.53 3.54)	7, 11
11	Yellow needles (aq. EtOH)	258 (256—258)	$C_{13}H_8O_4$	68.42 (68.69	3.53 3.63)	7, 11
12	Yellow needles (EtOH)	245 (238)	$C_{13}H_8O_3$	73.58 (73.37	3.80 3.76)	8
13	Orange needles (EtOH)	144 (148)	$C_{13}H_8O_3$	73.58 (73.46	3.80 3.90)	8, 11
14	Colorless needles (EtOH)	174 (174)	$C_{13}H_8O_2$	79.58 (79.50	4.11 4.08)	9, 11

(1H, s, C_8–H), 13.26 (1H, br, C=O---HO). ^{13}C-NMR (DMSO-d_6) δ: 93.57 (d), 97.69 (d), 101.54 (s), 102.40 (d), 107.66 (d), 111.34 (s), 144.01 (s), 151.21 (s), 155.01 (s), 157.28 (s), 162.54 (s), 164.65 (s), 178.74 (s). This was identical with authentic norathyriol[3] on the bases of mp, spectral comparisons and elemental analysis.

Mangiferin (2-*C*-Glucosyl-1,3,6,7-tetrahydroxyxanthone, 2), Athyriol (3-Methoxy-1,6,7-trihydroxyxanthone, 3) and Isoathyriol (6-Methoxy-1,3,7-trihydroxyxanthone, 4)——Compounds **2**, **3** and **4** which were isolated previously,[3] were tested for XO-inhibitory activity.

Hydroxyxanthone Derivatives (5—13)——The hydroxyxanthone derivatives which were assayed for XO-inhibitory activity were synthesized by the reported methods[5-8] and the results are summarized in Table II.

Xanthone (14)[9]——This material was obtained from Wako Pure Chemical Industries, Ltd. and was purified by recrystallization from EtOH. The elemental analysis data and mp are shown in Table II.

Lineweaver–Burk Plots[4]——The Lineweaver–Burk plots for XO with xanthine as a substrate under our assay conditions in the absence and in the presence of **1** are shown in Fig. 1.

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