Quinolone Alkaloids with Nitric Oxide Production Inhibitory Activity from *Orixa japonica*[1]

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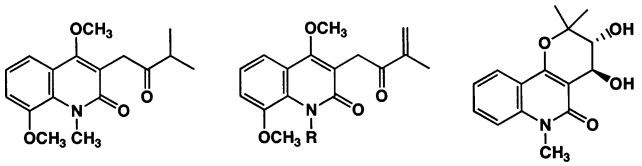
Four new quinolone alkaloids, orixalone A (**1**), B (**2**), C (**3**), and D (**4**), together with 12 known compounds were isolated from the stems of *Orixa japonica*. Orixalone A (**1**) inhibited nitric oxide production in murine macrophage-like RAW 264.7 cells stimulated with interferon-γ and lipopolysaccharide.

As a part of our systematic investigations on the constituents of Rutaceous plants, we have identified various types of chemical compounds.[2] *Orixa japonica* Tunberg (Rutaceae) is regarded as one of the constituents of the crude drug "Johzan", and the stems and leaves of this plant were formerly used in Japan as an insecticide for livestock.[3] In China and other Asian countries, the roots of this plant have been used in folk medicine as febrifuges and analgesics.[4] The chemical constituents of *O. japonica* have been previously investigated for the presence of quinoline and quinolone alkaloids.[5−10] We reported the isolation of several quinolones (preskimmianine and folimine) and furoquinolines (dictamine and evolitrine) from *Boronia* species belonging to the same family (Rutaceae), and these compounds were found to exhibit the inhibitory effects of tumor-promoter-induced Epstein−Barr virus early antigen activation on Raji cells and might be valuable as potential cancer chemopreventive agents (antitumor promoters).[11] In our search for other classes of effective chemopreventive agents, we have recently used a new bioassay system measuring the inhibition of nitric oxide (NO) generation. Nitric oxide is synthesized by the enzyme family NO synthase (NOS) and plays an important role in tumor growth, invasion, and angiogenesis.[12] The expression of inducible NOS (iNOS) in various tumor cell lines has been recently documented, and the selective inhibition of excessive iNOS-induced NO generation is widely accepted as a new paradigm for cancer chemoprevention.[13]

This paper describes the isolation and identification of four new quinolones, orixalone A (**1**), B (**2**), C (**3**), and D (**4**), together with nine known alkaloids and three furocoumarins from the stems of *O. japonica* collected at Kani in Japan and the results of a primary screening of several alkaloids isolated from this plant for their possible inhibitory effects on nitric oxide production.

Results and Discussion

The acetone extract of stems of *O. japonica* was fractionated by silica gel column chromatography and preparative TLC to obtain four new 2-quinolone alkaloids (**1**−**4**) along with 12 known compounds.



1 **2, R = CH_3** **4**

3, R = H

Orixalone A (**1**) was obtained as a colorless oil. The molecular formula was established as $C_{17}H_{21}NO_4$ by HREIMS. The UV spectrum (see Experimental Section) was similar to that of 4,8-dimethoxy-3-(3-methylbut-2-enyl)-*N*-methyl-2-quinolone,[14] which had previously been isolated from *Glycosmis arborea*.[15] In the ^{1}H NMR spectrum, ABC-type signals at δ_H 7.44, 7.18, and 7.07 assignable to H-5, H-6, and H-7 on the 2-quinolone, respectively, were observed in the aromatic region, along with three methyl groups at δ_H 3.94, 3.90, and 3.86. On the basis of the analysis of the HMQC spectrum, the proton in one of the three methyls at δ_H 3.94 showed a cross-peak with the carbon at δ_C 35.6, indicating the presence of an *N*-methyl group. The presence of an amide carbonyl group was indicated in the IR spectrum (ν_{max} 1644 cm^{-1}) and the ^{13}C NMR spectrum (δ_C 164.5). The remaining signals [δ_H 3.82 (2H, s), δ_C 37.0; δ_C 211.6; δ_H 2.89 (1H, sept, J = 7.0 Hz), δ_C 41.2; δ_H 1.22 (6H, d, J = 7.0 Hz), δ_C 18.4] in the ^{1}H and ^{13}C NMR spectra and an IR band at ν_{max} 1714 cm^{-1} indicated the presence of a C5 isoprenoid moiety [$-CH_2C(=O)CH(CH_3)_2$]. The C−H long-range correlation between H-1′ and C-2, and H-1′ and C-4, in the HMBC spectrum suggested the location of this side chain at C-3 on the 2-quinolone nucleus. Irradiation of OMe signals at δ_H 3.86 and 3.90 showed nuclear Overhauser effect (NOE) enhancements at H-5 (δ 7.44) and H-7 (δ 7.07), respectively. NOE enhancements were not observed at any proton signal on irradiation of the NMe signal at δ 3.94. On the basis of these results and other HMBC correlations (Figure 1), we assigned structure **1** to orixalone A.

Orixalone B (**2**) was isolated as a colorless oil, and its molecular formula was found to be $C_{17}H_{19}NO_4$ by HREIMS. The UV spectrum showed close resemblance to that of **1**, suggesting a 4,8-dioxygenated 2-quinolone structure. The ^{1}H NMR spectrum was also similar to that of **1**, except for the signals due to a side chain at C-3 on the 2-quinolone nucleus. The observed ^{1}H and ^{13}C NMR (Table 1) signals

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Table 1. ^{1}H and ^{13}C NMR Data of Quinolones (**1**−**4**) from *Orixa japonica*[a]

	orixalone A (**1**)		orixalone B (**2**)		orixalone C (**3**)	orixalone D (**4**)	
	δ_C	δ_H	δ_C	δ_H	δ_H	δ_C	δ_H
1-NMe	35.6 (q)	3.94 (3H, s)	35.6 (q)	3.94 (3H, s)		28.9 (q)	3.74 (3H, s)
1-NH					9.13 (br s)		
2	164.5 (s)		164.5 (s)			163.6 (s)	
3	117.8 (s)		118.0 (s)			106.0 (s)	
4	161.7 (s)		161.7 (s)			155.0 (s)	
4-OMe	62.0 (q)	3.86 (3H, s)	61.9 (q)	3.86 (3H, s)	3.89 (3H, s)		
4a	119.8 (s)		119.9 (s)			115.9 (s)	
5	116.0 (d)	7.44 (br d, 8.1)	116.0 (d)	7.45 (br d, 8.1)	7.35 (br d, 8.1)	123.9 (d)	8.00 (br d, 8.1)
6	122.5 (d)	7.18 (t, 8.1)	122.5 (d)	7.19 (t, 8.1)	7.16 (t, 8.1)	122.2 (d)	7.27 (dd, 8.1, 8.1)
7	113.9 (d)	7.07 (br d, 8.1)	113.9 (d)	7.07 (br d, 8.1)	6.97 (br d, 8.1)	131.3 (d)	7.60 (dd, 8.1, 8.1)
8	148.9 (s)		148.9 (s)			114.1 (d)	7.35 (br d, 8.1)
8-OMe	56.7 (q)	3.90 (3H, s)	56.7 (q)	3.90 (3H, s)	3.98 (3H, s)		
8a	131.0 (s)		131.0 (s)			139.2 (s)	
1′	37.0 (t)	3.82 (2H, s)	34.5 (t)	4.08 (2H, s)	4.09 (2H, s)	67.7 (d)	4.75 (d, 7.7)
2′	211.6 (s)		198.9 (s)			75.3 (d)	3.84 (d, 7.7)
3′	41.2 (d)	2.89 (sept., 7.0)	144.4 (s)			80.8 (s)	
4′	18.4 (q)	1.22 (3H, d, 7.0)	17.4 (q)	1.94 (3H, s)	1.94 (3H, s)	26.1 (q)	1.63 (3H, s)
5′	18.4 (q)	1.22 (3H, d, 7.0)	124.9 (t)	5.85 (br s)	5.84 (br s)	19.3 (q)	1.32 (3H, s)
				6.19 (br s)	6.17 (br s)		
others							5.56 (br s)
							2.82 (br s)

[a] Values in (δ_H and δ_C) ppm. All signals correspond to 1H, unless otherwise stated. Figures in parentheses are coupling constants (J) in Hz.

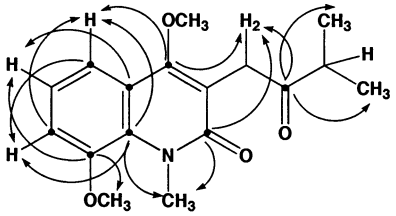

Figure 1. C−H long-range correlations in the HMBC spectrum of orixalone A (**1**).

assignable to the side chain comprised a vinyl methyl group [δ_H 1.94 (3H, s); δ_C 17.4], a vinyl methylene [δ_H 5.85 and 6.19; δ_C 124.9], a conjugated ketone carbonyl group [δ_C 198.9], a methylene [δ_H 4.08; δ_C 34.5], and a quaternary vinyl carbon [δ_C 144.4]. In the HMBC spectrum, C−H long-range correlations were detected between C-2′ at δ_C 198.9 and a vinyl methyl at δ_H 1.94 (H-4′) and methylene protons at δ_H 4.08 (H-1′), 5.85 (H-5′), and 6.19 (H-5′). These results indicated the structure of the side chain as $-CH_2-C(=O)-CCH_3(=CH_2)$, and the location of this side chain at C-3 was suggested by distinct C−H three-bond correlations between H-1′ and C-2 and between H-1′ and C-4. On the basis of these spectral data and NOE experiments (see Experimental Section), we assigned structure **2** to orixalone B.

Orixalone C (**3**) was isolated as a colorless oil. The close resemblance of the ^{1}H NMR spectrum to that of **2** (Table 1), with the exception of a missing *N*-methyl signal in the spectrum of **2**, coupled with the presence of an IR band at ν_{max} 3394 cm^{-1} and ^{1}H NMR D_2O exchangeable signal at δ 9.13 (1H, s) indicated structure **3** for orixalone C.

trans-Orixalone D (**4**) was obtained as a colorless oil with an optical rotation of $[\alpha]_D^{24}$ +3.1° (MeOH), and its molecular formula was found to be $C_{15}H_{17}NO_4$ by HREIMS. The presence of a 4-oxygenated *N*-methyl-2-quinolone nucleus in the molecule was suggested by UV absorptions (λ_{max} 230, 274, 284, 318, 332 nm), an IR band (ν_{max} 1635 cm^{-1}), ^{13}C NMR [δ 163.6 (an amide carbonyl group) and 28.9 (*N*-Me)], and ^{1}H NMR signals at δ 3.74 (3H, s), 8.00 (1H, br d, J = 8.1 Hz), 7.27 (1H, dd, J = 8.1, 8.1 Hz), 7.60 (1H, dd, J = 8.1, 8.1 Hz), and 7.35 (1H, br d, J = 8.1 Hz). The remaining ^{1}H NMR signals were assignable to *geminal* methyls [δ 1.63 and 1.32 (each 3H, s)] attached to an oxygenated carbon and *vicinal* protons [δ 4.75 and 3.84 (each 1H, d, J = 7.7 Hz)] attached to carbinol carbons, thus indicating the presence of a 3,4-dihydroxy-2,2-dimethylpyran ring in the molecule. The *trans* configuration of the glycol system was proposed on the basis of observations of the large coupling constant (J = 7.7 Hz) of the carbinol protons, the large chemical shift difference between the *geminal* methyls (Δ 0.31 ppm) on the pyran ring,[16] and the lack of a NOE between the two carbinol protons. Further in the NOE experiments, irradiation of the proton in one of the two methyls at δ 1.32 (H-4′) resulted in 4% enhancement of one of the two carbinol protons at δ 4.75 (H-1′). No NOE enhancement between the proton at δ 1.32 (H-4′) and one of the two carbinol protons at δ 3.84 (H-2′) was observed. Irradiation of the proton in one of the two methyls at δ 1.63 (H-5′) resulted in 4% enhancement of the carbinol proton at δ 3.84 (H-2′). On the basis of these spectral data and other HMBC data, structure **4** was assigned to orixalone D.

The known compounds described below were also isolated and characterized by analyses of their spectral data, revealing the alkaloids preorixine,[5] orixinone,[6] isopteleflolidine,[7] *N*-methylflindersine,[7] 3′-*O*-acetylisopteleflorine,[8] orixine,[9] kokusagine,[10] γ-fagarine,[17] and skimmianine[14] and the furocoumarins imperatorin,[18] bergapten,[18] and oxyimperatorin.[19]

Inhibitory Effect of Orixalone A on Nitric Oxide Production. The new quinolone, orixalone A (**1**), together with three previously reported quinolines (kokusagine, preorixine, and orixinone) were tested for their effects on NO production in LPS/IFN-γ-stimulated RAW 264.7 cells. Orixalone A (**1**) strongly inhibited LPS/IFN-γ-induced NO production in RAW 264.7 cells with 47.3% and 54.8% inhibition at 10 and 50 μM, respectively (Figure 2). Cell viability experiments, measured by the MTS assay, showed that orixalone A (**1**) was not significantly cytotoxic to RAW 264.7 cells at the effective concentration for the inhibition of NO production. Conversely, three quinolines (kokusagine, preorixine, and orixinone) had no inhibitory effect on the accumulation of NO in RAW 264.7 cells at 30 μM. Preorixine and orixinone showed a weak cytotoxic effect against RAW 264.7 cells at 30 μM. The present study suggests that inhibition of NO production in macrophage cells by orixa-

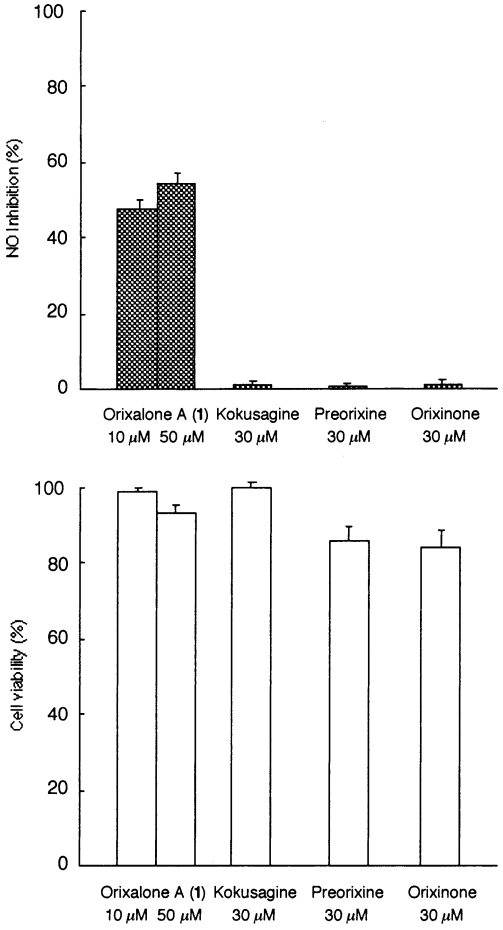

Figure 2. Inhibitory effects of quinolone and quinolines on NO production induced by LPS/IFN-γ. Macrophages (1×10^5 cells/dish) were incubated with LPS (100 ng/mL) and IFN-γ (100 U/mL) on a 96-well tissue culture plate in the absence or presence of test compounds for 18 h. After incubation, NO content in the supernatant was measured using the Griess reagent, and the cell viability was measured by MTS assay. Results are the means ± SE of four separate experiments.

lone A (**1**) may have potential cancer-preventive activity through reduction of excess NO production.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR, COSY, HMQC, HMBC (J = 8 Hz), and NOE were measured on a JNM A-400, A-600, and/or ECP-500 (JEOL) spectrometer. Chemical shifts are shown in δ (ppm) with tetramethylsilane (TMS) as an internal reference. Mass spectra were recorded under EI conditions, unless otherwise stated, using HX-110 (JEOL) and/or JMS-700 (JEOL) spectrometers with a direct inlet system. UV spectra were recorded on a UVIDEC-610C double-beam spectrophotometer (JASCO) in MeOH and IR spectra on an IR-230 (JASCO) spectrometer in $CHCl_3$. Preparative TLC was done on Kieselgel 60 F_{254} (Merck).

Plant Materials. The plant materials used in this study, *Orixa japonica* Tunberg, were collected at Kani, Gifu, Japan, during April 2001. A voucher specimen has been deposited at Meijo University under number MUY0112.

Isolation of Orixalones A (1), B (2), C (3), and D (4) from *O. japonica*. The dried stems (527 g) of *Orixa japonica* were extracted with acetone (2 L × 3) at room temperature, and the solvent was evaporated under reduced pressure to give the acetone extract (11 g). The acetone extract was fractionated by silica gel column chromatography, eluting with $CHCl_3$–acetone (100:1, 4 L), to obtain fractions 1–6. Fraction 2 (1.4 g) was chromatographed on silica gel, eluting with hexane–EtOAc (3:1), to yield preorixine (144 mg), orixinone (165 mg), imperatorin (5.5 mg), and bergapten (3.6 mg). Fraction 3 (1.87 g) was chromatographed on silica gel, eluting with hexane–EtOAc (3:1), to obtain fractions a–g. Fraction b (103.0 mg) was chromatographed on silica gel, eluting with benzene–MeOH (10:1), to yield isoptelefolidine (2.1 mg) and orixalone B (**2**, 1.8 mg). Fraction c (197.0 mg) was subjected to preparative silica gel TLC (PTLC), developing with hexane–EtOAc (3:1), to afford *N*-methylflindersine (32 mg) and orixalone A (**1**, 16.1 mg). Fraction e (193.0 mg) was subjected to PTLC, developing with benzene–acetone (6:1), to afford oxyimperatorin (7.4 mg) and orixalone C (**3**, 1.0 mg). Fraction f (160.0 mg) was subjected to PTLC, developing with benzene–MeOH (5:1), to afford kokusagine (98 mg). Fraction 4 (957 mg) was chromatographed on silica gel, eluting with hexane–EtOAc (3:2), to yield orixalone D (**4**, 1.7 mg), γ-fagarine (2.0 mg), and 3′-*O*-acetylisopteleflorine (3.1 mg), which were further purified by PTLC eluting with benzene–acetone (10:1). Fraction 5 (67 mg) was subjected to PTLC, developing with $CHCl_3$–acetone (30:1), to afford skimmianine (3.2 mg). Fraction 6 (55 mg) was subjected to PTLC, developing with iPr$_2$O–acetone (10:1), to afford orixine (15.0 mg).

Identification of the known compounds to be tested in the NO assay was done by comparison of their NMR data with those reported in the literature.

Orixalone A (1): colorless oil; UV (MeOH) λ_{max} 212, 238, 256, 284, 292, 332 nm; IR ($CHCl_3$) ν_{max} 1714, 1644, 1619 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); Differential NOE, irradiation of 4-OMe (δ 3.86), 7% enhancement of H-5 (δ 7.44); irradiation of 8-OMe (δ 3.90), 15% enhancement of H-7 (δ 7.07); irradiation of 1-*N*Me (δ 3.94), no NOE enhancement at any proton signal; EIMS *m/z* 303 (M^+, 29), 260 (71), 232 (100), 218 (63), 202 (32); HRMS *m/z* 303.1465 (calcd for $C_{17}H_{21}NO_4$, 303.1471).

Orixalone B (2): colorless oil; UV (MeOH) λ_{max} 218, 230, 256, 284, 294, 328 nm; IR ($CHCl_3$) ν_{max} 1716, 1682, 1645, 1621 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); Differential NOE, irradiation of 4-OMe (δ 3.86), 6% enhancement of H-5 (δ 7.45); irradiation of 8-OMe (δ 3.90), 10% enhancement of H-7 (δ 7.07); irradiation of 1-*N*Me (δ 3.94), no NOE enhancement at any proton signal; EIMS *m/z* 301 (M^+, 27), 256 (46), 232 (100), 217 (31), 202 (56); HRMS *m/z* 301.1295 (calcd for $C_{17}H_{19}NO_4$, 301. 1314).

Orixalone C (3): colorless oil; UV (MeOH) λ_{max} 226, 234sh, 254, 280, 292, 326 nm; IR ($CHCl_3$) ν_{max} 3394, 1650 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); Differential NOE, irradiation of 4-OMe (δ 3.89), 6% enhancement of H-5 (δ 7.35); irradiation of 8-OMe (δ 3.98), 11% enhancement of H-7 (δ 6.97); EIMS *m/z* 287 (M^+, 66), 272 (18), 259 (28), 244 (100), 218 (98), 203 (55); HRMS *m/z* 287.1158 (calcd for $C_{16}H_{17}NO_4$, 287.1158).

Orixalone D (4): colorless oil; $[\alpha]_D^{24}$ +3.1° (*c* 0.064, MeOH); UV (MeOH) λ_{max} 230, 274, 284, 318, 332 nm; IR ($CHCl_3$) ν_{max} 3406, 1635, 1615, 1579 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); Differential NOE, irradiation of 1-*N*Me (δ 3.74), 11% enhancement of H-8 (δ 7.35); irradiation of H-1′ (δ 4.75), no NOE enhancement at any proton signal; irradiation of H-2′ (δ 3.84), no NOE enhancement at any proton signal; irradiation of H-5′ (δ 1.63), 4% enhancement of H-2′ (δ 3.84); irradiation of H-4′ (δ 1.32), 4% enhancement of H-1′ (δ 4.75); EIMS *m/z* 275 (M^+, 8), 256 (15), 234 (11), 205 (73), 204 (100), 202 (68), 175 (39); HRMS *m/z* 275.1125 (calcd for $C_{15}H_{17}NO_4$, 275.1158).

Assay of NO Production in RAW 264.7 Cells. The murine macrophage cell line, RAW 264.7, was cultured in RPMI 1640 medium (without phenol red), supplemented with 10% heat-inactivated fetal bovine serum, and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells (0.2 mL, 1×10^6 cells/mL) were placed in 96-well tissue culture plates and treated with LPS (100 ng/mL), INF-γ (100 U/mL), and the test compounds. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and were then added to the culture medium, giving a final DMSO concentration of 0.5 v/v %. After 18 h incubation, the level of NO was measured by adding Griess reagent (NO_2/NO_3 assay kit, Dojindo, Kumamoto, Japan). Nitrite concentration was determined by measuring the absorbance at EX 365 nm and EM 450 nm using a Wallac 1420 ARVOsx microplate counter (Applied Biosystems, Foster City, CA).

Cytotoxicity Assay. The cell viability was determined using a CellTiter 96 Aqueous assay kit (Promega, Madison, WI). Briefly, after incubation for 18 h with the test compounds, MTS solution was added to the wells. After 1 h at 37 °C, the absorbance was measured at a wavelength of 490 nm with a Wallac 1420 ARVOsx microplate counter (Applied Biosystems, Foster City, CA).

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