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BIOCONVERSION OF RHODODENDROL BY *ACER NIKOENSE*

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Key Word Index—*Acer nikoense*; Aceraceae; callus culture; suspension culture; rhododendrol; bioconversion.

Abstract—Callus derived from *Acer nikoense* tissue contained the same compounds as the parent plants, but (+)-rhododendrol, which has hepatoprotective activity, was not detected in the methanol extract of the callus. Cells in suspension culture converted (*RS*)-rhododendrol into (*RS*)-rhododendrol 2-*O*-β-D-glucopyranoside and (*R*)-rhododendrol 2-*O*-β-D-xylopyranosyl-(1 → 6)-β-D-glucopyranoside. This is the first time that the latter compound has been isolated from a natural source.

INTRODUCTION

Acer nikoense Maxim. is a plant whose bark and leaves have been used as a flok medicine in Japan to improve hepatic function. Shinoda *et al.* reported that (+)-rhododendrol [(+)-4-(*p*-hydroxyphenyl)-2-butanol] (**1**) isolated from the bark of original plant provided protection against tetrachloromethane-induced hepatotoxicity in rats [1]. Furthermore, the methanol extract of callus derived from young branches of this plant was also found to have the same effects against liver injuries induced by tetrachloromethane or α-naphthylisothiocyanate [2, 3]. However, their active fractions did not contain **1**.

In this paper, we investigated the formation of callus from *A. nikoense* tissue and the production of glycosides from supplied rhododendrol by its cultured cells. In addition, the differences between original plants and cell cultures with regard to production of rhododendrol and its glycosides are discussed.

RESULTS AND DISCUSSION

The effects of explant type, phytohormones, and light on callus formation were investigated. Callus derived from petioles and young branches grew well, while callus from leaves did not grow because the explants secreted polyphenols. Good callus formation was obtained with explants taken in early spring because when the buds are bursting into leaves this decreases the chance of contamination and increases the viability of the callus. To induce callus formation, Murashige and Skoog's medium [4] was supplemented with 2,4-D and kinetin (DK-medium) or NAA and a cytokinin [6-benzylaminopurine (BA), kinetin, N^6-(2-isopentenyl) adenine (2ip), zeatin, or zeatin riboside]. Callus formation was induced on the DK-medium containing 0.21–2.15 ppm kinetin or the medium containing NAA and a cytokinin (BA, kinetin, 2ip, zeatin, or zeatin riboside). The callus on the medium supplemented with NAA and BA grew especially well. Light had no affect on callus formation. NB-R strain (NAA 1.86 ppm, BA 0.23 ppm) derived from petiols and NB-N strain (NAA 0.19 ppm, BA 2.25 ppm) from young branches which had been cultured in the dark since 1987 were used in the experiments reported below.

Next, the chemical components of the callus, NB-R strain, were investigated. In the ether-soluble fraction from the methanol extract, β-sitosterol, campesterol, stigmasterol, and scopoletin, all of which are found in the bark of the plant, were detected by TLC and GC-MS, but **1**, which protects against tetrachloromethane-induced hepatotoxicity in rats [1], was not detected. TLC of the ethylacetate-soluble fraction indicated catechin was present as a major compound. On acid hydrolysis, this fraction yielded rhododendrol (TLC and GC-MS). Thus the callus was shown to contain a small amount of rhododendrol glycoside(s).

In an attempt to produce **1** *in vitro*, the ability of cells (NB-N strain) in suspension culture, to convert **1** into other compounds was examined. At the end of the cultivation period, the fed compound, (*RS*)-rhododendrol, was not detected in the cells or medium. In the *n*-BuOH-soluble fraction from the methanol extract, the presence of two glycosides, **2** and **3**, which showed a positive reaction on TLC to diazo and orcinol reagents was confirmed. On acid hydrolysis, each fraction which contained **2** and **3** yielded rhododendrol. These compounds were purified by silica gel column chromatography followed by preparative HPLC.

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Table 1. ^{1}H NMR spectral data of compounds of **2** and **3** (in pyridine-d_5)

		2		3
	H	*S*	*R*	
	1	1.40 *d* (*J* = 6.2 Hz)	1.28 *d* (*J* = 6.2 Hz)	1.24 *d* (*J* = 6.2 Hz)
	2	4.08 *m*	4.18 *m*	4.23–4.20[b]
	3a	2.08 *m*		2.10 *m*
	3b	1.86 *m*		1.85 *m*
	4	2.88 *m*	2.80 *m*	2.91 *m*
	6,10	7.26 *m*		7.34 *m*
	7,9	7.18 *m*		7.15 *m*
Glc	1′	4.96 *d* (*J* = 7.8 Hz)	4.92 *d* (*J* = 7.7 Hz)	4.85 *d* (*J* = 7.8 Hz)
	2′	4.04 *m*		4.00 *m*
	3′	4.30[a]		4.23–4.20[b]
	4′	4.30[a]		4.23–4.20[b]
	5′	3.96 *m*		4.10–4.05[c]
	6′a	4.58 *m*		4.85 *m*
	6′b	4.42 *m*		4.38 *dd* (*J* = 11.3, 5.8 Hz)
Xyl	1″			5.04 *d* (*J* = 7.4 Hz)
	2″			4.10–4.05[c]
	3″			4.17 *m*
	4″			4.23–4.20[b]
	5″a			4.34 *dd* (*J* = 11.2, 5.1 Hz)
	5″b			3.69 *dd* (*J* = 11.2, 9.9 Hz)

[a–c]Overlapping signals.

Assignments of proton signals were achieved by analysis of ^{1}H–^{1}H COSY and HMQC spectra.

Table 2. ^{13}C NMR spectral data of compounds **2** and **3** (in pyridine-d_5)

		2		3
	C	*S*	*R*	
	1	22.3	20.1	20.2
	2	75.8	73.6	73.6
	3	39.6	40.1	40.3
	4	30.9	31.3	30.0
	5	133.4	133.4	133.5
	6,10	130.1	130.2	130.2
	7,9	116.2	116.2	116.2
	8	157.0	157.0	156.9
Glc	1′	104.6	102.5	102.3
	2′	75.5	75.3	75.1
	3′	78.7	78.7	78.6
	4′	71.8	71.9	71.2[a]
	5′	78.4	78.4	77.1
	6′	62.9	63.0	70.0
Xyl	1″			106.0
	2″			74.9
	3″			78.3
	4″			71.7[a]
	5″			67.2

[a]Assignments may be interchangeable within the same column.

Assignments of carbon signals were achieved by analysis of ^{1}H–^{1}H, ^{1}H–^{13}C COSY, and HMQC spectra.

Compound **2** gave $[M]^+$peak at *m/z* 328 (FD-MS), and acid hydrolysis gave rhododendrol and D-glucose. The ^{1}H NMR spectrum showed two anomeric proton signals at δ4.96 (1H, *d*, *J* = 7.8 Hz) and 4.92 (1H, *d*, *J* = 7.7 Hz) (Table 1). The ^{13}C NMR spectrum showed 24 carbon signals corresponding to 28 carbons and two anomeric carbon signals (δ104.6 and 102.5) (Table 2). In addition, C-2 showed *ca* 9 ppm downfield shift in the ^{13}C NMR spectrum when compared with C-2 of **1** [15]. From the above results, **2** is rhododendrin [(*RS*)-rhododendrol 2-*O*-β-D-glucopyranoside]. (*S*)-Rhododendrol 2-*O*-β-D-glucopyranoside has been isolated from the bark of the parent plant [6] and the *R* derivative from several plants. Compound **2** was a mixture of the C-2 diastereomers; the ratio *R*/*S* was 5:2 from the signal intensity in the ^{13}C NMR spectrum.

Compound **3** gave rise to a $[M + Na]^+$at *m/z* 483 (FD-MS). Because the ^{13}C NMR spectrum was similar to that of **2**, except for the signals of the sugar moiety, **3** was presumed to be a rhododendrol glycoside whose hexose and pentose units were attached at C-2. On partial hydrolysis with 0.5 M TFA, **3** gave **2** and D-xylose. The ^{1}H NMR spectrum showed two anomeric proton signals at δ5.04 (1H, *d*, *J* = 7.38 Hz) and 4.85 (1H, *d*, *J* = 7.78 Hz). The ^{13}C NMR spectrum showed two anomeric carbon signals at δ106.0 and 102.3. Furthermore, the detection of 19 carbon signals in the ^{13}C NMR spectrum indicated that only one C-2 stereomer of **3** had

been produced. On acid hydrolysis, **3** gave (*R*) but not (S)-rhododendrol (shown by HPLC). On the basis of these results, **3** is (*R*)-rhododendrol 2-*O*-*β*-D-xylopyranosyl-(1 → 6)-*β*-D-glucopyranoside. This is the first time that **3** has been isolated from a natural source.

Another example of the stereoselective formation of a glycoside from a racemic mixture is the bioconversion of (*RS*)-2-phenylpropionic acid by *Panax ginseng* root cultures [7]. The absolute configuration of (−)-rhododendrol has been demonstrated to be *S* [8], and **1** and its derivatives in plants have been determined to have the *S* configuration at C-2 [6, 9 and 10]. Our results showed that **3** had the *R* configuration at C-2, but production of the C-2 diastereomer was not observed. It is possible, however, that any (2*S*)-**3** is immediately converted into other compounds as soon as it is produced. The bioconversion of rhododendrol into its glycosides by *A. nikoense* cultured cells was presumed to be as shown in Fig. 1.

In this study, the ability of cells to produce **1** and its glycosides *in vitro* was found to be different from that of the parent plant. *Acer nikoense* callus contained a small amount of rhododendrol glycoside(s), but did not contain rhododendrol. Because the presence of rhododendrol glycoside(s) was confirmed in the methanol extracts of the callus, it is obvious that the enzymes responsible for its biosynthesis were expressed. We infer from the fact that *A. nikoense* callus did not contain **1** that (1) **1** and its glycosides are rapidly converted into other compounds in cell cultures, (2) if these compounds are accumulated in the bark which is an inactive tissue, cell cultures may not have a such ability, or (3) there is a rate-determining enzyme in its biosynthesis in cell cultures.

EXPERIMENTAL

General. ^{1}H NMR: 500 MHz; ^{13}C NMR: 125 MHz. TMS as int. standard. 2D NMR was performed with ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and HMQC. TLC: silica gel HPTLC plate.

Tissue cultures. Leaves, petioles, and young branches of *A. nikoense* (from Tochigi pref.) were used as sources of explants. Leaves were successively sterilized in 70% EtOH for 2–3 sec and in 1.2% NaClO for 15 min; other tissues were sterilized in 70% EtOH for 3 min. They were then rinsed (× 3) in sterile H_2O. In the case of petioles and young branches, the superficial layer was peeled off. They were then placed on Murashige and Skoog's medium containing 3% sucrose, 0.2% gelrite, and phytohormones, pH 5.8. In the DK-medium, 2,4-D (0, 0.02, 0.22, 2.21 ppm) and kinetin (0, 0.02, 0.21, 2.15, and 21.5 ppm) were added together. In the other medium used, NAA (from 0 to 2 ppm) and a cytokinin [BA, kinetin, 2ip, zeatin, or zeatin riboside (from 0.02 to 10 ppm)] were added. The cultures were maintained in the

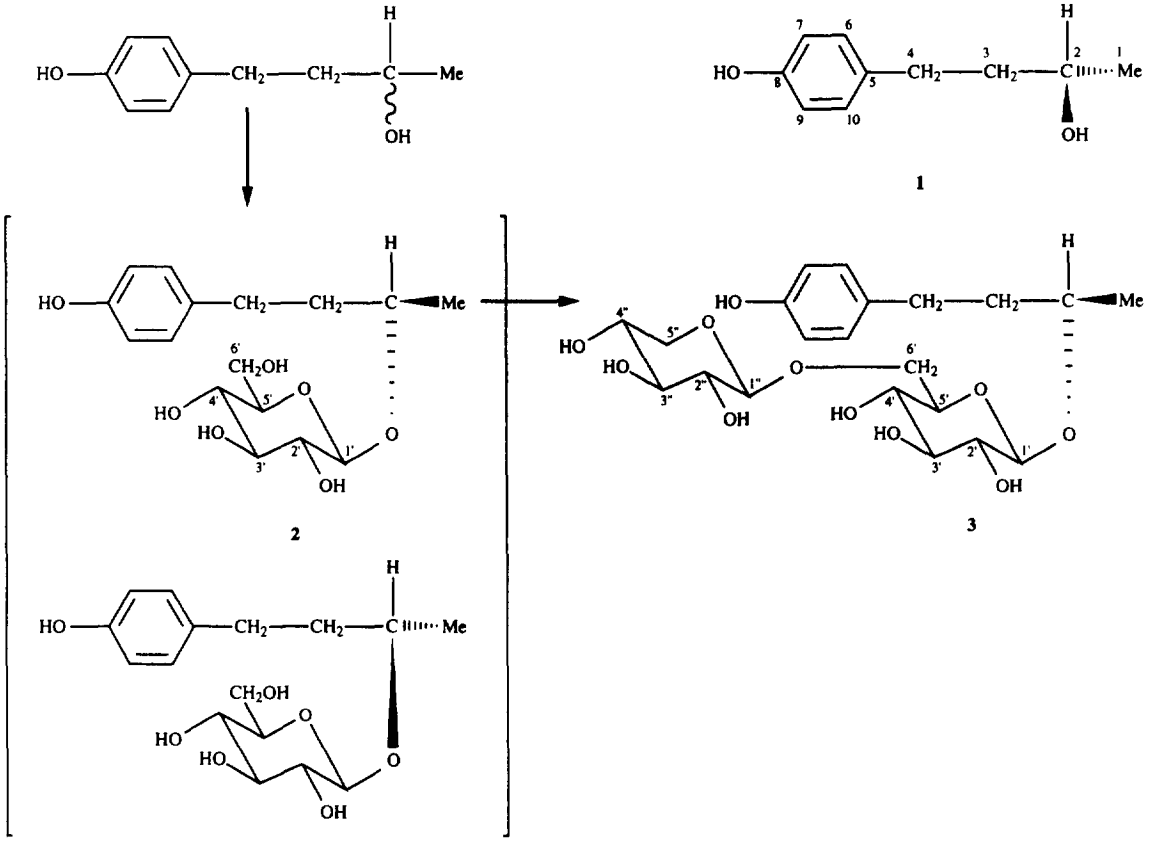

Fig. 1. Scheme for the bioconversion of rhododendrol by *Acer nikoense* cultured cells.

dark or under continuous illumination (3000 lux) at 26°. The callus formed on the surface or cut ends of the explants. Established cell cultures were subcultured every 4 weeks.

Chemical components of the callus. Lyophilized callus (NB-R strain, 300 g) were extracted (×3) with MeOH under reflux for 3 hr and the extract evapd *in vacuo*. After solubilizing the extract in H_2O, it was subjected to Et_2O (9.83 g), EtOAc (1.38 g), and *n*-BuOH (8.74 g) extraction. Subsequently, these fractions were subjected to silica gel CC.

(a) The Et_2O-soluble fraction was separated by elution with C_6H_6–EtOAc (19:1, 83:17, 4:1, 7:3) and EtOAc–MeOH (9:1, 0:1) into nine fractions (F_1–F_9) each of one l. Phytosterols were obtained from F_2 and F_3; scopoletin was detected in F_6 and F_7. The presence of these compounds were confirmed by TLC [solvent, $CHCl_3$–MeOH, 20:1; R_f 0.58 (phytosterols), 0.47 (scopoletin)] or GC-MS [dual FID; column, DB-1 (0.32 mm × 30 m); injector heater temp., 250°; detector heater temp., 280°; column temp., programmed 220°–280° at 3° min^{-1}; R_t 17.8 (campesterol), 18.3 (stigmasterol), and 19.5 (β-sitosterol) min; ionization voltage, 70 eV].

(b) The EtOAc-soluble fraction was also separated using $CHCl_3$–MeOH (19:1, 9:1, 0:1) as the eluent into seven fractions (F_1–F_7) each of 400 ml. Catechin was detected in F_2 and F_3 by TLC (solvent, $CHCl_3$–MeOH, 3:1; R_f 0.29), rhododendrol glycoside(s) was detected in F_3.

(c) A mixture of the EtOAc-soluble fraction (50 mg) or EtOAc–F_3 (52 mg) dissolved in a small amount of MeOH and 5% H_2SO_4 (2 ml) was heated for 1 hr under reflux. After cooling, the reaction mixture was diluted with H_2O and extracted with Et_2O. Rhododendrol in the organic layer was confirmed by TLC (solvent, $CHCl_3$–MeOH, 9:1; R_f 0.33) and GC-MS [dual FID; column, OV-1 (0.53 mm × 30 m); injector and detector heater temp., 280°; column temp., programmed 120°–280° at 2° min^{-1}; R_t 17.6 min; ionization voltage, 70 eV].

Bioconversion of rhododendrol by cultured cells. (a) (*RS*)-Rhododendrol was prepared by the reduction of 4-(*p*-hydroxyphenyl)-2-butanone (purchased from Tokyo Kasei Kogyo Co., Ltd, Tokyo) with excess $NaBH_4$.

(b) Callus (NB-N strain) was transferred to liquid media and cultured on a reciprocal shaker at 80 strokes min^{-1}. An ethanolic soln of (*RS*)-rhododendrol (5 mg ml^{-1}) was added to 3-week-old suspension cultures (100 ml) and these were then further incubated for 7 days.

(c) Isolation of **2** and **3**. The harvested cells were lyophilized and extracted with MeOH under reflux. The MeOH extract was evapd *in vacuo* and a mixture of the residue and H_2O was successively extracted with Et_2O and *n*-BuOH. The *n*-BuOH-soluble fraction was chromatographed on silica gel with $CHCl_3$–MeOH (19:1, 9:1, 4:1, 0:1) and $CHCl_3$–MeOH–MeOAc–H_2O (5:3:6:1) as the eluents to fractionate the diazo and orcinol positive compounds observed on TLC. Compounds **2** and **3** were finally isolated by prep. HPLC [column, μBondasphere (150 × 19 mm); mobile phase, 16 or 20%MeCN; flow rate, 5 ml min^{-1}; detector, UV 277 nm; temp., 25°].

(d) Hydrolysis of **2** was carried out in a manner similar to that described above. Rhododendrol in the Et_2O layer was confirmed by TLC and GC-MS. Subsequently, the alditol acetate derivative was prepared from the sugar fraction in the aq layer as previously reported [11] and shown to be glucitol acetate by GC comparison with a standard [dual FID; column, DB-1 (0.53 mm × 30 m); injector and detector heater temp., 280°; column temp., isothermal 190°; R_t 22.8 min)].

(e) Partial hydrolysis of **3** with 0.5 M TFA. A soln of **3** (0.5 mg) in 0.5 M TFA (200 μl) was heated at 100°. The reaction mixture was applied to TLC. After 15 min and up to 2 hr, **2**, D-xylose, and D-glucose were detected by TLC [**2**:$CHCl_3$–MeOH–MeOAc–H_2O (5:3:6:1), R_f 0.38; D-xylose and D-glucose, *n*-BuOH–2-propanol–H_2O (3:12:4), R_f 0.56 (xylose), 0.48 (glucose)]. D-Xylose and D-glucose were also confirmed by preparation of the corresponding alditol acetates; xylitol acetate R_t 10.9 min.

(f) Determination of the absolute configuration of **3** by HPLC. Rhododendrol prepared by hydrolysis of **3** with 5% H_2SO_4 was purified by prep. TLC [solvent, $CHCl_3$–MeOH (9:1); TLC plate, 0.25 mm thick; detection, UV 254 nm; solvent for recovery, Et_2O]. HPLC on a Chiralcel OD (250 × 4.6 mm) column [mobile phase, 3% isopropylamine in *n*-hexane–2-propanol (19:1); flow rate, 1 ml min^{-1}; detector, UV 277 nm; temp., 20°] showed that the (2*R*) (33.0 min) but not the (2*S*) (42.7 min) from of rhododendrol was present.

(RS)-*Rhododendrol* 2-O-β-D-glucopyranoside (**2**). Needles. FD-MS, *m/z*: 328 $[M]^+$; ^1H NMR (pyridine-d_5): Table 1; ^{13}C NMR (pyridine-d_5): Table 2.

(R)-*Rhododendrol* 2-O-β-D-*xylopyranosyl*-(1→6)-β-D-*glucopyranoside* (**3**). Powder. FD-MS, *m/z*: 483 $[M + Na]^+$; ^1H NMR (pyridine-d_5): Table 1; ^{13}C NMR (pyridine-d_5) Table 2.

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REFERENCES

1. Shinoda, M., Ohta, S., Fujita, M., Nagai, M. and Inoue, T. (1986) *Shoyakugaku Zasshi* **40**, 177.
2. Nakamura, H., Kumazawa, N., Ohta, S., Fujita, T., Iwasaki, T. and Shinoda, M. (1991) *Yakugaku Zasshi* **111**, 585.
3. Nakamura, H., Kumazawa, N., Ohta, S., Fujita, T., Iwasaki, T. and Shinoda, M. (1992) *Yakugaku Zasshi* **112**, 115.
4. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **135**, 473.
5. Kubo, M., Nagai, M. and Inoue, T. (1983) *Chem. Pharm. Bull.* **31**, 1917.

6. Inoue, T., Ishidate, Y., Fujita, M., Kubo, M., Fukushima, M. and Nagai, M. (1978) Yakugaku Zasshi **98**, 41.
7. Furuya, T., Ushiyama, M., Asada, Y. and Yoshikawa, T. (1989) *Phytochemistry* **28**, 483.
8. Klischies, M. and Zenk, M. H. (1978) *Phytochemistry* **17**, 1281.
9. Fujita, T., Miyamoto, M. and Iwasaki, T. (1992) *Jpn. Kokai Tokyo Koho* Jp 04 75, 587; *Chem. Abst.* **117**, 110110c).
10. Das, B., Padma Rao, S., Srinivas, K.V. N. S. and Yadav, J. S. (1993) *Phytochemistry* **33**, 1529.
11. Asada, Y., Ueoka, T. and Furuya, T. (1990) *Chem. Pharm. Bull.* **38**, 142.