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BIOTRANSFORMATION OF (−)-NOPOL BY *GLOMERELLA CINGULATA*

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Abstract—The biotransformation of (−)-nopol to (4*R*)-(−)-4-hydroxynopol, 4-oxonopol and 5-hydroxynopol by *Glomerella cingulata* has been demonstrated. The structures of the biotransformation products were determined by spectral methods.

INTRODUCTION

As part of a programme concerned with the use of specific microorganisms for the production of fine chemicals, we have been studying the biotransformation of terpenoids by the plant pathogenic microorganisms, *Glomerella cingulata, Rhizoctonia solani* and *Botrytis allii*. In our previous publications we reported that, (+)-cedrol [1], (−)-α-bisabolol [2], 1,8-cineole [3], (−)-globulol [4] and (+)-ledol [4] are transformed to novel terpenes by *G. cingulata*. (−)-Nopol (**1**) has been isolated from the essential oil of carrot root [5], and is easily synthesized from β-pinene [6]. This paper deals with the microbial oxidation of (−)-nopol (**1**) to (4*R*)-(−)-4-hydroxynopol (**2**), 4-oxonopol (**3**) and 5-hydroxynopol (**4**) by *G. cingulata*. The results show that **1** is predominantly oxidized at the 4-position.

RESULTS AND DISCUSSION

The time course of metabolite production following the addition of a small amount of (−)-nopol (**1**) to a culture of *G. cingulata* was monitored by TLC and quantitatively measured by GC (Fig. 1). The results suggested that the metabolic route leading to compounds **2** and **3** was the major one by which compound **1** was metabolized by *G. cingulata* and that the route leading to compound **4** was of minor importance in quantitative terms. The major products, **2** and **3**, each accounted for some 40% of the total monoterpenoid content of the cultures after 8 days whereas compound **4** accounted for only *ca* 5% after the same period of time. In order to isolate these metabolic products (**2–4**), **1** (3.60 g) was incubated with *G. cingulata* for 8 days. At the end of this time, the culture media and mycecia were extracted with CH_2Cl_2 and the extract (3.92 g) worked up to give **2** (326 mg), **3** (349 mg) and **4** (88 mg).

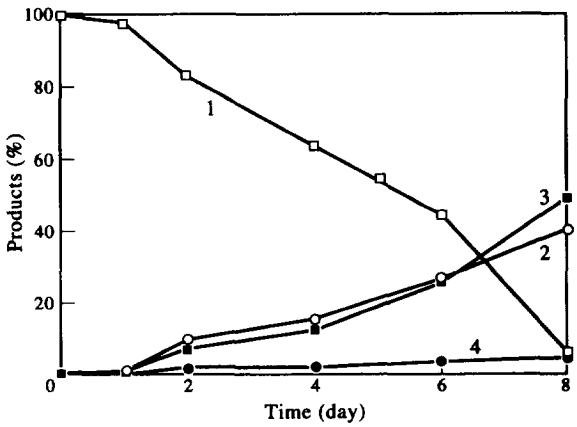
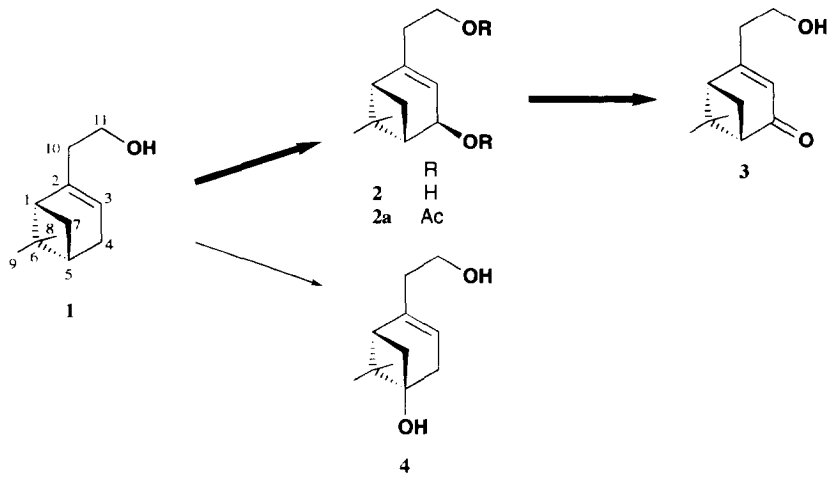


Fig. 1. Time course of metabolic products of (−)-nopol (**1**) by *G. cingulata*. □: (−)-nopol (**1**); ○, (4*R*)-(−)-4-hydroxynopol (**2**); ■: 4-oxonopol (**3**); ●, 5-hydroxynopol (**4**).

Compound **2** showed a specific ion peak at *m/z* 164 $[M - H_2O]^+$ in its GC-mass spectrum. The IR spectrum contained a C–O absorption band (1006 cm^{-1}) due to the presence of a secondary alcohol group as well as the primary alcohol absorption band (1049 cm^{-1}) of the parent compound. The ^1H NMR spectral data contained the signals (overlapped) for two hydroxyl groups at δ1.73 and a methine proton adjacent to the secondary hydroxyl group at δ4.32. The ^{13}C NMR spectral data showed the signals for a new methine carbon in place of the 4-methylene carbon present in **1**. Furthermore, the signals for C-3 and C-5 of **2** were shifted to lower field from those of **1**. These spectral data indicated that the new hydroxyl group was located at C-4. The structure of **2** was confirmed by the assignment of the NMR spectral data of its 4, 11-diacetate (**2a**). The absolute configuration at C-4 was established by comparison of the NMR spectral data with those of the known related compounds,



Scheme 1. The metabolism of (−)-nopol (**1**) by *G. cingulata*.

(−)-*cis*-verbenol and (−)-*trans*-verbenol [7, 8]. The ^{1}H NMR data showed that the signals for the proton at C-4 of **2**, (−)-*cis*-verbenol and (−)-*trans*-verbenol were at δ4.32, 4.45 and 4.25, and the signals for the proton at C-8 were at δ0.88, 1.07 and 0.87, respectively. The ^{13}C NMR data showed that the signals for C-4 of **2**, (−)-*cis*-verbenol and (−)-*trans*-verbenol were at δ70.2, 73.3 and 70.3, and those for C-6 were at δ46.3, 39.0 and 46.1, respectively. Consideration of the above data suggested that the chemical shifts of **2** were very similar to those of (−)-*trans*-verbenol. In addition, the other signals for C-2, C-5, C-7, C-8 and C-9 of **2** were almost identical with those of (−)-*trans*-verbenol. These results allowed us to determine the absolute configuration of C-4 as *R*. The optical rotation of **2** had a large laevorotatory value (− 121.4 °). Therefore, compound **2** was determined to be (4*R*)-(−)-4-hydroxynopol.

The metabolic product **3** contained specific ion peaks at *m/z* 180 $[M]^+$, 165 $[M - Me]^+$ and 149 $[M - CH_2OH]^+$ in the GC-mass spectrum. The IR spectrum showed a strong absorption band at 1665 cm^{-1} (C=O) due to the presence of a carbonyl group. The ^{13}C NMR spectral data also indicated the presence of a newly introduced carbonyl group in place of the 4-methylene group of **1**. In addition, the signals for H-3, H-5 and H-7 in the ^{1}H NMR spectrum and those for C-2, C-3, C-5, C-6, and C-7 in the ^{13}C NMR spectrum were shifted to lower field than the corresponding signals of **1**. These spectral data established that compound **3** was 4-oxonopol.

The mass spectral data of the minor metabolic product **4** showed specific ion peaks at *m/z* 182 $[M]^+$, 167 $[M - Me]^+$ and 137 $[M - CH_2CH_2OH]^+$. The IR spectrum of **4** contained the novel absorption at 1134 cm^{-1} (C–O) due to the presence of a tertiary hydroxy group. The ^{1}H NMR spectral data contained the signals (overlapped) for two hydroxyl groups at δ1.68, however, there were no signals for a proton adjacent to a newly introduced hydroxy group. This indicated the presence of a tertiary alcohol group in **4**. The ^{13}C NMR data showed the presence of a quaternary carbon instead of the 4-methine C present in **1**. In addition, movement of the chemical shifts between **1** and **4** was observed for the 4-position but not the 2-position. Therefore, compound **4** was shown to be 5-hydroxynopol.

This study established that compound (−)- **1** is oxidatively biotransformed to compounds **2**–**4** by the fungus *G. cingulata* (Scheme 1). The oxidations were confined mainly to the 4-position to give (4*R*)-(−)-4-hydroxy- and 4-oxo-nopol. This enantioselective hydroxylation might be due to steric hindrance by the geminal dimethyl group at C-6 dictating that the hydroxyl group is incorporated from the opposite side of the molecule to the dimethyl group.

EXPERIMENTAL

General. (−)-Nopol was purchased from Fluka Chem. ^{1}H and ^{13}C NMR: 270.05 and 67.80 MHz, respectively. GC-MS: 20 eV (ion voltage), 250° (ion source), OV-1(0.25 mm × 30 m) capillary column; TLC: silica gel 60 F_{254} pre-coated (layer thickness 0.25 mm, Merck); CC: silica gel with *n*-hexane–EtOAc gradients.

Cultivation of G. cingulata. Spores of *G. cingulata* (provided by Dr M. Hyakumachi, Gifu University) which had been preserved at low temp. were inoculated into sterilized culture media in an Erlenmeyer flask and shaken at 27° for 2 days. The components of the culture medium (g/250 ml) were: sucrose 3.75, glucose 3.75, polypeptone 1.25, $MgSO_4 \cdot 7H_2O$ 0.125, KCl 0.125, K_2HPO_4 0.25, $FeSO_4 \cdot 7H_2O$ 0.0025. The mycelia were then transplanted in to petri dishes which contained 15 ml of the same sterilized culture media (pH 7.2) and incubated at 27° without shaking for 3 days. After growth of *G. cingulata*, **1** (3.60 g) was added directly to the medium (15 mg/15 ml) and the cultures further incubated under the same conditions for 8 days.

Table 1. ^{1}H NMR spectral data for (−)-nopol (**1**) and its metabolic products (**2**–**4**) and derivative **2a** (270.05 MHz, $CDCl_3$, TMS as int. standard)

H	1	2	2a	3	4
1	2.04 *ddd* (1.5, 5.8,5.8)	2.12 *ddd* (1.5, 5.3, 5.5)	2.15 *ddd* (1.2, 5.3, 5.5)	2.52 *ddd* (1.3, 5.8,5.8)	2.00 *dd* (2.0, 6.3)
3	5.34 *m*	5.46 *m*	5.42 *m*	5.80 *dd* (1.3, 1.5)	5.48 *m*
4	2.24 *m*	4.32 *dd* (2.8, 3.0)	5.37 *dd* (2.8, 3.0)	—	2.24–2.31 *m*
5	2.15 *m*	2.20 *dddd* (1.5, 3.0, 5.5, 5.7)	2.24 *dddd* (1.5, 3.0, 5.5, 5.5)	2.68 *ddd* (1.5, 5.8, 5.8)	—
7_a	1.15 *d* (8.5)	1.32 *d* (7.8)	1.45 *d* (8.5)	2.10 *d* (9.5)	1.59 *d* (8.2)
7_b	2.39 *ddd* (5.8, 5.8, 8.5)	2.30 *ddd* (5.3, 5.7, 7.8)	2.33 *ddd* (5.3, 5.5, 8.5)	2.84 *ddd* (5.8, 5.8, 9.5)	2.34 *ddd* (0.8, 6.3, 8.2)
8	0.85 *s*	0.88 *s*	0.91 *s*	1.02 *s*	0.87 *s*
9	1.28 *s*	1.35 *s*	1.35 *s*	1.51 *s*	1.23 *s*
10	2.24 *dt* (1.0, 6.0)	2.28 *t* (6.5)	2.37 *ddd* (1.0, 6.5, 7.0)	2.55 *t* (6.5)	2.26 *dt* (1.0, 6.5)
11	3.61 *dt* (1.0, 6.0)	3.67 *t* (6.5)	4.08 *ddd* (6.5, 7.0, 11.5)	3.82 *t* (6.5)	3.64 *dt* (1.0, 6.5)
			4.14 *ddd* (6.5, 7.0, 11.5)		
4-OH	—	1.73 *br s*	—	—	—
5-OH	—	—	—	—	1.68 *br s*
11-OH	1.64 *br s*	1.73 *br s*	—	1.73 *br s*	1.68 *br s*
4-COMe	—	—	2.03[a] *s*	—	—
11-COMe	—	—	2.04[a] *s*	—	—

Coupling constants in Hz.
[a] Values are interchangeable within each column.

Table 2. ^{13}C NMR spectral data for (−)-nopol (**1**) and its metabolic products (**2**–**4**) and derivative **2a** (67.80 MHz, $CDCl_3$, $CHCl_3$, as int. standard)

C	1	2	2a	3	4
1	45.6 *d*	46.8 *d*	46.2[b] *d*	48.7 *d*	40.8 *d*
2	144.7 *s*	148.7 *s*	149.9 *s*	170.5 *s*	143.7 *s*
3	119.3 *d*	121.1 *d*	117.4 *d*	121.5 *d*	121.8 *d*
4	31.3 *t*	70.2 *d*	73.3 *d*	204.2 *s*	38.0[f] *t*
5	40.7 *d*	47.1[a] *d*	44.4[b] *d*	57.8 *d*	75.0 *s*
6	37.9 *s*	46.3 *s*	46.2 *s*	54.2 *s*	44.7 *s*
7	31.7 *t*	28.9 *t*	29.6 *t*	41.2[e] *t*	39.4[f] *t*
8	21.1 *q*	20.8 *q*	21.4 *q*	22.2 *q*	18.6 *q*
9	26.2 *q*	26.6 *q*	26.4 *q*	26.5 *q*	20.7 *q*
10	40.2 *t*	39.7 *t*	35.4 *t*	40.0[e] *t*	41.2[f] *t*
11	59.9 *t*	60.0 *t*	62.0 *t*	59.5 *t*	60.1 *t*
4-COMe			20.9[c] *q*		
4-COMe			170.9[d] *s*		
11-COMe			20.8[c] *q*		
11-COMe			170.9[d] *s*		

[a–f] Values are interchangeable within each column.

Purification of the metabolic products (**2**–**4**). After incubation, the culture media were collected, acidified to pH 2 with HCl, saturated with NaCl and extracted with CH_2Cl_2 for 3 days. The mycelia were also collected and extracted with CH_2Cl_2 for 3 days. Both CH_2Cl_2 extracts were mixed, and the solvent evapd under red. press. The extract (3.92 g) was dissolved in CH_2Cl_2 and separated into neutral and acid fractions in the usual manner. The neutral fraction (3.55 g) was chromatographed over silica gel with a hexane–EtOAc gradient repeatedly to give the metabolic products **2** (326 mg), **3** (349 mg) and **4** (88 mg).

(4R)-(−)-*Hydroxynopol* (**2**). Crystal; mp. 111.5–112.4°; $[\alpha]_D^{20}$ − 121.4° (MeOH; *c* 1.0); EIMS *m/z* (rel. int.): 164 $[M - H_2O]^+$ (17), 149 (25), 133 (30), 131 (36), 119 (46), 104 (45), 91 (100), 69 (36), 59 (24), 43 (47), 40 (67); IR ν_{max} cm^{-1}: 3255, 2929, 1440, 1049, 1006, 929, 858, 766, 652; ^{1}H and ^{13}C NMR: Tables 1 and 2.

(4R)-(−)-4,11-*Diacetoxy*-4-*hydroxynopol* (**2a**). Compound **2** (30 mg) was acetylated in the usual manner to yield **2a** (43 mg). Oil; $[\alpha]_D^{20}$ − 125.9° ($CHCl_3$; *c* 1.0); EIMS *m/z* (rel. int.): 224 (0.8), 223 $[M - Ac]^+$ (0.4), 181 (0.7), 180 (0.2), 163 (17), 147 (47), 131 (75), 121 (52), 105 (91), 91 (56), 43 (100); IR ν_{max} cm^{-1}: 2938, 1741, 1472, 1370, 1240, 1087, 1019, 972; ^{1}H and ^{13}C NMR: Tables 1 and 2.

4-*Oxonopol* (**3**). Oil; $[\alpha]_D^{20}$ − 78.2° ($CHCl_3$; *c* 0.5); EIMS *m/z* (rel. int.): 180 $[M]^+$ (42), 165 (58), 149 (33), 147 (50), 135 (48), 121 (65), 91 (71), 79 (80), 55 (82), 41 (100); IR ν_{max} cm^{-1}: 3411, 2950, 1665, 1610, 1042, 986, 865, 748; ^{1}H and ^{13}C NMR: Tables 1 and 2.

5-*Hydroxynopol* (**4**). Oil; $[\alpha]_D^{20}$ − 45.0° ($CHCl_3$; *c* 0.30); EIMS *m/z* (rel. int.): 182 $[M]^+$ (2), 167 (2), 164 (2), 149 (10), 137 (11), 126 (34), 121 (34), 95 (48), 91 (48), 79 (45), 43 (100), 41 (85); IR ν_{max} cm^{-1}: 3326, 2943, 1723, 1667, 1134, 1042, 755, 659; ^{1}H and ^{13}C NMR: Tables 1 and 2.

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