

Repandiol, a New Cytotoxic Diepoxide from the Mushrooms *Hydnum repandum* and *H. repandum* var. *album*

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Repandiol, a new cytotoxic diepoxide has been isolated from the mushrooms *Hydnum repandum* and *H. repandum* var. *album*. The chemical structure was elucidated as (2*R*,3*R*,8*R*,9*R*)-4,6-decadiyne-2,3:8,9-diepoxy-1,10-diol on the basis of spectroscopic analysis. The structure was confirmed by the synthesis of optically active repandiol. Repandiol displayed potent cytotoxic activity against various tumor cells.

Keywords cytotoxic diepoxide; repandiol; (2*R*,3*R*,8*R*,9*R*)-4,6-decadiyne-2,3:8,9-diepoxy-1,10-diol; mushroom; basidiomycete; *Hydnum repandum*; *Hydnum repandum* var. *album*; structure elucidation; total synthesis

In the course of our screening program for biologically active metabolites from mushrooms (Basidiomycetes), a new cytotoxic diepoxide, designated repandiol, has been isolated from the mushrooms *Hydnum repandum* (Japanese name; kanoshita) and *H. repandum* var. *album* (Japanese name; shirokanoshita).¹ Herein, we report the isolation, structure elucidation, total synthesis and cytotoxic activity of repandiol.

Results and Discussion

Isolation The fruiting bodies of *H. repandum* var. *album* (160 g), collected at Miyagi prefecture in September 1989, were extracted twice with MeOH (400 ml) at room temperature for 2 d. After removal of the solvent, the residue was separated into EtOAc (964.6 mg), *n*-BuOH (126.1 mg) and H₂O-soluble portions, and the EtOAc fraction (484.0 mg) was subjected to column chromatography on silica gel (10 g; 1 cm i.d. × 21 cm). The active fractions (60.2 mg) eluted with CHCl₃-MeOH (19:1; 9:1) were submitted to preparative TLC [Merck, Kieselgel 60 F₂₅₄, 20 cm × 20 cm × 0.25 mm; CHCl₃-MeOH (10:1)], followed by recrystallization from MeOH to afford repandiol (**1**, 5.3 mg) as colorless leaflets. Repandiol was also obtained from the MeOH extract of *H. repandum* (88 g) in the yield of 13.2 mg, after purification by the same procedure as described above.

Structure Elucidation The physico-chemical properties of repandiol (**1**) are summarized in Table I. It showed a molecular ion peak at *m/z* 194 in the electron impact mass spectrum (EI-MS). The molecular formula was determined to be C₁₀H₁₀O₄ based on the high resolution EI-MS (HREI-MS). The IR spectrum of **1** showed an absorption at 3600—2600 cm⁻¹ due to two hydroxyl groups, which was confirmed by the formation of the corresponding

diacetate (**2**) on treatment of **1** with acetic anhydride in pyridine (Ac₂O/Py). The UV spectrum of **1** exhibited characteristic absorptions at 203, 229, 241, 255 and 269 nm indicative of the presence of a diyne chromophore.²⁾

The ¹H- and ¹³C-NMR spectral data for **1** are shown in Table II. The ¹³C-NMR spectrum (CD₃OD) revealed only five signals assignable to two acetylenic carbons (δ 68.8, 77.6), two oxymethine carbons (δ 44.1, 62.4) and one oxymethylene carbon (δ 62.1), which account for half of the carbons, implying that **1** has a symmetrical structure. The ¹H-NMR spectrum (dimethylsulfoxide-*d*₆ (DMSO-*d*₆), Fig. 1) also showed five signals due to oxymethylene protons (δ 3.44; 3.60), two oxymethine protons (δ 3.32; 3.60) and one deuterium-exchangeable proton (δ 4.98), and detailed analysis of the coupling constants for these protons led us to the partial structure I. Further, the ¹H-¹H correlation spectroscopy (COSY) spectrum (DMSO-*d*₆) revealed the H-H relationships between H-1/OH, H-1/H-2, H-2/H-3, H-8/H-9, H-9/H-10 and H-10/OH. Taking into account the degree of unsaturation (U.N.=6), the planar structure of **1** was concluded to be 4,6-decadiyne-2,3:8,9-diepoxy-1,10-diol (**3**). The structure of **3** was also substantiated by formation of the tetraol (**4**) upon hydrogenation over PtO₂ (Fig. 2). Finally, the structure of **1**, as well as the stereochemistry, was established by the total synthesis of **1**.

Total Synthesis of (+)- and (-)-Repandiol Repandiol (**1**) is an optically active compound, although it has a symmetrical structure. Further, the coupling constant, *J*=2.0 Hz, suggested the stereochemistry of the oxirane ring protons to be *trans*. The configuration at C-2, C-3, C-8 and C-9 was, therefore, deduced to be all-*S* or all-*R*. Thus, the synthesis of **1** was carried out as follows (Chart 1).

TABLE I. Physico-Chemical Properties of Repandiol (**1**)

Appearance	Colorless leaflets
mp (°C)	168—169
[α] _D ²⁰	+40.0° (c=0.12, MeOH)
Formula	C ₁₀ H ₁₀ O ₄
HR EI-MS	Calcd 194.0579 Found 194.0616
IR ν _{max} ^{KBr} cm ⁻¹	3600—2600 (OH)
UV λ _{max} ^{MeOH} nm (log ε)	203 (4.46), 229 (3.65), 241 (3.79), 255 (3.86), 269 (3.74)

TABLE II. ¹H- and ¹³C-NMR Spectral Data for Repandiol (**1**)^{a)}

No.	¹ H	¹³ C ^{b)}
1, 10	3.44 (each 1H, ddd, <i>J</i> =12.7, 5.9, 4.4) 3.60 (each 1H, ddd, <i>J</i> =12.7, 5.9, 2.9)	62.1 (each 1C, t)
2, 9	3.32 (each 1H, br s)	44.1 (each 1C, d)
3, 8	3.60 (each 1H, d, <i>J</i> =2.0)	62.4 (each 1C, d)
4, 7		77.6 (each 1C, s)
5, 6		68.8 (each 1C, s)
<u>OH</u>	4.98 (each 1H, t, <i>J</i> =5.9)	

a) ¹H- and ¹³C-NMR spectra were recorded in DMSO-*d*₆ and CD₃OD, respectively. b) Multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) experiments.

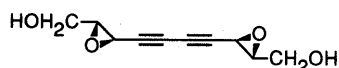


Fig. 3. Chemical Structure of Repandiol (1)
(2*R*,3*R*,8*R*,9*R*)-4,6-Decadiyne-2,3:8,9-diepoxy-1,10-diol.

TABLE III. Cytotoxic Activity of Repandiol (1) *in Vitro*

Cell line	IC ₅₀ (μg/ml)
P388	1.88
L1210	1.20
Colon 26	0.30
DLD-1	0.66
A549	1.35

value of specific rotation (+38.0°) which was nearly equal to that of **1**, and an optical purity of 95% was assessed by comparison of the $[\alpha]_D$ value with that of **1**. (–)-Repandiol was obtained by the same procedure described above except that (+)-DET was used as the chiral auxiliary.

Thus the structure and stereochemistry of **1** were concluded to be (2*R*,3*R*,8*R*,9*R*)-4,6-decadiyne-2,3:8,9-diepoxy-1,10-diol, as depicted in Fig. 3.

Cytotoxic Activity The *in vitro* cytotoxic activity of repandiol is shown in Table III. Repandiol displayed potent cytotoxic activity against various tumor cells, especially colon adenocarcinoma cells. Repandiol is regarded as a bifunctional alkylating agent like DAG (dianhydrogalactitol)⁶ and WF-3405,⁷ which are promising anticancer agents. Therefore, a more detailed evaluation of the antitumor activity for repandiol is being undertaken, and the results will be published in the near future.

Experimental

Melting points were measured on a Yanagimoto micro hot plate and are uncorrected. IR spectra were recorded on a JASCO A-100S IR spectrometer with polystyrene calibration at 1601 cm⁻¹. UV spectra were recorded on a Hitachi U-3200 spectrophotometer. Optical rotation values were measured on a JASCO DIP-370 polarimeter. ¹H- and ¹³C-NMR spectra were recorded on a JEOL GX-500 (500 and 125 MHz, respectively) using tetramethylsilane (TMS) as an internal standard. Chemical shifts are shown in δ (ppm) and multiplicities are indicated as follows: singlet=s, doublet=d, triplet=t, multiplet=m and broad=br. Coupling constants (*J*) are shown in hertz (Hz). EI and HREI-MS were taken on a JEOL DX-303 spectrometer. TLC analyses were performed on Kieselgel 60F₂₅₄ (Merck) and spots were detected under UV irradiation and by heating on a hot plate after spraying sulfuric acid and anisaldehyde-sulfuric acid reagents.

Acetylation of Repandiol (1) A solution of repandiol (**1**: 3.1 mg) in pyridine (200 μl) was treated with Ac₂O (150 μl), and the mixture was left to stand at room temperature overnight, then poured into ice-water and extracted with EtOAc (10 ml × 2). The combined extracts were washed with 1*N* HCl, saturated aqueous NaHCO₃, water and saturated NaCl, then dried over anhydrous MgSO₄. After evaporation of the solvent, the residue was chromatographed on silica gel with CHCl₃ as the eluent to give the diacetate (**2**, 2.9 mg) as an amorphous powder. $[\alpha]_D^{25} +63.4^\circ$ (*c*=0.12, CHCl₃). FD-MS *m/z*: 279 [(M+H)⁺]. EI-MS *m/z*: 205, 149, 74. IR $\nu_{\max}^{\text{CHCl}_3}$ cm⁻¹: 3025, 2960, 2860, 2170, 1750 (ester C=O). ¹H-NMR (CDCl₃) δ: 2.10 (6H, s, 2 × CH₃CO), 3.40 (2H, brs, H-3, 8), 3.41 (2H, m, H-2, 9), 4.03 (2H, dd, *J*=12.7, 4.9 Hz, H-1, 10), 4.34 (2H, dd, *J*=12.7, 3.4 Hz, H-1, 10).

Hydrogenation of Repandiol (1) A solution of repandiol (**1**: 3.0 mg) in EtOH (3 ml) was hydrogenated over PtO₂ (1.0 mg) at room temperature. After 1 h, the catalyst was filtered off and the filtrate was concentrated under reduced pressure. The resulting oil was purified by silica gel chromatography using CHCl₃-MeOH (4:1) as eluent to afford the tetraol (**4**, 2.3 mg). $[\alpha]_D^{25} -13.7^\circ$ (*c*=0.19, MeOH). FD-MS *m/z*: 207 [(M+H)⁺].

EI-MS *m/z*: 207 [(M+H)⁺], 175, 157, 139, 121. ¹H-NMR (DMSO-*d*₆) δ: 1.15–1.32 (8H, brs), 1.32–1.45 (4H, brs), 3.23 (4H, m), 3.36 (2H, brs), 4.31 (2H, d, *J*=5.4 Hz, exchangeable with D₂O, 2 × OH), 4.39 (2H, t, *J*=5.9, 5.4 Hz, exchangeable with D₂O, 2 × OH). ¹H-NMR (DMSO-*d*₆+D₂O) δ: 1.15–1.30 (8H, brs), 1.33–1.44 (4H, brs), 3.21 (2H, dd, *J*=10.8, 5.9 Hz, H-1, 10), 3.25 (2H, dd, *J*=10.8, 5.9 Hz, H-1, 10), 3.37 (2H, brs, H-2, 9).

Synthesis of (+)- and (–)-Repandiol. 2,4-Hexadiyne-1,6-diol (6) Bistriphenylphosphine palladium(II) chloride [(Ph₃P)₂PdCl₂: 140 mg, 0.2 mmol], CuI (40 mg, 0.2 mmol) and Et₃N (4.2 ml) were sequentially added to a solution of propargyl alcohol (**5**: 0.58 ml, 10 mmol) in *N,N*-dimethylformamide (DMF) (20 ml), then the resulting mixture was stirred at room temperature under an O₂ atmosphere. After 6 h, the reaction mixture was poured into ice-water and then extracted with EtOAc (50 ml × 4). The combined extracts were washed with 0.5*N* HCl, saturated aqueous NaHCO₃, water and saturated NaCl, dried over anhydrous MgSO₄ and evaporated under reduced pressure. The residue was chromatographed on silica gel with CHCl₃-MeOH as the eluent to give the diol (**6**), 299.5 mg (54.5%) as pale brown needles (from CHCl₃-MeOH). mp 113–115 °C. EI-MS *m/z*: 110 (M⁺). IR ν_{\max}^{NaCl} cm⁻¹: 3400–3000 (OH). ¹H-NMR (DMSO-*d*₆) δ: 4.17 (4H, d, *J*=5.6 Hz), 5.40 (2H, t, *J*=6.0 Hz, OH).

Diethyl (2*E*,8*E*)-2,8-Decadiene-4,6-diyne-1,10-dicarboxylate (8a) Dry DMSO (0.57 ml, 8.0 mmol) was added dropwise to a stirred solution of oxalyl chloride (0.52 ml, 6.0 mmol) in CH₂Cl₂ (20 ml) at –78 °C. After 10 min, a solution of the diol (**6**: 440 mg, 4.0 mmol) in CH₂Cl₂-DMSO (1:1 (v/v) 2 ml) was added slowly to the above mixture over 3 min, and then the mixture was stirred at –78 °C for 20 min. The resulting mixture was treated with Et₃N (2.4 ml, 16.0 mmol), and allowed to warm to 0–5 °C. After 10 min, the reaction mixture was recooled to –78 °C and then a solution of ethyl(triphenylphosphoranylidene)acetate (2.1 g, 6.0 mmol) in CH₂Cl₂ (10 ml) was added dropwise. This reaction mixture was stirred at the same temperature for 10 min and allowed to warm to room temperature over 50 min. The resulting mixture was poured into saturated aqueous NH₄Cl and extracted with CH₂Cl₂ (10 ml). The organic phase was washed with 0.5*N* HCl, saturated aqueous NaHCO₃, water and saturated NaCl, dried over anhydrous MgSO₄, and evaporated. The residual oil was subjected to chromatography on silica gel using *n*-hexane-EtOAc as the eluent to afford the diesters (**8a**, **8b** and **8c**) in 54.3% (534.7 mg), 10.6% (104.5 mg) and 0.5% (5.2 mg) yields, respectively.

8a: Pale yellow waxy solid. mp <31 °C. EI-MS *m/z*: 246 (M⁺), 217, 201, 189. HR-MS *m/z*: 246.0897 (M⁺); Calcd for C₁₄H₁₄O₄: 246.0892. IR $\nu_{\max}^{\text{CHCl}_3}$ cm⁻¹: 3025, 2985, 2940, 2140, 1720 (sh), 1715 (ester C=O), 1610. ¹H-NMR (CDCl₃) δ: 1.30 (6H, t, *J*=7.3 Hz, CH₃CH₂), 4.25 (4H, q, *J*=7.3 Hz, CH₃CH₂), 6.38 (2H, d, *J*=15.4 Hz, H-2, 9), 6.82 (2H, d, *J*=15.4 Hz, H-3, 8).

8b: Pale yellow waxy solid. EI-MS *m/z*: 246 (M⁺), 217, 201, 189, 133. HR-MS *m/z*: 246.0878 (M⁺); Calcd for C₁₄H₁₄O₄: 246.0892. IR $\nu_{\max}^{\text{CHCl}_3}$ cm⁻¹: 3025, 2985, 2940, 2135, 1720 (sh), 1715 (ester C=O), 1615, 1605. ¹H-NMR (CDCl₃) δ: 1.31 (3H, t, *J*=7.1 Hz, CH₃CH₂), 1.33 (3H, t, *J*=7.1 Hz, CH₃CH₂), 4.23 (2H, q, *J*=7.1 Hz, CH₃CH₂), 4.25 (2H, q, *J*=7.1, CH₃CH₂), 6.23 (1H, dd, *J*=11.5, 0.9 Hz, H-8), 6.29 (1H, d, *J*=11.5 Hz, H-9), 6.36 (1H, d, *J*=15.8 Hz, H-2), 6.83 (1H, dd, *J*=15.8, 0.9 Hz, H-3).

8c: EI-MS *m/z*: 246 (M⁺), 217, 201, 189, 133. HR-MS *m/z*: 246.0858 (M⁺); Calcd for C₁₄H₁₄O₄: 246.0892. IR $\nu_{\max}^{\text{CHCl}_3}$ cm⁻¹: 3025, 3000, 2985, 2125, 1720 (sh), 1715 (ester C=O), 1600. ¹H-NMR (CDCl₃) δ: 1.33 (6H, t, *J*=7.3 Hz, CH₃CH₂), 4.25 (4H, q, *J*=7.3 Hz, CH₃CH₂), 6.23 (2H, d, *J*=11.1 Hz, H-2, 9), 6.26 (2H, d, *J*=11.5 Hz, H-3, 8).

(2*E*,8*E*)-2,8-Decadiene-4,6-diyne-1,10-diol (9) DIBAH (1.0*M* *n*-hexane solution: 10.4 ml, 4.8 mmol) was added dropwise to a stirred solution of the diester (**8a**: 534.7 mg, 2.2 mmol) in dry benzene (10 ml) at 0 °C. The mixture was stirred at the same temperature for 1 h, then excess reducing agent was decomposed by addition of MeOH and water. The reaction mixture was diluted with 0.5*N* HCl and extracted with EtOAc (50 ml × 2). The combined extracts were washed with saturated aqueous NaHCO₃, water, and saturated NaCl, dried over anhydrous MgSO₄ and evaporated, then the residue was chromatographed on silica gel with CHCl₃-MeOH as the eluent to give the diol (**9**), 276.7 mg (78.6%) as pale yellow leaflets. mp 147–150 °C (<136 °C dec.). EI-MS *m/z*: 162 (M⁺), 133, 115, 89. HR-MS *m/z*: 162.0696 (M⁺); Calcd for C₁₀H₁₀O₂: 162.0681. IR ν_{\max}^{KBr} cm⁻¹: 3500–3000 (OH), 2125, 1625. ¹H-NMR (CD₃OD) δ: 4.14 (4H, dd, *J*=4.7, 2.1 Hz, H₂-1, H₂-10), 5.84 (2H, dt, *J*=15.4, 2.1 Hz, H-2, 9), 6.39 (2H, dt, *J*=15.4, 5.1, 4.7 Hz, H-3, 8).

(+)-Repandiol (10) D-(–)-DET (0.17 ml, 0.96 mmol) and titanium

tetraisopropoxide [$\text{Ti}(\text{O}^i\text{Pr})_4$: 0.24 ml, 0.8 mmol] were added to a stirred suspension of 4A molecular sieves (0.6 g) in CH_2Cl_2 (20 ml) at -20°C , then the mixture was stirred for 5 min. A suspension of the diol (**9**: 65 mg, 0.4 mmol) in CH_2Cl_2 was added dropwise to the above mixture and then *tert*-butylhydroperoxide (TBHP, *ca.* 2.0 M CH_2Cl_2 solution: 0.8 ml, 1.6 mmol) was added. The mixture was stirred at -20°C for 3 h, then diluted with saturated aqueous NaCl and extracted with CH_2Cl_2 (50 ml \times 2). The combined extracts were washed with water, dried over anhydrous MgSO_4 and evaporated. The residue was subjected to successive chromatography on silica gel using CHCl_3 -MeOH as the eluent to give (+)-repandiol (**10**), 29.1 mg (37.4%) as colorless leaflets. mp 165 – 167°C . $[\alpha]_D^{27} + 38.0^\circ$ ($c=0.14$, MeOH). EI-MS m/z 194 (M^+), 163, 74. HR-MS m/z : 194.0592 (M^+); Calcd for $\text{C}_{10}\text{H}_{10}\text{O}_4$: 194.0579.

(-)-Repandiol (**11**) (-)-Repandiol (**11**) was obtained from **9** (81.0 mg, 0.5 mmol) in 36.0% (34.9 mg) yield by the same procedure as described above except for the use of L-(+)-DET as a chiral auxiliary. $[\alpha]_D^{24} - 37.5^\circ$ ($c=0.14$, MeOH). EI-MS m/z : 194 (M^+), 163, 74. HR-MS m/z : 194.0615 (M^+); Calcd for $\text{C}_{10}\text{H}_{10}\text{O}_4$: 194.0579.

Assay for Cytotoxic Activity against Ascites Tumor Cells RPMI 1640 Medium (Nissui) supplemented with 10% fetal bovine serum (Whittaker), 20 μM 2-mercaptoethanol and 100 $\mu\text{g}/\text{ml}$ of kanamycin was used for cell culture. The sample was dissolved in DMSO at a concentration of 100, 10, 1.0 or 0.1 $\mu\text{g}/\text{ml}$. A suspension (2 ml) of tumor cells (10^4 cells/ml) in the medium was prepared in a culture tube (Falcon No. 2054) and incubated in a CO_2 (5%) incubator at 37°C for 5 h, then a sample solution was added. Incubation was continued at 37°C for a further 72 h, then the cell number in the culture suspension was determined with a Coulter counter (model ZB). The control cell suspension was similarly prepared without the test sample. All assays were performed in triplicate and the 50%-inhibitory concentration (IC_{50}) was calculated from the differences of the cell numbers in the culture suspensions in the presence and absence of the sample.

Assay for Cytotoxic Activities against Solid Tumor Cells MEM medium (Nissui) supplemented with 10% fetal bovine serum and 60 $\mu\text{g}/\text{ml}$ of kanamycin was used for cell culture. A suspension (2 ml) of tumor cells (5×10^3 cells/ml) in the medium was prepared in a culture dish (Costar No. 3512) and incubated at 37°C for 24 h in a 5% CO_2 incubator, then a sample solution in DMSO was added. Incubation at 37°C for a further 72 h, then the cells were treated with trypsin and the cell number was counted with a Coulter counter. The control cell suspension was prepared without the test sample. The 50%-inhibitory concentration (IC_{50}) was calculated in the same manner as described above.

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