PRODUCTION, ISOLATION AND STRUCTURE DETERMINATION OF A NOVEL β-GLUCOSIDASE INHIBITOR, CYCLOPHELLITOL, FROM *PHELLINUS* SP.

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In the course of our screening of β-glucosidase inhibitor, a culture filtrate of a mushroom, *Phellinus* sp. strongly inhibited the enzyme activity. The active substance was isolated through charcoal separation, column chromatography and crystallization. Spectroscopic and crystallographic analysis revealed that it had a novel cyclitol structure, (1*S*,2*R*,3*S*,4*R*,5*R*,6*R*)-5-hydroxymethyl-7-oxabicyclo[4,1,0]heptane-2,3,4-triol, and we named it cyclophellitol. It inhibited almond-derived β-glucosidase with an IC_{50} of 0.8 μg/ml.

β-Glucosidase inhibitors such as castanospermine and 1-deoxynojirimycin have been reported to inhibit syncytium formation and infection of human immunodeficiency virus (HIV), possibly by perturbing gp120-linked glycan structure[1,2]. Castanospermine is also known to suppress experimental metastasis possibly by changing the saccharide structure on tumour cell surface[3]. Therefore, glucosidase inhibitors may inhibit HIV infection and metastasis. Consequently we screened culture filtrates of microorganisms for inhibitory activity against β-glucosidase. Among a thousand strains of bacteria, Actinomycetes and mushrooms, we found that a culture filtrate of a mushroom strain, *Phellinus* sp., showed inhibitory activity against almond β-glucosidase. Isolation and structure determination of the active principle, established that it was a novel compound. We have named it cyclophellitol.

Materials and Methods

General

p-Nitrophenyl-β-D-glucopyranoside and almond β-glucosidase were purchased from Sigma.

NMR spectra were recorded on a Jeol JNM-GX400. The MS spectra were taken by a Hitachi M-80H spectrometer. The mp was measured by the micro mp apparatus, MP-S3 (Yanagimoto). The UV and IR spectra were measured by a Hitachi 220S and a 260-10 spectrophotometer, respectively. Optical rotations were taken by a Perkin-Elmer 241 polarimeter using micro-cell (light path 10 cm).

β-Glucosidase Assay

The enzyme activity was assayed by the method described by SAUL *et al.*[4], with slight modifications. The reaction mixture contained 25 mM sodium acetate buffer (pH 5.3), 5 mM *p*-nitrophenyl-β-D-glucopyranoside, 46 mU/ml almond β-glucosidase and the sample in 0.5 ml volume. The reaction was started by the addition of *p*-nitrophenyl-β-D-glucopyranoside. After incubation for 10 minutes at 37°C, 2.5 ml of 0.4 M glycine - NaOH buffer (pH 10.4) was added to stop the reaction, and the liberated *p*-nitrophenol was

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measured by the spectrometer at 410 nm.

Fermentation of *Phellinus* sp.

Hyphae of *Phellinus* sp. grown on a potato-glucose agar were inoculated into a medium containing glucose 1%, Polypeptone 0.5%, yeast extract 0.3%, KH_2PO_4 0.3% and $MgSO_4 \cdot 7H_2O$ 0.1%. The flask was incubated without shaking for 2 weeks at 28°C, and this preparation used as a seed culture. Then, 5 ml of the seed culture was inoculated into Sakaguchi flasks containing 125 ml of the same medium. After standing for 15 days, the flasks were shaken at 130 rpm on a reciprocal shaker at 28°C. The quantity of total reducing sugars were measured by the anthrone method[5]. The amount of cyclophellitol was calculated from the inhibitory activity of cultured broth.

Isolation of Cyclophellitol

Activated charcoal (3.15 g) was added to the culture filtrate (315 ml) and the mixture was allowed to stand for 1 hour at room temperature. After removing the charcoal by filtration, the filtrate contained 450 mg of cyclophellitol. It was passed through 24 ml of Dowex 50W-X4 column (50~100 mesh, H form). The effluent was neutralized with Dowex 1-X4 (100~200 mesh, free form) and the resin was separated by filtration with glass filter. The solution, which contained 355 mg of cyclophellitol, was evaporated, dried, and extracted with 20 ml of methanol. The extract was concentrated and kept in the refrigerator for 3 hours. The active substance (240 mg) was obtained as colorless crystals.

X-Ray Crystallographic Analysis

Crystals of cyclophellitol were grown in an aqueous solution as rather thick colorless plates. An X-ray specimen of approximate dimensions $0.20 \times 0.28 \times 0.23$ mm was cut and mounted on a Philips PW1100 diffractometer.

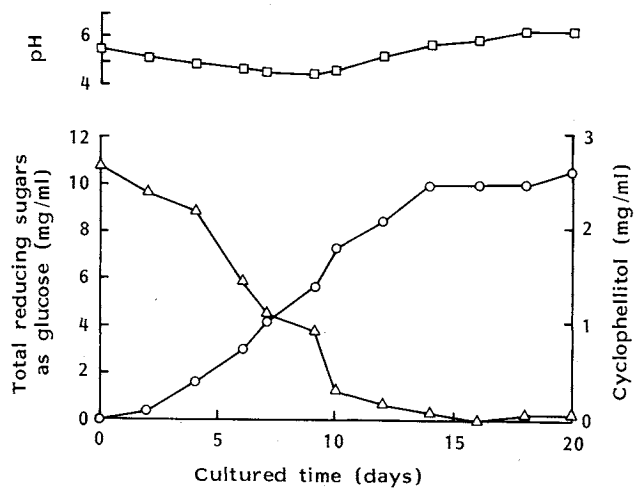
Results

Production of Cyclophellitol

Fig. 1 shows the time course of cyclophellitol production in the glucose-peptone-yeast extract medium. The pH became slightly acidic during incubation and after 10 days it began to rise slightly. Production of cyclophellitol was linearly increased to 2.5 mg/ml on day 14, as the amount of glucose decreased.

Fig. 1. The time course of *Phellinus* sp. fermentation.

Each sample was assayed for production of cyclophellitol (○), total amount of reducing sugars (△) and pH (□).



Physico-chemical Properties of Cyclophellitol

Cyclophellitol exist as colorless plates, mp 149~151°C, $[\alpha]_D^{27}$ +103° (*c* 0.5, H_2O), FD-MS *m/z* 177 (M+1), end absorption in UV spectrum, and the following absorption in IR spectrum (KBr) cm^{-1} 3400 (br), 2940, 1315, 1105, 1085, 1060, 985, 905, 830, 710. It gave a single spot at Rf 0.76 (BuOH-pyridine-AcOH-H_2O, 6:4:1:3) or at Rf 0.72 (PrOH-H_2O, 8:2) on a silica gel TLC (Merck, 60 F_{254}).

Structure of Cyclophellitol Enantiomers

The molecular formula of cyclophellitol was deduced to be $C_7H_{12}O_5$ by FD-MS and elemental analysis (Calcd for $C_7H_{12}O_5$: C 47.72, H 6.87, Found: C 47.96, H 6.87). The ^{13}C NMR spectrum of cyclophellitol showed the presence of three methine groups bearing hydroxyl groups at δ 67.4, 71.6 and 77.0, and a hydroxymethyl carbon at δ 61.2. Two methines at δ 56.7 and 56.9 suggested the presence of an epoxide ring in the molecule (Table 1). Each connectivity of ^{13}C-^1H was confirmed by the ^{13}C-^1H COSY spectrum.

The analysis of ^1H-^1H COSY spectrum revealed that cyclophellitol was a six membered ring compound. The result of ^1H NMR spectrum is shown in Table 1. The small coupling constant ($J_{1,2}$ = <0.2 Hz) shows the dihedral angle between 1-H and 2-H is nearly 90°. NOE experiment on irradiation at δ 2.12 (5-H) caused the enhancements of signal intensities of δ 3.55 (6-H) and 3.37 (3-H) arising from their spatial proximities.

From the above described data, we propose the structure of cyclophellitol as shown in Fig. 2 or its enantiomer.

Structure and Configuration of Cyclophellitol

Crystal data (Table 2) and intensities were measured using CuKα radiation monochromated by a graphite plate. A total of 1,793 reflections including 705 Friedel reflections and 211 symmetry equivalent reflections were measured within the 2θ range of 6° through 156°, which correspond to about 98% of the possible reflections in the same angular range. The R_F values for Friedel pairs and symmetry equivalent reflections were 0.021 and 0.016, respectively.

Table 1. ^{13}C and ^1H NMR data of cyclophellitol in D_2O. RM 6.50″.

Position	Chemical shifts		Coupling constants (Hz)
	δ_C[a]	δ_H[b]	
1	56.7	3.26 (1H, d)	$J_{1,6}$=3.8
2	71.6	3.78 (1H, br d)	$J_{1,2}$=<0.2
3	77.0	3.37 (1H, dd)	$J_{2,3}$=8.4
4	67.4	3.25 (1H, t)	$J_{3,4}$=10.2
5	44.2	2.12 (1H, m)	$J_{4,5}$=10.0
6	56.9	3.55 (1H, br dd)[c]	$J_{5,6}$=1.8
8a	61.2	4.00 (1H, dd)	$J_{8a,8b}$=11.3, $J_{5,8a}$=4.0
8b		3.82 (1H, dd)	$J_{5,8b}$=7.5

[a] Dioxane was used as an internal reference (δ 67.4) at 100 MHz.
[b] DOH as a reference (δ 4.8) at 400 MHz.
[c] 6-H couples with 2-H, 4-H and 8-H_a in very small *J* values.

Fig. 2. Structure of cyclophellitol.

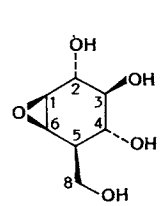


Table 2. Crystal data.

Cyclophellitol, $C_7H_{12}O_5$, MW 176.2
Crystal system: Monoclinic, space group: $P2_1$
Lattice constants: a=6.914(4)Å, b=8.513(5), c=6.850(5), β=104.29(5)°, U=390.7Å^3, Z=2.
D_x=1.497 gcm^{-3}, μ for CuKα=10.6 cm^{-1}

The crystal structure was determined by the direct method based on MULTAN[6] and refined by the method of least-squares with block-diagonal matrix approximations. Final R factor was 0.037 for 877 independent reflections (excluding Friedel reflections) in which anisotropic thermal vibrations were assumed for heavier atoms and isotropic ones for hydrogen atoms. Twelve hydrogen atoms were located on the difference electron density map and their coordinates were, at first, set at the calculated positions†.

Absolute configuration was determined by the anomalous dispersion method. The dispersion corrections for C and O atoms for CuKα radiation were taken from International Tables for X-Ray Crystallography[7]. Seven reflections for which both the Qo and Qc values (Qo=|Fo(+)|/|Fo(−)|, Qc=|Fc(+)|/|Fc(−)|) were calculated to differ by more than 4% from 1.00, and for which their ||Fo(+)|−|Fo(−)|| values were estimated to exceed σ (Fo); six reflections showed definitely the right-hand system for the coordinates. Furthermore, this conclusion was confirmed by the ENGEL's test[8].

Fig. 3. Molecular structure and packing of cyclophellitol.

Dotted lines represented hydrogen bonds and their lengths (Å).

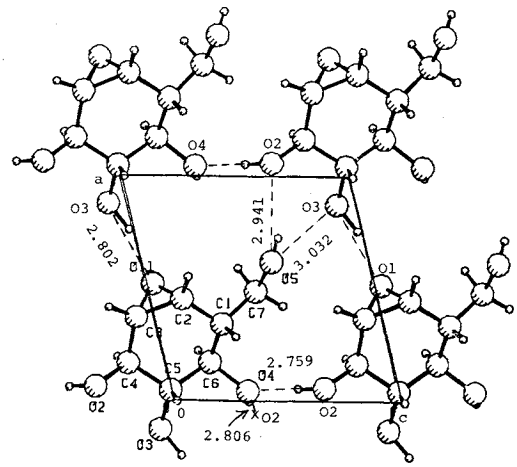


Fig. 3 shows the molecular structure drawn by PLUTO[9]. The absolute configuration was determined to be (1*S*,2*R*,3*S*,4*R*,5*R*,6*R*)-5-hydroxymethyl-7-oxabicyclo[4,1,0]heptane-2,3,4-triol (Fig. 2). The molecules are linked by several hydrogen bonds as shown in Fig. 3.

Biological Properties

Cyclophellitol inhibits almond β-glucosidase activity for 50% at 0.8 μg/ml. This value is lower than the IC_{50}s of 1-deoxynojirimycin (30 μg/ml) and of castanospermine (12 μg/ml) in our assay system. Cyclophellitol showed no antimicrobial activity, and no cytotoxicity on NIH3T3 cells, Molt-4 cells, and P388 cells at 100 μg/ml.

Discussion

Cyclophellitol has a unique cyclitol structure having an epoxide, in which the configuration of hydroxy groups in closely related to that of nojirimycin[10]. As expected, it has been shown to be a competitive inhibitor (manuscript in preparation).

It is not clear how castanospermine and 1-deoxynojirimycin suppress the HIV cytopathic effect. Having potent inhibitory activity without cytotoxicity, cyclophellitol might contribute to understanding the mechanism of infection. Sugar hydrolytic enzyme activities are involved in the mechanism of various viral diseases[11]. Cyclophellitol should be useful for studying the importance of sugar metabolism in these diseases.

† The atomic parameters, bond lengths and bond angles have been sent to the Cambridge Crystallographic Data Centre. The list of observed and calculated structure factors may be obtained from one of the authors (H.N.) upon request.

Acknowledgments

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