Panepophenanthrin, from a Mushroom Strain, a Novel Inhibitor of the Ubiquitin-Activating Enzyme

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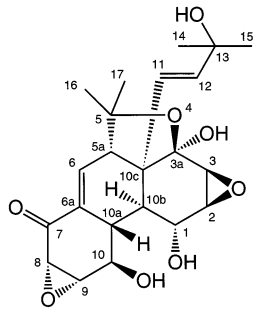
Screening for inhibitors of the ubiquitin-proteasome pathway, considered to regulate important cellular events and linked to serious diseases as well, led to isolation of a new compound, panepophenanthrin, from the fermented broth of a mushroom strain, *Panus rudis* Fr. IFO 8994. This is the first inhibitor of the ubiquitin-activating enzyme, which is indispensable for the ubiquitin-proteasome pathway. The structure of panepophenanthrin was determined by NMR and X-ray crystallographic analyses as 1,3a,10-trihydroxy-10c-(3-hydroxy-3-methylbut-1-enyl)-5,5-dimethyl-1,2,3,3a,5,5a,8,9,10,10a,10b,10c-dodecahydro-4-oxa-2,3,8,9-diepoxyacephenanthrylen-7-one.

The ubiquitin-proteasome pathway (UPP) has been determined to regulate important cellular functions via the degradation or processing of targeted proteins. Ubiquitin is first activated by a ubiquitin-activating enzyme (E1) prior to conjugating to a substrate through the ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) following degradation by proteasome.[1−3] E1 activates the C-terminal glycine of ubiquitin to an adenylated intermediate and transfers it to a thiol site in the enzyme.[4,5] This high-energy intermediate of ubiquitin is further transferred to the thiol site of an E2 and finally to the targeted protein by combination with an E3.[1] Recent observations suggested that ubiquitination of a protein is not only the tag for proteasomal degradation but also a modification state of the signal mediators. Some signal transducers are regulated by ubiquitination in response to activation signals for cellular functions.[6−10] Hence, E1 activity is indispensable for UPP and some signal transductions, and an inhibitor of E1 could be expected to block the UPP and ubiquitin functions that are linked to serious disease.[11]

To identify new microbial secondary metabolites that are biologically, as well as structurally, interesting, relevant to certain diseases or functions, and potential drug candidates, we screened the cultured broth of mushrooms, which include a variety interesting metabolic pathways,[12] for inhibition of E1, which is indispensable for the ubiquitinyl cascade. As a result of this study, we have identified a novel compound named panepophenanthrin (**1**) from the mushroom strain *Panus rudis* Fr. IFO8994. This first reported inhibitor of E1 from a natural source has a unique structure that was determined from NMR experiments and X-ray crystallographic analysis. We describe herein the isolation and structural determination of **1**, as well as its biological activity.

The UPP is initiated by the ubiquitin-activating enzyme called E1. To discover the inhibitors of the UPP process, we constructed E1-bearing *Escherichia coli* in which the human E1 gene was expressed via the pTV-118N vector and utilized the active enzyme to screen for inhibitors in microbial broths. The mushroom strain IFO8994 produced an inhibitory substance for the E1−ubiquitin complex formation, and a novel inhibitor, namely, panepophenanthrin (**1**), was isolated and identified as the first inhibitor of the ubiquitination process.



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The molecular formula of **1** was determined as $C_{22}H_{28}O_8$ from its HRFABMS. The ^1H and ^{13}C NMR data of **1** are shown in Table 1. The ^{13}C NMR and DEPT spectra indicated the presence of 22 carbons including four methyl groups, four olefinic carbons, and a carbonyl group. No methylene group was observed. On the basis of the chemical shifts in the ^{13}C NMR spectra, there were six methines and two quaternary carbons bearing oxygen atoms.

The analyses of the $^1H-^1H$ COSY spectrum suggested a partial structure composed of eight methine signals from 3-H to 8-H as shown in Figure 1. In the HMBC spectrum, the signal of 8-H was coupled to C-6a, and an olefinic proton of 6-H correlated to C-7 and C-10a. The signal of 3-H showed cross-peaks with signals for C-3a and C-10c, and 5a-H correlated with C-10b and C-10c. Other HMBC correlations were observed from 11-H to C-3a and from 10b-H to C-11. The presence of two oxirane rings was indicated by the large $^1J_{C,H}$ values of 185 Hz for C-2 (57.1 ppm) and C-3 (57.2 ppm) and 188 Hz for C-8 (55.1 ppm) observed in the HMBC spectrum. A hydroxyisopentenyl moiety was deduced from the long-range couplings from methyl groups 14 and 15. Connection of the hydroxyisopentenyl moiety at C-10c was revealed by the HMBC correlations of H-11 to C-10b, C-10c, and C-3a. A methyl signal of an oxyisopropyl (16-Me, δ_H 1.45 ppm) showed long-

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Table 1. ^{13}C and ^{1}H NMR Data of **1** in CD_3OD[a]

position	δ_C mult.	δ_H mult.	J (Hz)
1	69.0 d	4.35 br t	2.0, 2.0
2	57.1 d	3.50 br t	4.0, 4.0
3	57.2 d	3.32 d	4.0
3a	102.6 s		
5	79.2 s		
5a	57.3 d	3.35 dd	2.0, 5.0
6	139.9 d	6.81 dd	3.0, 5.0
6a	138.8 s		
7	196.2 s		
8	55.1 d	3.42 d	4.0
9	60.7 d	3.84 br t	4.0, 4.0
10	66.2 d	4.55 br t	2.0, 2.0
10a	51.2 d	2.33 m	10.0, 2.0, 2.0, 2.0, 3.0
10b	50.0 d	2.03 br d	10.0, 2.0, <1
10c	55.6 s		
11	129.2 d	5.99 d	16.0
12	143.0 d	5.68 d	16.0
13	71.8 s		
14	29.4 q	1.17 s	
15	30.3 q	1.20 s	
16	26.1 q	1.45 s	
17	32.3 q	1.35 s	

[a] Chemical shifts were determined in ppm.

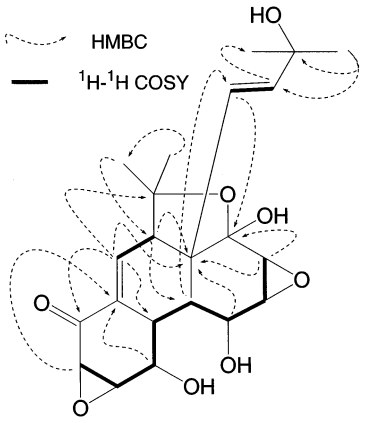

Figure 1. Two-dimensional correlation of **1**. The summarized results from $^{1}H-^{1}H$ COSY (bold) and HMBC experiments (dashed arrows) are shown.

range coupling with C-5a. The remaining unsaturation, deduced from the molecular formula, could be accounted for by an ether bond between C-5 (79.2 ppm) and C-3a (102.6 ppm) via an oxygen atom of the oxyisopropyl group. Thus, from analyses of the NMR data, **1** was deduced to be a derivative of hexadecahydroacephenanthrylene.

The conformations of rings A, B, and C were suggested as boat forms, based on the results of the NOESY and 1D NOE difference experiments (1-H/10-H, 1-H/10a-H, 10a-H/5a-H, 10b-H/12-H, indicated in Figure 2) and the large coupling constant between 10a-H and 10b-H (J = 10 Hz). The geometry of the double bond at C-11 was determined to be 11*E* by the large coupling constant ($J_{11,12}$ = 16.0 Hz).

The structure of **1** crystallized from a mixture of hexane−EtOAc at room temperature was confirmed by X-ray crystallographic analysis. The ORTEP drawing of **1** is shown in Figure 3. The result confirmed the relative structure of **1** as 1,3a,10-trihydroxy-10c-(3-hydroxy-3-methylbut-1-enyl)-5,5-dimethyl-1,2,3,3a,5,5a,8,9,10,10a,10b,10c-dodecahydro-4-oxa-2,3,8,9-diepoxyacephenanthrylen-7-one.

The effects of **1** on the formation of the E1−ubiquitin complex are shown in Figure 4. The 120 kDa spot disappeared with the addition of the compound in a dose-dependent manner. The IC_{50} value was 17.0 μg/mL from a

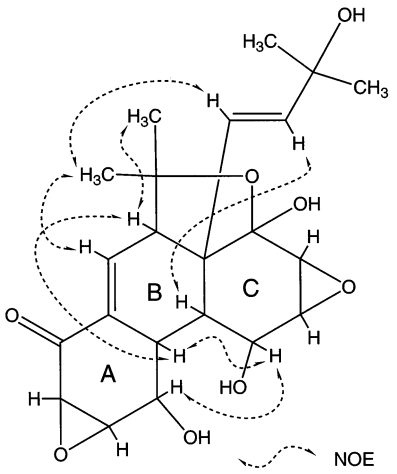

Figure 2. Summary of NOE experiments of **1**. Observed signals are indicated by dashed arrows.

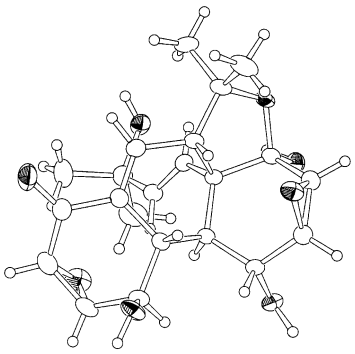

Figure 3. X-ray molecular structure of **1**.

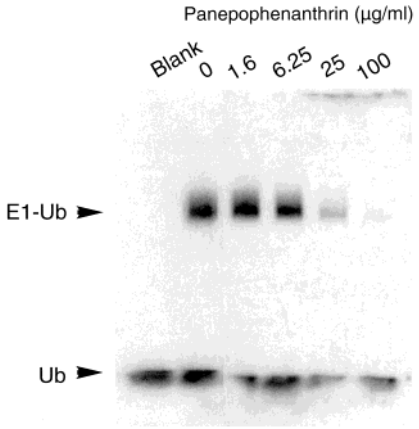

Figure 4. Inhibition of E1−ubiquitin complex formation by **1**. Recombinant human E1 and biotinylated ubiquitin were allowed to react with the indicated concentration of **1**. The reaction mixture was separated on an SDS-7%PAGE, and the biotin was traced by the ECL method.

densitometric analysis. Here, it is expected that E1 and ubiquitin should form a complex of which the molecular mass is about 120 kDa. Thus, the inhibition to the binding of ubiquitin and E1 should be observed as disappearance of the 120 kDa spot in this assay system. Actually, two spots around 120 and 7 kDa were detected on the lane for each reaction mixture. While the 7 kDa spots were considered to be the free biotinylated ubiquitin, the 120 kDa spots appeared in a time-dependent manner and the maximum signal was observed at the time point of less than a 30 min reaction. When nonlabeled ubiquitin was added to the reaction mixture, the outward inhibition was

observed as the disappearance of the 120 kDa spots in a dose-dependent manner (data not shown).

Although **1** inhibited the binding of ubiquitin to E1 in vitro, no significant inhibitory effect was observed in intact cells up to the concentration of 50 μg/mL. The cellular effects should be investigated further.

Experimental Section

General Experimental Procedures. The melting point was determined with a Yanagimoto micro-melting point apparatus, and the temperature is uncorrected. Optical rotation was measured with a Perkin-Elmer 241 polarimeter. The mass spectrum was obtained using a JEOL JMS-SX102 spectrometer. All NMR spectra were recorded using a JEOL JNM-A500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) in CD_3OD.

Primers for cloning the E1 gene were custom synthesized by Amersham-Biosciences.

Forward: 5′-CCCCATGGCCAGCTCGCCGCTGTCCAAGAA-3′

Reverse: 5′-GGGGATCCTCAGCGGATGGTGTATCGGACA-3′

Ubiquitin (Sigma, U6253) was biotinylated by the ECL protein biotinylation module (Amersham-Biosciences, RPN 2202).

Preparation of E1. A cDNA library was constructed from U937 cells, and the cDNA for the human E1 was cloned by using the specific primer pair described above. An amplified fragment (3200 bp) was digested and sequenced and was determined to code the human E1 gene.[13] This fragment was inserted into the *Nco* I-*Bam* HI site of the pTV118N vector, termed pTV-E1, and transfected into *E. coli* BL21.

The E1 protein was induced by the addition of IPTG (1 mM) to the culture of the pTV-E1 transfected BL21 when the OD_{595} of the culture reached 0.5. After 4 h, E1 was purified from the lysate of the bacterial cells. The purification procedure is as follows. The cells from a 500 mL culture were collected by centrifugation and incubated in 50 mL of 1% Triton X-100 and 0.5 mM EDTA containing Pi-buffer (3 mM potassium phosphate, pH 7.4, 1 mM DTT) for 30 min on ice. Further centrifugation (15000*g*, 20 min) was performed, and the supernatant was charged on a DEAE-Sephacel column (30 mm $\Phi \times$ 20 mm) equilibrated with Pi-buffer. The column was washed and eluted by 200 mL of KCl linear gradient from 0 to 0.5 M in Pi-buffer. Fractions containing the E1 protein were collected and desalted by Amicon, divided into running aliquots, and stored at −20 °C.

Detection of E1 Activity. Ten microliters of the E1 fraction described above was added to 10 μL of the reaction buffer (100 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$, 1 mM DTT) containing 50 ng of biotinylated ubiquitin and 2.5 mM ATP. This reaction mixture was incubated at 37 °C for 15 min, and the reaction was stopped by boiling with Laemmli's sample buffer (LSB). Fifteen microliters of mixture was loaded on an SDS-7% polyacrylamide gel, and electrophoresis was done under nonreducing conditions. The proteins were electrically transferred to a PVDF membrane (Millipore). The membrane was blocked and incubated with streptavidin-linked horseradish peroxidase to trace the biotinylated ubiquitin by the enhanced chemiluminescence method. The spot around 120 kDa was determined as the complex of E1 and the biotinylated ubiquitin.

Fermentation and Isolation of 1. The strain of *Panus rudis* Fr. IFO8994 grown on a slant culture (potato dextrose-agar, Difco) was inoculated into a 500 mL Erlenmeyer flask containing 200 mL of medium consisting of 1% glucose, 0.5% peptone, 0.3% KH_2PO_4, and 0.05% $MgSO_4$. After 3 days of stationary culture, the flask was shaken for 2 days at 27 °C. Seven milliliter portions of the culture were inoculated into 500 mL Erlenmeyer flasks containing 200 mL of the same medium. The growth was cultured for 14 days without shaking at 27 °C, and the mycelia were removed by filtration.

The filtered broth (9.2 L, pH 4.8) was applied to a Diaion HP-20 column (55 mm $\Phi \times$ 250 mm) and eluted with a mixture of MeOH−H_2O (1:1). Fractions containing **1** were evaporated and extracted with EtOAc. The organic layer was dried under reduced pressure to give 4.1 g of an oily residue. This material was charged on a silica gel column (35 mm $\Phi \times$ 100 mm) and chromatographed with the mixture of $CHCl_3$−MeOH (from 50:1 to 25:1 v/v) to give 550 mg of a residue mainly containing **1**. Further chromatography on a silica gel column (30 mm $\Phi \times$ 35 mm) was repeated with the solvent system $CHCl_3$−MeOH (20:1), and then, 135 mg of **1** was precipitated in $CHCl_3$ with a small amount of MeOH.

Physicochemical Properties. 1 was obtained as colorless needles (MeOH−$CHCl_3$): mp 144−146 °C; $[\alpha]_D^{26}$ +149.8° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 255 (4.20) nm, UV (MeOH−0.1 N HCl) λ_{max} (log ϵ) 258 (3.40) nm, UV (MeOH−0.1 N NaOH) λ_{max} (log ϵ) 255 (4.20) nm; IR ν_{max} (KBr) 2978, 1676, 1597, 1338, 1142, 997 cm^{-1}; HRFABMS *m*/*z* 419.1719 (calcd for $C_{22}H_{27}O_8$, 419.1706); TLC R_f 0.42 (Merck Art. 105715, $CHCl_3$−MeOH, 5:1).

X-ray Diffraction Analysis of 1. Suitable crystals were obtained from a solution in hexane−EtOAc. Crystal data for **1**: colorless crystal (0.05 × 0.08 × 0.25 mm); formula $C_{22}H_{28}O_8 \cdot 1/2C_4H_8O_2$, fw = 464.51, monoclinic, space group $P2_1$ with a = 9.708(1) Å, b = 20.008(1) Å, c = 11.894(1) Å, β = 90.424(9)°, V = 2310.4(4) Å^3, Z = 4, D_{calcd} = 1.34 g/cm^3, $F(000)$ = 992.00, μ = 8.5 cm^{-1}, λ(Cu Kα) = 1.5418 Å, 4324 measured intensities, 4051 unique (R_{int} = 0.022). The intensity data were collected on a Rigaku AFC7R diffractometer using graphite-monochromated Cu Kα radiation and a rotating anode generator with the $\omega-2\theta$ scan technique to a maximum 2θ of 130.1°. Cell constants were refined from 25 well-centered reflections in the range 41.63° < 2θ < 48.38°. The structure was solved by direct methods using SIR92[14] and refined by full-matrix least-squares refinement. The final least-squares cycle was based on 4051 reflections and 595 variable parameters and converged to $R(F^2)$ = 0.076, $R_w(F^2)$ = 0.123, and R_1 = 0.043.[15]

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(15) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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