Production, Isolation, and Identification of Dihydroepiepoformin as an IL-1 Receptor Antagonistic Component in *Penicillium patulum*

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In the course of screening soil microorganisms for interleukin-1 (IL-1) receptor binding antagonists[1], we discovered that *Penicillium patulum* produced a number of bioactive metabolites. Bioassay guided fractionation of fermentation beer led to the identification of gentisyl alcohol[2] and epiepoformin[3] (EEF), two metabolites reported previously to be made by this microorganism. A new metabolite, dihydroepiepoformin (DHEEF), was discovered when HP-20 resin was added to the fermentation in an attempt to increase the titer of EEF. This new metabolite was also found to have antagonistic activity for the IL-1 receptor. This report describes the isolation, identification, and IL-1 inhibitory activity of this newly discovered metabolite.

Materials and Methods

Fermentation of *P. patulum*

P. patulum, UC 4223, was obtained from the culture collection of The Upjohn Company. The organism was inoculated into a seed medium (GS-7) which contained Cerelose and Pharmamedia each added at 25 g/liter of tap water. The seed medium was adjusted to pH 7.2 with NH_4OH, and was autoclaved for 30 minutes. The inoculated 100 ml volumes of GS-7 were shaken in wide-mouth 500 ml fermentation flasks at 250 rpm for 72 hours at 28°C. The mature seed cultures were used as the source of inoculum (3% seed rate) for the fermentation medium (Medium A) containing washed HP-20 (Mitsubishi Chemicals, Ltd.) resin at 80 g/liter. HP-20 resin was employed as described previously[4]. Medium A also contained Wilson's Liquid Peptone (20 ml), Cerelose (20 g), malt extract (15 g), and K_2HPO_4 (3 g) per liter of tap water. This medium was adjusted to pH 6.8 with HCl and was autoclaved for 30 minutes. Inoculated Medium A containing resin was employed in the manner described for GS-7 for 4 days of fermentation.

Isolation of DHEEF

The isolation procedures used for EEF and DHEEF are described briefly as follows. Filtered beer was extracted twice with an equal volume of ethyl acetate. The organic layer after concentration was dissolved in MeOH/water and passed through a column of Diaion HP-20. The column was eluted with a stepwise water/MeOH gradient. Active fractions were combined, freeze-dried, and dry-packed to a silica column. Elution with a CH_2Cl_2/MeOH gradient resolved the activity into gentisyl alcohol and mixture of EEF and DHEEF. The separation of DHEEF from EEF was achieved by using preparative HPLC (Waters Prep 3000) procedures. The mixture was loaded onto a C-18 preparative radial compression cartridge (2.5 × 10 cm) *via* a 5 ml loop. The column was then developed isocratically with 15% MeOH in water for 15 minutes. The flow rate was 4.5 ml/minute. Since DHEEF does not have a UV chromophore, the fractions were collected with a ISCO 200 fraction collector using constant volume mode and peak separator. The fractions near the EEF peak were then analyzed by proton NMR (300 MHz, Bruker AM-300). The peak fraction of DHEEF was estimated to be 90% pure by measuring the NMR signal intensities.

NMR Spectroscopy Studies

One dimensional H-1 and C-13 NMR spectra were obtained with a Bruker AM-300 spectrometer. The sample was dissolved in deuterated methanol. Two-dimensional ^1H-^1H and ^1H-^{13}C COSY as well as phase-sensitive NOE spectra were obtained using a Varian XL-300 spectrometer. The ^1H-^1H spectra (COSY and NOE) were obtained with 256 increments and zero-filled to 1 K. The ^1H-^{13}C COSY spectrum was obtained with 512 increments and zero-filled to 1 K in the F1 dimension. Standard pulse sequences were used in all experiments. The proton coupling constants were obtained by analyzing the HMR spectrum with various decouplings.

IL-1 Receptor Binding Activity

The IL-1 receptor binding antagonist (IL-1 RBA) assay was performed as described previously[1]. The assay was based on the comparison of the binding of the labeled IL-1 to its receptor present in the YT-NCI cell with and without the presence of an inhibitor. Thus, EEF and DHEEF were dissolved in phosphate-buffered saline containing 1% DMSO at 2 mg/ml and 0.62 mg/ml, respectively. These solutions were tested along with eleven two-fold serial dilutions in the same buffer; EEF samples were tested in duplicate. Assay mixtures contained one million YT-NCI cells, 50 pM ^{125}I-IL-1, and 50 μl sample in a total volume of 200 μl. Control samples containing 50 nM unlabeled IL-1 were included to determine nonspecific binding. Mixtures were incubated 1 hour at room temperature with shaking, and unbound label was removed by filtration with a Skatron cell harvester. Filters were counted by liquid scintillation with an LKB Betaplate counter.

Results and Discussion

It has been demonstrated previously that the addition of resins capable of adsorption of metabolites during fermentations can improve the titers of these metabolites[4]. In this study, HP-20 resin was used since it was shown previously that EEF was reversibly adsorbed to this non-ionic resin. To compare the titers between the resin supplemented fermentation and the control, each broth was identically extracted and partially purified to remove gentisyl alcohol and then analyzed by a TLC assay. The results indicated that the titers from the resin process sample was approximately four times of that of the control sample which is estimated to be approximately 15 mg/ml. TLC analysis of the two samples also indicated that neither sample contained gentisyl alcohol. To obtain pure EEF, the two crude samples were combined and purified as described in Materials and Methods. However, a minor component was co-chromatographed with EEF as indicated by ^1H and ^{13}C NMR spectra. This minor component was never observed during the course of previous work-up of EEF. We attributed this new discovery to the HP-20 resin which may have stabilized this metabolite.

In order to elucidate the structure of the new metabolite, the new metabolite was then studied by NMR. The chemical shift and multiplicity of each NMR signal due to the minor component are listed in Table 1.

In reviewing the NMR data, it can be easily concluded that the new component contains an aliphatic methyl, methylene, and methine, a carbinol, an epoxy, and a ketone functional group. In contrast to EEF, the minor component does not have a C–C double bond as no olefinic carbon signals are detected. The hydroxyl and epoxy functional groups are still present in the molecule as evidenced by the proton signals at 4.30, 3.53, and 3.28 ppm. Furthermore, the latter two proton signals have a coupling constant of 3.7 Hz, which is characteristic of an epoxy functional group. A two-dimensional ^1H COSY experiment further established that the methyl, methine, methylene, and carbinol, are linked in sequence as would be expected for EEF. Although no cross peaks were observed between carbinol and the adjacent epoxy protons in the COSY spectrum due to zero coupling constant between them, the connection of the epoxy moiety to the carbinol carbon is deduced from the long-range coupling ($J_4 = 1.7$ Hz) between the equatorial methylene proton and the epoxy proton. The ketone group is placed between the methine and the epoxy function group as in EEF. This is supported by the fact that the methine proton is strongly deshielded and one of the epoxy protons is strongly shielded. The structure of the new metabolite is therefore elucidated as 5,6-epoxy-4-hydroxy-2-methyl-cyclohexanone, the dihydro analogue of EEF as shown in Fig. 1.

The stereochemistry of the methyl group of DHEEF was then investigated using ^1H NMR coupling constants and two-dimensional NOE experiments. The large coupling constants observed for H-2 ($J_{2,3ax} = 11.8$ Hz) and H-4 ($J_{3ax,4} = 8.4$ Hz) implied that H-2 and H-4 are axial and pseudo-axial protons, respectively. The methyl and hydroxy group, consequently, possess the equatorial positions. Assuming that DHEEF is derived from EEF whose hydroxyl group is *trans* to the epoxy group, the methyl group of DHEEF is therefore *cis* to the hydroxyl group and *trans* to the epoxide group. The complete structure of DHEEF is therefore elucidated as (*R*,*R*)-5,6-epoxy-(*S*)-4-hydroxy-(*S*)-2-methyl-cyclohexanone. In agreement with this structure are the results from 2D NOE experiment. The cross-peak intensities in the spectrum indicate that the internuclear distance is smallest between H-4 and H-3_{eq}, followed by H-4 and H-2, and then H-4 and H-3_{ax}.

The conformation of DHEEF can also be derived from the coupling constants. The lack of couplings between the carbinol proton (H-4) and the epoxy (H-5) proton suggests that the dihedral angle between the two is nearly 90°. In support of this conclusion are the values of $J_{3ax,4}$ and $J_{4,3eq}$ which imply that the dihedral angle between H-3_{ax} and H-4 is approximately 160° and 30°, respectively. The conformation of DHEEF can be deduced as a slightly twisted half-chair form with the methyl-bearing carbon being puckered downward and the methylene group benting slightly upward, as shown in Fig. 2.

Table 1. ^1H and ^{13}C NMR data of dihydroepiepoformin.

Carbon No.	^1H NMR Chemical shift in PPM (coupling constant in Hz)	^{13}C NMR Chemical shift (multiplicity)
1		196.0 (s)
2	2.71 (dqd, J=11.8, 6.7, 5.8)	35.6 (d)
3	ax 1.50 (ddd, J=13.5, 11.8, 8.7),	
	eq 2.28 (dtd, J=13.5, 5.8, 1.7)	42.1 (t)
4	4.30 (dd, J=8.4, 5.9)	65.5 (d)
5	3.53 (dd, J=3.8, 1.7)	64.5 (d)
6	3.28 (d, J=3.8)	56.2 (d)
7	0.90 (d, J=6.7)	15.0 (q)

Fig. 1. Structure of dihydroepiepoformin.

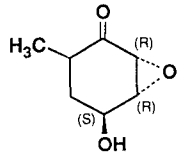
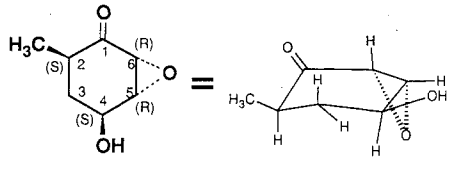


Fig. 2. Stereostructure of dihydroepiepoformin.



From the dose-response curve (data not shown) it was determined that DHEEF has IL-1 inhibition activity similar to that of EEF with an IC_{50} value 100 μg/ml.

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