VOL. 57 NO. 9, SEPT. 2004 THE JOURNAL OF ANTIBIOTICS pp. 564 — 568

Epohelmins A and B, Novel Lanosterol Synthase Inhibitors from a Fungal Strain FKI-0929

Yuichi Sakano[a], Masaaki Shibuya[a], Yuichi Yamaguchi[b], Rokuro Masuma[b], Hiroshi Tomoda[b], Satoshi Ōmura[b] and Yutaka Ebizuka[a]

[a] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[b] Kitasato Institute for Life Sciences, Kitasato University, and The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

(Received for publication May 10, 2004)

From a fungal strain FKI-0929, two compounds designated epohelmins A and B, were isolated as new natural products with inhibitory activity against recombinant human lanosterol synthase. The crude extract from the whole broth of this strain was fractionated by silica gel column chromatography and HPLC to afford two isolated inhibitors. Detailed spectroscopic analyses led to the identification of their structures. They are diastereomers of 4,5-epoxy-2-(4′-oxoundec-(5′*E*)-enyl)-heptamethylenamines, and their relative stereochemical configurations were determined as (2*R*, 4*R*, 5*R*) or (2*S*, 4*S*, 5*S*) for epohelmin A, and (2*R*, 4*S*, 5*R*) or (2*S*, 4*R*, 5*S*) for epohelmin B, respectively. These compounds inhibited recombinant human lanosterol synthase with IC_{50} values of 10 and 6.0 μM, respectively.

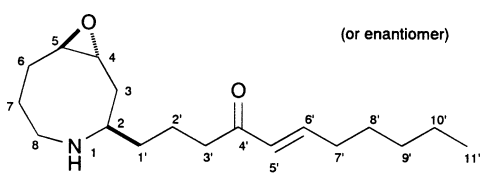
Lanosterol synthase inhibitors have a potential to reduce a risk of hypercholesterolemia. In the course of our screening project for recombinant human lanosterol synthase inhibitors, lanopylins A_1, B_1, A_2 and B_2 were isolated from *Streptomyces* sp. K99-5041, one of the 1,031 screened actinomycete strains[1]. Further screening of 280 fungal strains led us to find two new active compounds, designated epohelmins A and B (Fig. 1), from an unidentified fungus FKI-0929. The taxonomy and fermentation of the producing strain, compound isolation, biological properties, and structure elucidation of epohelmins A and B are described in this paper.

Materials and Methods

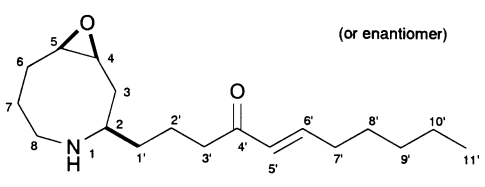
General

NMR spectra were recorded on a JEOL JNM-alpha 500 spectrometer. Mass spectrometry analysis utilized a JEOL JMS-SX102A spectrometer. UV and IR spectra were measured with a Hitachi U-2000 spectrophotometer and a Horiba FT-210 infrared spectrometer, respectively. Optical rotations were obtained with a JASCO P-1010 polarimeter. Preparative HPLC was carried out using the Tosoh CCPE-II pump under the following conditions: column, TSK gel ODS-80T_M (7.8×300 mm, Tosoh); eluent, 45% aq. MeOH containing 0.5% AcOH; flow rate, 1.5 ml/minute;

Fig. 1. Structures of epohelmins A and B.



epohelmin A



epohelmin B

* Corresponding author: yebiz@mol.f.u-tokyo.ac.jp

monitoring, UV absorbance at 254 nm.

Taxonomic Studies of the Producing Organism

The strain FKI-0929 was isolated from a soil sample collected at Miyakojima Island, Japan. Morphological observations were done under a microscope (Olympus Vanox-S AH-2).

Fermentation Media

The seed medium was composed of 2.0% glucose, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% polypeptone, 0.1% KH_2PO_4 and 0.1% agar in tap water (pH 6.0 prior to sterilization). The production medium in each of eleven 1-liter Roux flasks was composed of 150 g Vialone nano rice (Nichioushouji) and trace elements (each 0.9 mg $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 6H_2O$) in 90 ml tap water.

In Vitro Assay for Human Lanosterol Synthase

The procedures of preparation of cell-free extracts from recombinant *Saccharomyces cerevisiae* expressed human lanosterol synthase, preparation of [^{14}C] (3*S*)-2,3-oxidosqualene and the *in vitro* assay were described in the previous paper[1].

Results and Discussion

Taxonomy of the Producing Organism

For the taxonomic studies of the fungus, potato dextrose agar (PDA), malt extract agar, potato carrot agar, oat meal agar and Miura's medium were used. Colonies grown on each medium were 58～68 mm in diameter after incubation at 25°C for a week. This strain grew moderately to form white to brownish white colonies which were floccose. Hyphae were hyaline to light brown, septate and 2～4.5 μm in diameter. This strain has produced neither teleomorphic nor anamorphic structures on various media after incubation 25°C for over a month. From the lack of definitive characteristics, it was concluded that fungal strain might be classified as a member of the Agonomycetes (Mycelia sterilia). The fungus has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM P-19147.

Fermentation

A slant culture of the strain FKI-0929 grown on PDA was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium. The flask was shaken on a rotary shaker at 27°C for 3 days. The seed culture (2 ml) was transferred into each of eleven 1-liter Roux flasks containing the production medium. The stationary fermentation was carried out at 27°C for 14 days.

Isolation and Biological Properties

The cultured broth (3.0 kg) was extracted with 50% EtOH, concentrated and partitioned with $CHCl_3$ twice. The organic layer was combined, dried over Na_2SO_4 and concentrated *in vacuo* to dryness to afford 2.7 g of brown oily material. The crude extract was subjected to silica gel column chromatography (Wakogel® C-200, Wako) with a solvent system comprising $CHCl_3$ - MeOH - AcOH. Activity was concentrated to a single spot with Rf ～0.3 on TLC ($CHCl_3$ - MeOH - AcOH (60 : 10 : 1)) to yield 69.6 mg of oily material.

The two compounds, epohelmins A and B, were purified from the fraction by preparative HPLC. Epohelmins A and B were eluted at 15 and 17 minutes, respectively. The eluates were concentrated to dryness to yield colorless oils of epohelmin A (1.5 mg) and epohelmin B (2.9 mg). The IC_{50} values (10, 6.0 μM, respectively) of these compounds are on the order of those measured for lanopylins A_1, B_1, A_2 and B_2 (15, 18, 33 and 41 μM, respectively)[1].

Structure Elucidation

Physico-chemical properties of the two compounds are summarized in Table 1. HR-FABMS analysis revealed that they have identical molecular formulae of $C_{18}H_{31}O_2N$.

^{1}H NMR, ^{13}C NMR, DEPT, and HMQC spectra indicated the presence of one quaternary carbon, five methines, eleven methylenes and one methyl group in epohelmin A. Taken together with other NMR data, the structure of epohelmin A was finally determined to be 4,5-epoxy-2-(4′-oxoundec-(5′*E*)-enyl)-heptamethylenamine (Fig. 2). NMR assignment of this compound is shown in Table 2. The evidences were as follows;

1) The presence of a 4,5-epoxyheptamethylenamine moiety was identified by the following NMR data: the ^{1}H-^{1}H couplings between H-2 (δ_H 2.99) and H-3 (δ_H 2.06), between H-3 and H-4 (δ_H 4.17), between H-4 and H-5 (δ_H 4.11), between H-5 and H-6 (δ_H 2.26 and 1.72), between H-6 and H-7 (δ_H 2.04), and between H-7 and H-8 (δ_H 3.43 and 2.96), and the ^{1}H-^{13}C long-range couplings from H-2 to C-4 (δ_C 74.3), from H-3 to C-2 (δ_C 67.3), from H-4 to C-6 (δ_C 28.4), from H-5 to C-2 and C-4, from H-6 to C-4, C-5 (δ_C 73.9), C-7 (δ_C 24.2) and C-8 (δ_C 51.9), from H-7 to C-

Table 1. Physico-chemical properties of epohelmins A and B.

Epohelmin A
Appearance colorless oil
Molecular weight 293
Molecular formula $C_{18}H_{31}O_2N$
EIMS *m/z* (% rel. int.) 293 $[M]^+$ (8), 126 (100, epoxyheptamethylenamine moiety), 96 (29), 70 (19)
HR-FABMS *m/z* 294.2460 $[M+H]^+$, Δ +2.7 mmu
$[\alpha]_D^{22}$ +22 ° (*c* 0.14, MeOH)
UV λ_{max}^{MeOH} nm (ε) 224 (9,300)
IR ν_{max} (KBr) cm^{-1} 3437, 2956, 2931, 2872, 1662, 1633, 1566, 1458, 1408, 1267, 1107, 1024
TLC $CHCl_3$-MeOH-AcOH (60:10:1) Rf~0.3, H_2SO_4(+)

Epohelmin B
Appearance colorless oil
Molecular weight 293
Molecular formula $C_{18}H_{31}O_2N$
EIMS *m/z* (% rel. int.) 293 $[M]^+$ (19), 126 (100, epoxyheptamethylenamine moiety), 96 (69), 70 (31)
HR-FABMS *m/z* 294.2452 $[M+H]^+$, Δ + 1.9 mmu
$[\alpha]_D^{22}$ +25 ° (*c* 0.28, MeOH)
UV λ_{max}^{MeOH} nm (ε) 224 (11,800)
IR ν_{max} (KBr) cm^{-1} 3431, 2958, 2873, 2819, 1666, 1628, 1574, 1404, 1335, 1213, 1028
TLC $CHCl_3$-MeOH-AcOH (60:10:1) Rf~0.3, H_2SO_4(+)

Table 2. NMR data of epohelmins A and B.

	epohelmin A			epohelmin B	
Position	^{13}C δ ppm	^1H δ ppm (*J* in Hz)	Position	^{13}C δ ppm	^1H δ ppm (*J* in Hz)
1			1		
2	67.3	2.99 (1H, m)	2	66.7	3.30 (1H, m)
3	40.9	2.06 (2H, m)	3	42.3	2.22 (1H, m)
					2.10 (1H, m)
4	74.3	4.17 (1H, td, 6.3, 3.0)	4	70.2	4.38 (1H, br s)
5	73.9	4.11 (1H, td, 8.3, 3.3)	5	70.8	4.47 (1H, br t, 4.3)
6	28.4	2.26 (1H, m)	6	24.1	2.21 (1H, m)
		1.72 (1H, m)			1.88 (1H, m)
7	24.2	2.04 (2H, m)	7	26.8	2.04 (2H, m)
8	51.9	3.43 (1H, dt, 12.0, 7.8)	8	52.9	3.50 (1H, m)
		2.96 (1H, dt, 12.0, 6.0)			2.88 (1H, dt, 11.0, 6.0)
1'	31.2	1.92 (1H, m)	1'	30.4	1.96 (1H, m)
		1.82 (1H, m)			1.81 (1H, m)
2'	21.1	1.64 (2H, ddt, 7.7, 7.7, 7.7)	2'	21.3	1.65 (2H, ddt, 7.5, 7.5, 7.5)
3'	39.1	2.60 (2H, t, 7.0)	3'	39.3	2.61 (2H, td, 7.1, 2.1)
4'	199.6		4'	199.8	
5'	130.1	6.07 (1H, dt, 16.0, 1.5)	5'	130.2	6.07 (1H, dt, 15.5, 1.5)
6'	148.2	6.83 (1H, dt, 15.7, 6.9)	6'	148.1	6.84 (1H, dt, 16.0, 7.0)
7'	32.5	2.21 (2H, tdd, 7.0, 7.0, 1.5)	7'	32.5	2.21 (2H, tdd, 7.0, 7.0, 1.5)
8'	27.7	1.47 (2H, tt, 7.4, 7.4)	8'	27.7	1.46 (2H, tt, 7.4, 7.4)
9'	31.3	1.31 (2H, m)	9'	31.3	1.30 (2H, m)
10'	22.4	1.29 (2H, m)	10'	22.4	1.32 (2H, m)
11'	13.9	0.90 (3H, t, 7.0)	11'	13.9	0.89 (3H, t, 7.0)

^1H (500.00 MHz) and ^{13}C (125.65 MHz) NMR spectra were obtained in chloroform-*d*.

Fig. 2. Structure elucidation of epohelmin A by ^{1}H-^{1}H COSY and HMBC experiments.

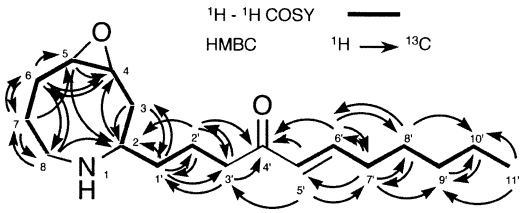
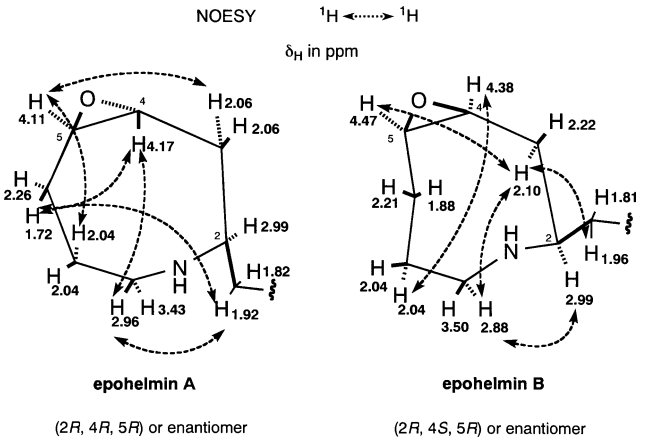


Fig. 3. Analyses of the relative stereochemical configuration of epohelmins A and B by NOESY experiments.



5, C-6 and C-8, and from H-8 to C-2, C-6 and C-7. The NMR data of this moiety in known synthetic compounds[2,3] support this structure. To the best of our knowledge, this is the first report of the isolation of natural products with this moiety.

2) Attachment of the 4′-oxoundec-(5′*E*)-enyl side chain at the position 2 was indicated by the following facts: the ^{1}H-^{1}H couplings between H-2 and H-1′ (δ_H 1.92 and 1.82), between H-1′ and H-2′ (δ_H 1.64), between H-2′ and H-3′ (δ_H 2.60), between H-5′ (δ_H 6.07) and H-6′ (δ_H 6.83) ($^{3}J_{H\text{-}H}$=16.0 Hz), between H-5′ and H-7′ (δ_H 2.21), between H-6′ and H-7′, between H-7′ and H-8′ (δ_H 1.47), between H-8′ and H-9′ (δ_H 1.31), between H-9′ and H-10′ (δ_H 1.29), and between H-10′ and H-11′ (δ_H 0.90), and the ^{1}H-^{13}C long-range couplings from H-3 to C-1′ (δ_C 31.2), from H-1′ to C-2, C-3, C-2′ (δ_C 21.1), and C-3′ (δ_C 39.1), from H-2′ to C-2, C-1′, C-3′ and C-4′ (δ_C 199.6), from H-3′ to C-1′, C-2′ and C-4′, from H-5′ to C-3′, C-4′ and C-7′ (δ_C 32.5), from H-6′ to C-4′, C-7′ and C-8′ (δ_C 27.7), from H-7′ to C-5′ (δ_C 130.1), C-6′ (δ_C 148.2), C-8′ and C-9′ (δ_C 31.3), from H-8′ to C-6′, C-7′ and C-10′ (δ_C 21.4), from H-9′ to C-10′, from H-10′ to C-9′, and from H-11′ to C-9′ and C-10′. This is also supported by the observation of the signals at *m/z* 293 ($[M]^{+}$) and 126 (base peak) in the EIMS spectrum (Table 1).

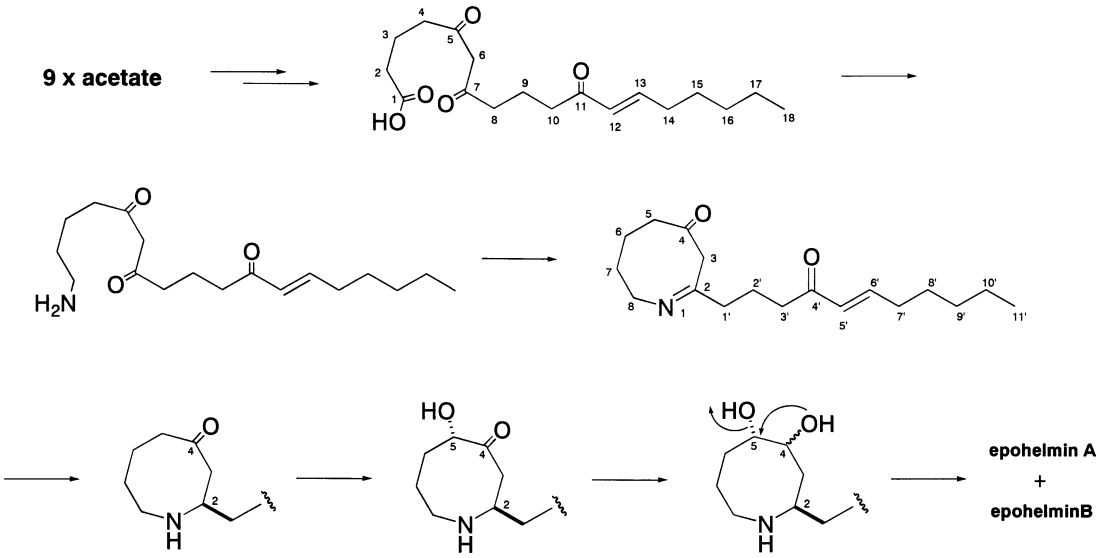
3) The relative stereochemical configuration of the 4,5-epoxyheptamethylenamine backbone was elucidated to be (2*R*, 4*R*, 5*R*) or (2*S*, 4*S*, 5*S*) by the coupling constant between H-4 and H-5 ($^{3}J_{H\text{-}H}$=3.0 Hz) and the NOEs among H-3, H-5 and H-7, and among H-4, H-6, H-8 and H-1′ as presented in Fig. 3.

In the same manner, the planer structure of epohelmin B was finally elucidated to be identical to that of epohelmin A. NMR assignment of epohelmin B is also presented in Table 2. The different chemical shifts at positions 2, 3, 4, 5 and 6 in epohelmin B from those of epohelmin A suggested that the compounds are diastereomers. The relative stereochemical configuration of epohelmin B was finally elucidated to be (2*R*, 4*S*, 5*R*) or (2*S*, 4*R*, 5*S*) by the coupling constant between H-4 and H-5 ($^{3}J_{H\text{-}H}$=4.3 Hz) and the NOEs among H-2, H-3, H-4, H-5, H-7, H-8 and H-1′ as presented in Fig. 4.

As described above, two diastereomers of, 4,5-epoxyheptamethylenamine derivatives (Fig. 1) have been isolated from a fungal strain, FKI-0929, as new lanosterol synthase inhibitors and their planer structures elucidated to be 4,5-epoxy-2-(4′-oxoundec-(5′*E*)-enyl)-heptamethylenamines by spectroscopic method. Their relative stereochemical configurations were determined to be (2*R*, 4*R*, 5*R*) or (2*S*, 4*S*, 5*S*) for epohelmin A, and (2*R*, 4*S*, 5*R*) or (2*S*, 4*R*, 5*S*) for epohelmin B, respectively. Absolute configurations were not determined, but these compounds are the first examples as the naturally-occurring 4,5-epoxyheptamethylenamine backbone. The epohelmins might be competitive inhibitors of hOSC by mimicking substrate molecules, *i.e.*, the combination of the epoxide, the secondary ammonium cation (generated by protonation), and the 4′-oxoundec-(5′*E*)-enyl side chain could respectively mimic the epoxide, the C-6 cation (presumably generated by cyclization of the A-ring), and the flanking non-polar hydrocarbons of the enzyme substrate, (3*S*)-2,3-oxidosqualene.

Understanding the biosynthesis of epohelmins will be important because this is the first isolation of the natural product with 4,5-epoxyheptamethylenamine structure. Hypothetical pathways can be derived from fundamental principals of biosynthesis (Fig. 4). The heptamethylen

Fig. 4. Proposed biosynthesis of epohelmins A and B.



amine moiety is unlikely to be derived from amino acids or polyamines. Acetate-derived polyketide chain with transamination is one of the possible biosynthetic routes. The fatty acid analog (C_{18}) from nine acetate units might be the first intermediate. Reduction and transamination at C-1 result in the formation of an amino group that reacts with the carbonyl group (C-7) to form a Schiff base. The resultant imine is stereospecifically reduced, and one of enatiomeric secondary amines is produced. The C-5 methylene carbon is probably hydroxylated from anti-direction against C-2 side chain due to less spatial hindrance. Then the carbonyl group (C-4) is reduced non-stereospecifically or stereospecifically by two independent reductases to produce one pair of epimers. The S_N2 reaction of epimeric C-4 hydroxyl groups to C-5 gives two epimeric epoxides. If it is the case, their possible absolute configurations of epohelmins A and B are restricted to a pair of (2*R*, 4*R*, 5*R*) and (2*R*, 4*S*, 5*R*), or (2*S*, 4*S*, 5*S*) and (2*S*, 4*R*, 5*S*). Stereospecificity of imine reductase is presumed to be the key reaction in determining the stereochemistry of epohelmins. Determination of absolute configurations and future biosynthesis studies will reveal the pathways utilized to construct epohelmins in this fungus.

Acknowledgements

A part of this research was financially supported by Grant-in-Aids for Scientific Research (B) (No. 14380285) to M.S. and (S) (No. 15101007) to Y.E from Japan Society for Promotion of Science, and a Grant-in-Aid for Scientific Research on Priority Area (A) (No. 12045213) to Y.E. from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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