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Lanopylins A_1, B_1, A_2 and B_2, Novel Lanosterol Synthase Inhibitors from *Streptomyces* sp. K99-5041

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From an actinomycete strain, *Streptomyces* sp. K99-5041, lanopylins A_1, B_1, A_2 and B_2 were isolated as new natural products that inhibited the reaction of recombinant human lanosterol synthase. The crude extract from the whole broth of this strain was fractionated by silica gel column chromatography to afford an active fraction that showed a single spot on TLC. Detailed analyses of this fraction with liquid chromatography-atmospheric pressure chemical ionization mass spectrometry revealed that it contained 20 homologous compounds with differing side chain lengths. The fraction was separated by preparative HPLC to afford four of these homologues, lanopylins A_1, B_1, A_2 and B_2. Detailed spectroscopic analyses of these isolated compounds led to the identification of their structures. Lanopylins A_1 and B_1 were (3*E*)-isohexadecylmethylidene-2-methyl-1-pyrroline and (3*E*)-hexadecylmethylidene-2-methyl-1-pyrroline, respectively, and lanopylins A_2 and B_2 were homologues with the insertion of one *cis*-ethylenylidene in the side chain of lanopylins A_1 and B_1, respectively. These compounds inhibited recombinant human lanosterol synthase with IC_{50} values of 15, 18, 33, and 41 μM, respectively.

Hypercholesterolemia is recognized as a risk factor for atherosclerotic disease, such as coronary heart disease. Moderate exercise and/or diets containing low cholesterol or which suppress cholesterol biosynthesis may prevent the development of hypercholesterolemia. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) is a clinically validated target for suppressing cholesterol biosynthesis. Inhibitors of this enzyme, for example, pravastatin and lovastatin, are the first choice for pharmaceutical treatment of hypercholesterolemia[1]. But since HMG-CoA reductase is located upstream in the cholesterol biosynthetic pathway, inhibition of this enzyme may cause a simultaneous reduction in the physiologically essential non-sterol isoprenoid metabolites such as dolichol, ubiquinone, and prenylated proteins. On the other hand, inhibition of the enzymes along the pathway from lanosterol through cholesterol will cause accumulation of steroidal intermediates or metabolites, with unexpected side effects.

Lanosterol synthase (EC 5.4.99.7) is viewed as more selective target for suppression of cholesterol biosynthesis[2,3] since it is located downstream of HMG-CoA reductase in the pathway. Reported lanosterol synthase inhibitors are lauryldimethylamine *N*-oxide (LDAO)[4], 29-methylidene-2,3-oxidosqualene (29-MOS)[5], BIBX 79[6], Ro 48-8071[7], BIBB 515[8] *etc.*, although no natural compounds have so far been reported to be lanosterol synthase inhibitors. Using recombinant human enzymes, screening of inhibitors of natural origin has been carried out with fermentation broths of 1,031 actinomycete strains. Four inhibitors, lanopylin A_1, B_1, A_2 and B_2, have been isolated as new natural products from the actinomycete strain *Streptomyces* sp. K99-5041 and their structures elucidated using spectroscopic methods. In this paper, we describe the taxonomy and fermentation of the producing strain, and isolation, biological properties, and structure elucidation of these

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compounds.

Materials and Methods

General

The actinomycete strain K99-5041 was isolated from a soil sample. The International *Streptomyces* Project (ISP) media recommended by SHIRLING and GOTTLIEB[9], and media recommended by WAKSMAN[10] were used to investigate the cultural and physiological characteristics. Cultures were routinely observed after incubation for two weeks at 27°C. Color names and hue numbers were determined according to the Color Harmony Manual[11]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon at 27°C[12]. The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL). The isomers of diaminopimelic acid (DAP) isomers were determined by the method of BECKER *et al.*[13]. Menaquinones were extracted and purified after COLLINS *et al.*[14] then analyzed by high performance liquid chromatography (HPLC) equipped with a CAPCELL PAK C18 column (Shiseido)[15].

NMR spectra were recorded using 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. Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS) analysis was carried out using a Thermo Quest LCQ equipped with a Hewlett Packard HP1100 series LC system under the following conditions: column, TSK gel ODS-80T_M (4.6×150 mm, Tosoh); eluent, 98% MeOH; flow rate, 0.8 ml/minute; and photodiode array detector, APCI (positive).

Fermentation Media

The seed medium was composed of starch 2.4%, glucose 0.1%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5% and $CaCO_3$ 0.4% in deionized water (pH 7.0 prior to sterilization). The production medium was composed of glucose 0.5%, corn steep powder 1.0%, oatmeal 1.0%, Pharmamedia 1.0%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, Sekado (Kanto Chemicals, Co. Ltd.) 0.5% and trace elements (each 0.0001%: $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 6H_2O$) in tap water (pH 7.0 prior to sterilization).

In Vitro Assay for Human Lanosterol Synthase

The details of procedures for the preparation of cell-free extracts from recombinant *Saccharomyces cerevisiae* expressing human lanosterol synthase, the preparation of [^{14}C] (3*S*)-2,3-oxidosqualene and the *in vitro* assay for human lanosterol synthase will be described elsewhere. The cDNA of human lanosterol synthase[16] was expressed in the mutant *S. cerevisiae* GIL77 (*gal2 hem3-6 erg7 ura3-167*)[17], and the cells from this recombinant yeast were collected and homogenized in a Waring blender with acid-washed glass beads in 0.1 M potassium-phosphate buffer (pH 7.4, containing 0.45 M sucrose, 1 mM EDTA and 1 mM dithiothreitol). The lysate was centrifuged at 8,600×*g* for 30 minutes and the resultant supernatant was adjusted to *ca.* 10 mg protein/ml with the same buffer. The amount of protein was estimated using the Protein Assay (Bio-Rad). The substrate [^{14}C] (3*S*)-2,3-oxidosqualene was prepared by feeding the mutant yeast strain GL7 (*MAT***a** *gal2 hem3-6 erg7*)[18] with [1-^{14}C] sodium acetate. The *in vitro* enzyme reaction was performed as follows: The cell-free extract (1 mg protein) was incubated with [^{14}C] (3*S*)-2,3-oxidosqualene (0.17 nmol, 4.5 nCi dissolved in 5 μl of 2-methoxyethanol) and testing samples (dissolved in 120 μl of 50% aqueous ethanol) in 1 ml of 0.1 M potassium phosphate buffer (pH 7.4, containing 0.1% Triton X-100) at 37°C for 1 hour. After incubation, the reaction was stopped by adding 6% KOH/EtOH followed by 10-minute incubation at 37°C. After extraction with cyclohexane, the lipids were concentrated and applied to a TLC plate (Merck), which was developed with benzene-acetone (19:1). The resultant TLC plates were exposed to an imaging plate and analyzed with a Photo Image Analyzer BAS-1500 (Fujifilm).

Results and Discussion

Taxonomy of the Producing Organism

The vegetative mycelia grew abundantly on yeast extract-malt extract agar and oatmeal agar, and did not show fragmentation into coccoid forms or bacillary elements. The aerial mycelia grew abundantly on yeast extract-malt extract agar. The spore chains were open loops and each had more than 20 spores per chain. The spores were cylindrical in shape, 0.9~1.1×0.6~0.7 μm in size and had a smooth surface (Fig. 1). Whirls, sclerotic granules, sporangia or flagellate spores were not observed.

The isomer of DAP in whole-cell hydrolysates of strain K99-5041 was determined to be LL-form. Major menaquinones were MK-9(H_6) and MK-9(H_8).

Table 1. Cultural characteristics of strain K99-5041.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract-malt extract agar [a]	G: Good, light wheat (2ea) R: Honey gold (2ic) AM: Abundant, dusk (10fe) SP: Trace, yellow	Tyrosine agar [a]	G: Moderate, light tan (3gc) to chestnut brown (4ni) R: Bamboo (2fb) to beaver (3li) AM: None SP: Trace, yellow
Oatmeal agar [a]	G: Good, honey gold (2ic) R: Honey gold (2ic) AM: Poor, orchid haze (10dc) SP: None	Sucrose-nitrate agar [b]	G: Moderate, ivory (2db) R: Ivory (2db) AM: Poor, alabaster tint (13ba) SP: None
Inorganic salts-starch agar [a]	G: Moderate, honey gold (2ic) R: Bamboo (2gc) AM: Moderate, alabaster tint (13ba) SP: None	Glucose-nitrate agar [b]	G: None R: None AM: None SP: None
Glycerol-asparagine agar [a]	G: Moderate, light wheat (2ea) to clove brown (3ni) R: Colonial yellow (2ga) to mustard brown (2ni) AM: None SP: Trace, yellow	Glycerol-calcium malate agar [b]	G: Poor, yellow maple (3ng) R: Cinamom (3le) AM: None SP: Trace, yellow
Glucose-asparagine agar	G: Poor, light ivory (2ca) R: Light ivory (2ca) AM: None SP: None	Glucose-peptone agar [b]	G: Moderate, honey gold (2ic) R: Mustard gold (2ne) AM: Scant, white (a) SP: None
Peptone-yeast extract-iron agar [a]	G: Moderate, light wheat (2ea) R: Bamboo (2gc) AM: None SP: Trace, yellow	Nutrient agar [b]	G: Moderate, honey gold (2ic) R: Bamboo (2gc) AM: Poor, alabaster tint (13ba) SP: None

a; Medium recommended by ISP
b; Medium recommended by S. A. Waksman.
Abbreviations: G, growth of vegetative mycelium; R, reverse side color; AM, aerial mycelium; SP, soluble pigment.

Fig. 1. Scanning electron micrograph of *Streptomyces* sp. K99-5041.

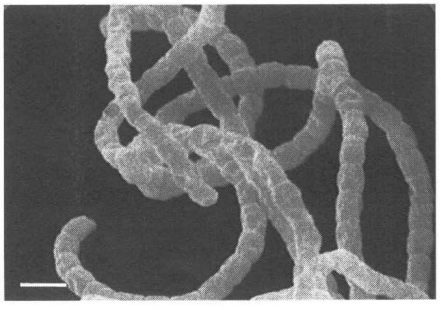


Table 2. Physiological properties of strain K99-5041.

Melanin formation	
Tyrosine agar	negative
Peptone-yeast extract-iron agar	negative
Tryptone-yeast extract broth	negative
Gelatin medium	negative
Reduction of nitrate	negative
Liquefaction of gelatin (21~23°C)	negative
Hydrolysis of starch	negative
Coagulation of milk (27°C)	negative
Peptonization of milk (27°C)	negative
Decomposition of cellulose	negative
Temperature range for growth	12~33°C

Table 3. Utilization of carbon sources by strain K99-5041.

Utilized:	D-Glucose, *myo*-Inositol
Weakly utilized:	Raffinose
Not utilized:	L-Arabinose, D-Fructose, D-Mannitol, Melibiose , L-Rhamnose, Sucrose, D-Xylose

The cultural characteristics, the physiological properties and the utilization of carbon sources are shown in Tables 1~3. The color of vegetative mycelia was yellow to brown and the aerial mass was white to gray. Melanoid pigment was not produced but yellowish pigment was produced.

Based on the taxonomic properties described above, strain K99-5041 is considered to belong to the genus *Streptomyces*[19].

Fermentation

A slant culture of strain K99-5041 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 (1 ml) was transferred into 70 separate 500-ml Erlenmeyer flasks containing 100 ml of the production medium. The fermentation was carried out on a rotary shaker at 27°C for 6 days.

Isolation and Biological Properties

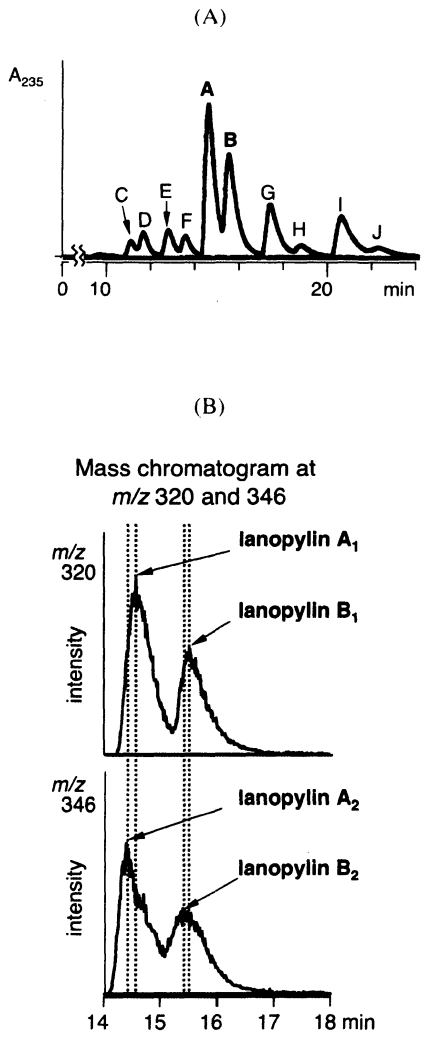
The cultured broth (7 liters) was extracted with ethyl acetate and centrifuged. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to afford 5.5 g of red-brown oily material. The crude extract was subjected to silica gel column chromatography (Wakogel® C-200, Wako) with a $CHCl_3$-MeOH solvent system. Activity was concentrated to the single spot of Rf ~0.4 on TLC [$CHCl_3$-MeOH (19:1)] to yield 97.3 mg of oily material (labeled fraction N).

Fraction N was analyzed by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS) with an ion-trap mass analyzer. The UV and total-ion chromatograms of this fraction comprised 10 peaks, all of which had maximal absorbance at 235 nm. The molecular masses (*m/z*) of these peaks were observed, in order from the peak with the earliest retention time, 317, 317, 331, 331, 345, 345, 359, 359, 373 and 373. We labeled these peaks C, D, E, F, A, B, G, H, I and J, respectively (Fig. 2-(A)).

The two peaks A and B were purified from fraction N by preparative HPLC using a Tosoh CCPE-II pump under the following conditions: column, TSK gel ODS-80T_M (7.8×300 mm, Tosoh); eluent, 98% MeOH; flow rate, 2.0 ml/minute; and detection, 234 nm. Under these conditions, peaks A and B were eluted at 29 and 31 minutes, respectively. Each of the eluates was concentrated to dryness. These two HPLC fractions were essentially equipotent in the enzyme assay (data not shown). NMR analyses revealed that they were mixtures because the signals δ_H 5.35, 2.01 and δ_C 129.9, 27.2 were observed at decimal intensities (data not shown).

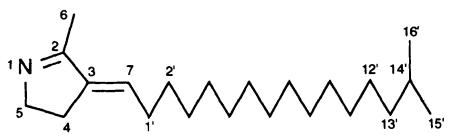
Next, we performed detailed LC-APCIMS analysis of fraction N. For example, in the mass spectra of peaks A and B, a signal was observed at *m/z* 320 (26 mass units smaller than the molecular mass *m/z* 346) and further investigation of the mass chromatograms at *m/z* 320 and 346 revealed a *ca.* 0.1-minute time lag in the retention time (Fig. 2-(B)). A similar time lag was observed in other HPLC peaks (data not shown). These findings led us to consider that each of the above-mentioned ten peaks (from A to J) was composed of two compounds, differing in molecular mass by 26 mass units, and thus that fraction N contained 20 types of homologous compounds as shown in Fig. 3. Therefore for

Fig. 2.

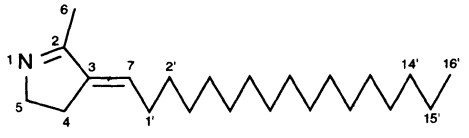


(A) The UV (235 nm) and (B) mass chromatograms from analytical LC-APCIMS of the active silica-gel fraction N.

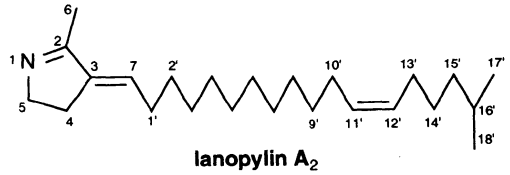
Fig. 3. Structures of lanopylins A_1 and B_1, and putative structures of A_2, B_2 and C~J.



lanopylin A_1

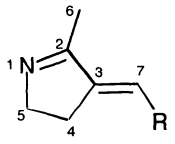


lanopylin B_1



lanopylin A_2

lanopylin B_2



Compound	R	Compound	R
Lanopylin C_1	Isotetradecyl	Lanopylin C_2	Isohexadecenyl
Lanopylin D_1	Tetradecyl	Lanopylin D_2	Hexadecenyl
Lanopylin E_1	Isopentadecyl	Lanopylin E_2	Isoheptadecenyl
Lanopylin F_1	Pentadecyl	Lanopylin F_2	Heptadecenyl
Lanopylin A_1	Isohexadecyl	Lanopylin A_2	Isooctadecenyl
Lanopylin B_1	Hexadecyl	Lanopylin B_2	Octadecenyl
Lanopylin G_1	Isoheptadecyl	Lanopylin G_2	Isononadecenyl
Lanopylin H_1	Heptadecyl	Lanopylin H_2	Nonadecenyl
Lanopylin I_1	Isooctadecyl	Lanopylin I_2	Isoicosenyl
Lanopylin J_1	Octadecyl	Lanopylin J_2	Icosenyl

The ten peaks identified with LC-APCIMS (Fig. 2-(A)) comprise lanopylins C_1 and C_2 (for peak C), D_1 and D_2 (for peak D), E_1 and E_2 (for peak E), F_1 and F_2 (for peak F), A_1 and A_2 (for peak A), B_1 and B_2 (for peak B), G_1 and G_2 (for peak G), H_1 and H_2 (for peak H), I_1 and I_2 (for peak I), and J_1 and J_2 (for peak J), respectively.

Table 4. Physicochemical properties of lanopylins A_1, B_1, A_2 and B_2.

Lanopylin A_1

Appearance: colorless oil

Molecular weight: 319

Molecular formula: $C_{22}H_{41}N$

EIMS: *m/z* (% rel. int.) 319 $[M]^+$ (70), 304 (30), 290 (0), 276 (42), 262 (19), 248 (15), 234 (14), 220 (11), 206 (6), 192 (5), 178 (4), 164 (5), 150 (5), 136 (11), 122 (100), 96 (66), 67 (66)

HR-FABMS: *m/z* 320.3321 $[M+H]^+$, Δ +0.3 mmu

UV λ_{max}^{MeOH} nm (ε): 235 (14,500)

IR ν_{max} (KBr) cm^{-1}: 3435, 2924, 2854, 1606, 1466, 1385

TLC: $CHCl_3$-MeOH (19:1) Rf~0.4, H_2SO_4(+), Ninhydrin(+), Dragendorff(+)

Lanopylin B_1

Appearance: colorless oil

Molecular weight: 319

Molecular formula: $C_{22}H_{41}N$

EIMS: *m/z* (% rel. int.) 319 $[M]^+$ (77), 304 (12), 290 (20), 276 (23), 262 (20), 248 (17), 234 (16), 220 (12), 206 (7), 192 (5), 178 (5), 164 (5), 150 (5), 136 (10), 122 (100), 96 (67), 67 (61)

HR-FABMS: *m/z* 320.3324 $[M+H]^+$, Δ +0.6 mmu

UV λ_{max}^{MeOH} nm (ε): 235 (13,900)

IR ν_{max} (KBr) cm^{-1}: 3431, 2924, 2852, 1606, 1466, 1385, 1261, 1099, 1032, 804

TLC: $CHCl_3$-MeOH (19:1) Rf~0.4, H_2SO_4(+), Ninhydrin(+), Dragendorff(+)

Lanopylin A_2

Appearance: colorless oil

Molecular weight: 345

Molecular formula: $C_{24}H_{43}N$

EIMS: *m/z* (% rel. int.) 345 $[M]^+$ (71), 330 (24), 316 (1), 302 (23), 288 (38), 274 (43), 260 (24), 246 (15), 234 (4), 220 (12), 206 (10), 192 (6), 178 (5), 164 (4), 150 (5), 136 (7), 122 (100), 96 (40), 67 (31), 55 (34)

HR-FABMS: *m/z* 346.3449 $[M+H]^+$, Δ –2.5 mmu

TLC: $CHCl_3$-MeOH (19:1) Rf~0.4, H_2SO_4(+), Ninhydrin(+), Dragendorff(+)

^{1}H-NMR (500.00 MHz, $CDCl_3$): δ_H 0.87 (6H, d, *J* = 6.5 Hz), 1.15 (2H, br dt, *J* = 6.8, 6.8 Hz), 1.2–1.4 (16H, m), 1.43 (2H, br tt, *J* = 7.3, 7.3 Hz), 1.51 (1H, sept, *J* = 6.6 Hz), 2.01 (4H, br dt, *J* = 7.0, 7.0 Hz), 2.06 (3H, t, *J* = 1.8 Hz), 2.10 (2H, br dt, *J* = 7.3, 7.3 Hz), 2.48 (2H, m), 3.86 (2H, m), 5.35 (2H, t, *J* = 5.5 Hz), 5.75 (1H, sept-like)

Lanopylin B_2

Appearance: colorless oil

Molecular weight: 345

Molecular formula: $C_{24}H_{43}N$

EIMS *m/z* (% rel. int.): 345 $[M]^+$ (55), 330 (12), 316 (10), 302 (13), 288 (31), 274 (31), 260 (19), 246 (11), 234 (5), 220 (10), 206 (9), 192 (6), 178 (5), 164 (4), 150 (5), 136 (7), 122 (100), 96 (43), 67 (37), 55 (45)

HR-FABMS: *m/z* 346.3499 $[M+H]^+$, Δ +2.5 mmu

TLC: $CHCl_3$-MeOH (19:1) Rf~0.4, H_2SO_4(+), Ninhydrin(+), Dragendorff(+)

^{1}H-NMR (500.00 MHz, $CDCl_3$): δ_H 0.88 (3H, t, *J* = 7.0 Hz), 1.2–1.4 (22H, m), 1.43 (2H, br tt, *J* = 7.2, 7.2 Hz), 2.01 (4H, br dt, *J* = 6.3, 6.3 Hz), 2.06 (3H, t, *J* = 1.8 Hz), 2.10 (2H, br dt, *J* = 7.2, 7.2 Hz), 2.48 (2H, m), 3.86 (2H, m), 5.35 (t, *J* = 5.5 Hz), 5.76 (1H, sept-like)

peaks A and B, we called the two compounds with molecular mass of 319 lanopylins A_1 and B_1, and the other two compounds with molecular mass of 345 were called lanopylins A_2 and B_2, as shown in Fig. 2-(B).

Lanopylins A_1 and A_2 were purified from fraction A with further preparative HPLC using the Tosoh 8020 system under the following conditions: column, TSK gel ODS-120T (4.6×150 mm, Tosoh); eluent, MeOH; flow rate, 1.5 ml/minute; and detection, 234 nm. Lanopylin A_2 was eluted at 8 minutes, followed by an extended 30~40 minutes elution of lanopylin A_1. Lanopylin B_1 and B_2 were similarly purified from fraction B under the above conditions. Lanopylin B_2 was eluted at 8 minutes, followed by a 30~40 minutes elution of lanopylin B_1. The eluates were concentrated to dryness to yield lanopylin A_1 (3.5 mg), B_1 (2.3 mg), A_2 (0.6 mg) and B_2 (0.5 mg) as colorless oil. The IC_{50} values of these compounds were 15, 18, 33 and 41 μM, respectively, and their potency was comparable to the known oxidosqualene cyclase inhibitors, LDAO (purchased from Sigma; IC_{50}=0.84 μM) and AMO1618 (purchased from Wako; IC_{50}=120 μM).

Structure Elucidation

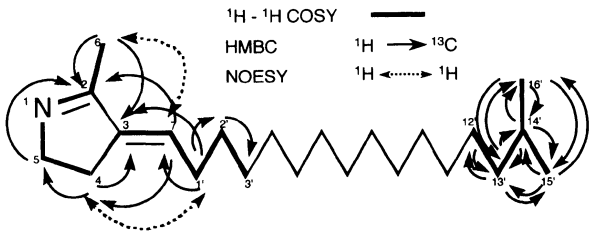
The physicochemical properties of the isolated compounds are summarized in Table 4. The molecular formulae of lanopylin A_1 and B_1 were determined to be $C_{22}H_{41}N$, and the molecular formulae of lanopylin A_2 and B_2 were determined to be $C_{24}H_{43}N$ by HR-FABMS. The 26-mass unit differences between lanopylins A_1 and A_2 and between lanopylins B_1 and B_2 therefore corresponded to C_2H_2.

The structure of lanopylin A_1 was elucidated by NMR and MS analyses. Analyses of the ^{1}H-NMR, ^{13}C-NMR, DEPT, and HMQC spectra revealed the presence of two quaternary carbons, two methines, 15 methylenes and three methyl groups. Taken together with the results of analyses of various NMR investigations and the EIMS spectra, the structure of lanopylin A_1 was finally elucidated to be (3*E*)-isohexadecylmethylidene-2-methyl-1-pyrroline (Fig. 4). The NMR assignment of lanopylin A_1 is presented in Table 5. The evidence was as follows:

1) The ^{1}H homonuclear decoupling measurement by irradiation of H-4, H-7 or H-1′ indicated that the ^{1}H-^{1}H coupling constants are 3.0 Hz between H-4 and H-7, and 7.3 Hz between H-7 and H-1′, respectively.

2) The presence of a 3-methylidene-2-methyl-1-pyrroline moiety was indicated by the following NMR data: The ^{1}H-^{1}H couplings between H-4 (δ_H 2.48) and H-5 (δ_H 3.86), between H-4 and H-7 (δ_H 5.75) and between H-5 and H-6 (δ_H 2.06), and the ^{1}H-^{13}C long-range couplings from H-4 to C-3 (δ_C 143.2) and C-5 (δ_C 57.8), from H-5 to C-2 (δ_C 171.7), from H-6 to C-2 and C-3 and from H-7 to C-2 and C-4 (δ_C 26.8). This is supported by the NMR data of this moiety in the known synthetic compounds[20]. To the best of our knowledge, no natural products have been previously reported to have this moiety.

Fig. 4. Key ^{1}H-^{1}H COSY, HMBC and NOESY connectivities observed in lanopylin A_1.



3) The doublet signal at δ_H 0.86 with the intensity of 6 protons showed the presence of an *iso*-branched propyl moiety (gem-dimethyl).

4) That the isohexadecyl side chain is attached to the 3-methylidene-2-methyl-1-pyrroline moiety at position 7 was indicated by the following: The ^{1}H-^{1}H couplings between H-4 and H-1′ (δ_H 2.10), between H-7 and H-1′, between H-1′ and H-2′ (δ_H 1.43), between H-2′ and H-3′ (δ_H 1.2~1.4), between H-12′ (δ_H 1.2~1.4) and H-13′ (δ_H 1.15), between H-13′ and H-14′ (δ_H 1.51), between H-14′ and H-15′ (δ_H 0.86) and between H-14′ and H-16′ (δ_H 0.86), and the ^{1}H-^{13}C long-range couplings from H-1′ to C-3, C-7 (δ_C 126.3) and C-2′ (δ_C 29.0), from H-2′ to C-3′ (δ_C 29.4~30.0), from H-12′ to C-13′ (δ_C 39.1), from H-13′ to C-12′ (δ_C 27.4), C-14′ (δ_C 28.0), C-15′ (δ_C 22.7) and C-16′ (δ_C 22.7), from H-14′ to C-13′, C-15′ and C-16′, from H-15′ to C-13′, C-14′ and C-16′ and from H-16′ to C-13′, C-14′ and C-15′. This is also supported by the observation of a series of fragmentations of the isoalkyl chain from *m/z* 319 ($[M]^+$) to 122 (base peak), with weak relative intensity at *m/z* 290 ($[M]^+-29$) in the EIMS spectrum (Table 4).

5) The geometrical configuration of the double bond between positions 3 and 7 was elucidated to be *E* by the NOEs between H-4 and H-1′, and between H-6 and H-7.

In the same manner, the structure of lanopylin B_1 was finally elucidated to be (3*E*)-hexadecylmethylidene-2-methyl-1-pyrroline. The NMR assignment of lanopylin B_1 is also presented in Table 5. The evidence was as follows:

Table 5. NMR data of lanopylins A_1 and B_1.

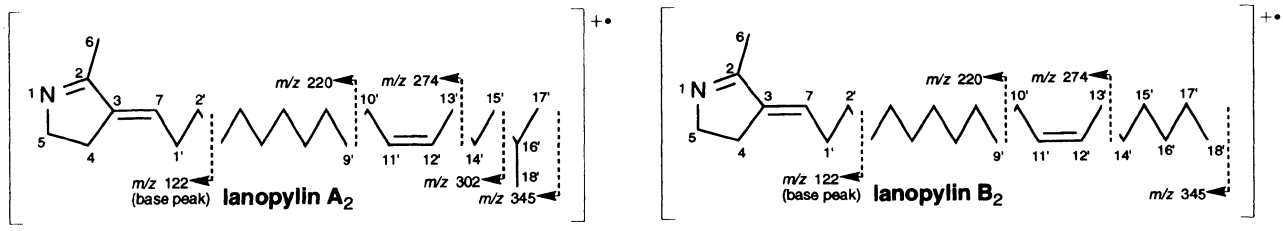
	Lanopylin A_1			Lanopylin B_1	
Position	^{13}C δ ppm	^{1}H δ ppm (J in Hz)	Position	^{13}C δ ppm	^{1}H δ ppm (J in Hz)
1			1		
2	171.7		2	171.7	
3	143.2		3	143.2	
4	26.8	2.48 (2H, m)	4	26.8	2.48 (2H, m)
5	57.8	3.86 (2H, m)	5	57.8	3.86 (2H, m)
6	15.9	2.06 (3H, t, 1.5)	6	15.9	2.06 (3H, t, 1.5)
7	126.3	5.75 (1H, tt, 7.3, 3.0)	7	126.2	5.75 (1H, tt, 7.3, 3.0)
1'	30.5	2.10 (2H, br dt 7.5, 7.5)	1'	30.5	2.10 (2H, br dt 7.5, 7.5)
2'	29.0	1.43 (2H, br tt, 7.1, 7.1)	2'	29.0	1.43 (2H, br tt, 7.3, 7.3)
3'–11'	29.4–30.0	1.2–1.4 (20H, m)	3'–13'	29.4–29.7	1.2–1.4 (26H, m)
12'	27.4				
13'	39.1	1.15 (2H, br dt, 6.8, 6.8)	14'	31.9	
14'	28.0	1.51 (1H, sept, 6.6)	15'	22.7	
15',16'	22.7	0.86 (6H, d, 6.5)	16'	14.1	0.88 (3H, t, 7.0)

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

1) Signals and correlations obtained from NMR analyses were almost identical to those of lanopylin A_1.

2) The evidence that a hexadecyl side chain is attached to the (3*E*)-methylidene-2-methyl-1-pyrroline moiety at position 7 is as follows: Increase of two methylene carbons, decrease of one methine and one methyl carbons, the triplet signal at δ_H 0.88 with the intensity of 3 protons in place of the doublet signal at δ_H 0.86 with the intensity of 6 protons,

Fig. 5. EIMS fragmentation of lanopylins A_2 and B_2.



the ^{1}H-^{1}H coupling between H-15′ (δ_H 1.2~1.4) and H-16′ (δ_H 0.88) and the ^{1}H-^{13}C long-range couplings from H-16′ to C-14′ (δ_C 31.9) and C-15′ (δ_C 22.7). This is also supported by the fact that a series of fragmentations of the alkyl chain from *m/z* 319 ($[M]^+$) to 122 (base peak) were observed in the EIMS spectrum (Table 4).

The ^{1}H-NMR spectra of lanopylins A_2 and B_2 appeared almost the same as those of lanopylins A_1 and B_1, respectively, except for the presence of the signals δ_H 5.35 (2H, brt, J=4~5 Hz) and 2.01 (4H, dt, J=~7, ~7 Hz) (Table 4). Preliminary NMR analyses of fractions A and B showed that these protons were connected to the carbons at δ_C 129.9 (d) and 27.2 (t), respectively, and these two methines and two methylenes correlate to the side chain methylenes [δ_H 1.2~1.4 (m), δ_C 29~30 (t)] (data not presented). Thus, the structure of lanopylin A_2 was elucidated to be the same as that of lanopylin A_1 except that one ethylenylidene (C_2H_2=26 mass units) was inserted in the isohexadecyl side chain of lanopylin A_1. The geometrical configuration of the double bond was assumed to be *cis* from the δ_C value (27.2 ppm) of the neighboring methylene[21], although a spin-spin coupling was not observed between olefinic protons. Similarly, the structure of lanopylin B_2 was elucidated to be the same as that of lanopylin B_1 except that one *cis*-ethylenylidene (C_2H_2=26 mass units) was inserted in the hexadecyl side chain of lanopylin B_1. The fragmentation of the *cis*-alkenyl side chain in lanopylin A_2 and B_2 appeared to resemble that of *N*-monounsaturated fatty acyl pyrrolidides[22].

The position of the *cis*-double bond in the side chain was assumed to be follows: The fragment peak at *m/z* 274 in the EIMS spectrum of lanopylin A_2 was intense, and two ions at 12-mass unit intervals were observed between *m/z* 234 and 246 (Table 4), and therefore lanopylin A_2 might be (3*E*)-isooctadec-(11′*Z*)-enylmethylidene-2-methyl-1-pyrroline (Figs. 3 and 5). Similarly, the fragment peak at *m/z* 274 in the EIMS spectrum of lanopylin B_2 was intense and one pair of ions at 12-mass unit intervals was observed between *m/z* 234 and 246 (Table 4), and therefore lanopylin B_2 might be (3*E*)-octadec-(11′*Z*)-enylmethylidene-2-methyl-1-pyrroline (Figs. 3 and 5). However, the position of the *cis*-double bond in the side chain could be elucidated by analyzing the fatty acids that might be produced by ozonolysis of these compounds or fractions.

In conclusion, four compounds, lanopylins A_1, B_1, A_2 and B_2 (Fig. 3) were isolated from the Actinomycete strain *Streptomyces* sp. K99-5041 as new lanosterol synthase inhibitors and their structures were elucidated using spectroscopic methods to be (3*E*)-isohexadecylmethylidene-2-methyl-1-pyrroline, (3*E*)-hexadecylmethylidene-2-methyl-1-pyrroline, (3*E*)-isooctadec-(11′*Z*)-enylmethylidene-2-methyl-1-pyrroline and (3*E*)-octadec-(11′*Z*)-enylmethylidene-2-methyl-1-pyrroline, respectively. On the basis of the structures of lanopylins A_1, B_1, A_2 and B_2, the structures of the other 16 homologues in fraction N were tentatively elucidated by preliminary NMR and LC-APCIMS analyses. All these homologues have the (3*E*)-methylidene-2-methyl-1-pyrroline moiety as a common building block and differ in the length of side chains from lanopylins A_1, B_1, A_2 and B_2 (Fig. 3). These compounds are the first examples of lanosterol synthase inhibitors of natural origin. They are also the first examples with a naturally occurring (3*E*)-methylidene-2-methyl-1-pyrroline backbone. We hypothesize that the (3*E*)-methylidene-2-methyl-1-pyrroline homologues might inhibit lanosterol synthase production by mimicking substrate molecules: the iminium cation generated by protonation and the long side chains might mimic the C-2 cation generated by opening the epoxide and the flanking non-polar hydrocarbons, respectively. We also hypothesize that these homologues might be biosynthesized

via condensation of malonyl-CoA and alanine to fatty acyl-CoA or *via* metabolism from arginine and/or proline through coupling with alkyl or alkenyl side chains. These hypotheses could be confirmed in biochemical and biogenetical investigations using the producer strain. The assay for inhibition of cholesterol biosynthesis within the Chang liver cells[23] and extensive structure-activity relationship studies are in progress.

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