PANNORIN, A NEW 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE INHIBITOR PRODUCED BY *Chrysosporium pannorum*

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Pannorin, a naphthopyrone that inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, was isolated from a culture broth of *Chrysosporium pannorum* M10539 by solvent extraction, Bio-Gel P-6 column chromatography and reverse phase HPLC (Silica ODS). Spectroscopic analyses of the compound yielded 4,8,10-trihydroxy-5-methyl-2*H*-naphtho[1,2-*b*]pyran-2-one as the proposed structure. Pannorin inhibited HMG-CoA reductase and *in vitro* sterol synthesis 50% at a concentration of 160 μM.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in cholesterol synthesis. Compactin and its analogs have been isolated from microorganisms as specific inhibitors of HMG-CoA reductase. These compounds have a potent cholesterol-lowering effect in animals and humans.[1~3]

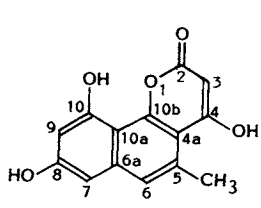
The present communication deals with the isolation, characterization and biological activity of pannorin (Fig. 1), a new type of HMG-CoA reductase inhibitor produced by a strain of *Chrysosporium pannorum*.

Taxonomy of Producing Organism

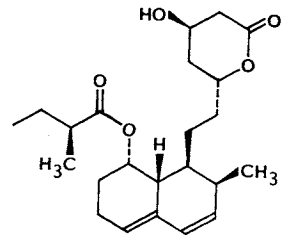
Fungal strain M10539 that produces pannorin was isolated from a soil sample collected in Fussa City, Tokyo. The colonies on potato-glucose agar grow restrictedly, reaching a diameter of 2 cm after 2 weeks at 25°C, glabrous to powdery with scattered tufts of aerial growth, irregularly heaped, surface in brown; exudate in brown; reverse in light brown to brown. The fungus can grow at temperature range from 4 to 25°C with the growth optimum at about 15°C.

The aleuriospores are borne at the tips, along the slides, or in an intercalary position on more or less erect conidiophores which branch verticillately at an acute angle. The conidia are subglobose to pyriform with smooth and hyaline walls, 2.5~3.5 × 2~3 μm in size (Fig. 2). From the characteristics described, the fungus was identified as a strain of *C. pannorum* (Link) Hughes M10539.[4,5]

Fig. 1. Structures of pannorin and compactin.



Pannorin (**1**)



Compactin (**2**)

Fig. 2. Micrograph of aerial mycelia and conidiophores of *Chrysosporium pannorum* M10539 on potato-glucose agar

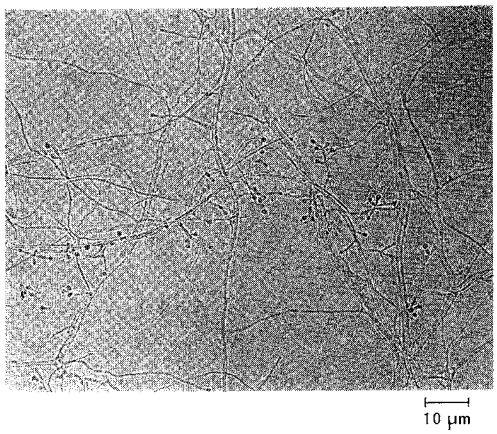
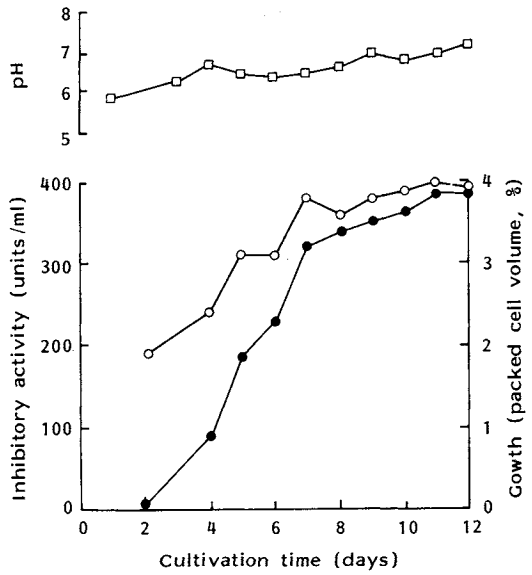


Fig. 3. Fermentation profile of *Chrysosporium pannorum* M10539.

□ pH, ○ growth, ● inhibitory activity.



Fermentation

Pannorin (**1**) was produced in shake flask culture of *C. pannorum* M10539, grown at 25°C for 10 to 12 days. Potato-glucose medium (potato 200 g, glucose 20 g in 1 liter tap water) was used for both inoculation and production. Seed medium (100 ml in 500-ml Sakaguchi flasks) was inoculated with mycelial and spore scrapings from slant surfaces and was incubated on a reciprocal shaker (200 rpm) at 25°C for 4 days. A one-ml portion of the seed was inoculated into the same medium (100 ml in 500-ml Sakaguchi flasks), which was then incubated under the same conditions and harvested after 12 days. A typical flask fermentation is shown in Fig. 3, where production of active compound(s) in culture filtrate was monitored by determining inhibitory activity against *in vitro* sterol biosynthesis.[6]

Isolation

A portion of the fermentation broth (2.7 liters) was centrifuged and the supernatant was adjusted to pH 2.5 by adding 6 N HCl, followed by extraction with 2.7 liters of ethyl acetate (twice). The organic extracts were pooled and concentrated to 2.5 liters under reduced pressure and then mixed with 2.5 liters of 5% $NaHCO_3$ (pH 8.2). The aqueous layer was pooled and adjusted to pH 2.5 with 6 N HCl. The resultant precipitate was collected by centrifugation, washed with water and then lyophilized, giving 1.8 g of a brownish residue. This residue was suspended in 180 ml of water and extracted with 180 ml of *n*-butyl acetate and pH 3.0 (twice). The solvent extract was dried over sodium sulfate and concentrated under reduced pressure to give 679 mg of a brownish powder.

The brownish powder was dissolved in a small volume of water by adjusting to pH 8.5 with NaOH and then submitted to gel filtration in seven batches over a Bio-Gel P-6 column (2.6 × 40 cm), equilibrated and developed with 20 mM potassium phosphate buffer, pH 7.4 containing 0.3 M NaCl and 0.02% sodium azide. Fractions containing inhibitory activity were pooled, adjusted to pH 3.0 with HCl and then extracted twice with an equal volume of ethyl acetate. The solvent layer pooled was dried with sodium sulfate and concentrated to dryness, giving 220 mg of dark-brownish powder. Final purification of pannorin was achieved by reverse phase HPLC (octadecylsilyl). The column (1.0 × 30 cm) was developed with a mixture

of acetonitrile - 0.1% H_3PO_4 (1 : 1) at a flow rate of 3 ml/minute. The active fractions were extracted with ethyl acetate. The solvent layer was dried with sodium sulfate and evaporated to dryness to give 20 mg of pure pannorin.

Physico-chemical Properties and Structural Determination

Pannorin (**1**) is a yellow amorphous solid. It is soluble in methanol and ethanol but not in water. Physico-chemical data of **1** are summarized in Table 1. The EI-MS, HREI-MS spectrum and ^{13}C NMR data for **1** established $C_{14}H_{10}O_5$ (*m/z* 258) as the molecular formula. The IR spectrum of **1** (Fig. 4) suggested the presence of an α-pyrone ring at 1695, 1680, 1633 and 1560 cm^{-1}.[6,7] The UV spectra (Fig. 5) and IR absorption at 3250, 1599 and 1498 cm^{-1} showed the existence of phenolic functions. The ^1H NMR data of **1** revealed 5 singlet proton signals due to one methyl proton at δ 2.66, one olefinic proton at δ 5.55, and 3 aromatic protons at δ 6.54, 6.56 and 7.19 and 3 exchangeable proton signals resulting from 3 hydroxy protons at δ 9.88, 10.03 and 12.18 (Table 2). The decoupled ^{13}C NMR of **1** revealed the presence of 14 carbons (Table 2). The ^{13}C-^1H COSY and deuterium-induced differential ^{13}C-isotope shifts indicated one methyl carbon at δ 23.33, one olefinic methine carbon at δ 88.81, three aromatic methine carbons at δ 100.88, 102.64 and 124.04, four aromatic quaternary carbons at δ 106.39, 107.46, 132.25 and 138.02, one quaternary oxycarbon at δ 154.46, three aromatic carbons bearing a hydroxyl group at δ 156.46, 158.85 and 169.50 and one ester carbonyl carbon at δ 161.03.

In the ^1H detected heteronuclear multiple-bond correlation (HMBC) spectroscopy and correlation

Table 1. Physico-chemical properties of pannorin.

Appearance	Yellow powder
Molecular formula	$C_{14}H_{10}O_5$
HREI-MS (*m/z*)	
Calcd:	258.0527
Found:	258.0520 (M^+)
EI-MS (*m/z*)	258 (M^+), 216, 187, 160, 132, 103
UV λ_{max}^{MeOH} nm (ε)	230 (36,000), 252 (29,000), 275 (18,000), 288 (16,000), 338 (5,900), 352 (6,500)
IR (KBr) cm^{-1}	3250, 1695, 1680, 1633, 1599, 1560, 1498, 870, 835, 820

Fig. 4. IR spectrum of pannorin (**1**) (KBr).

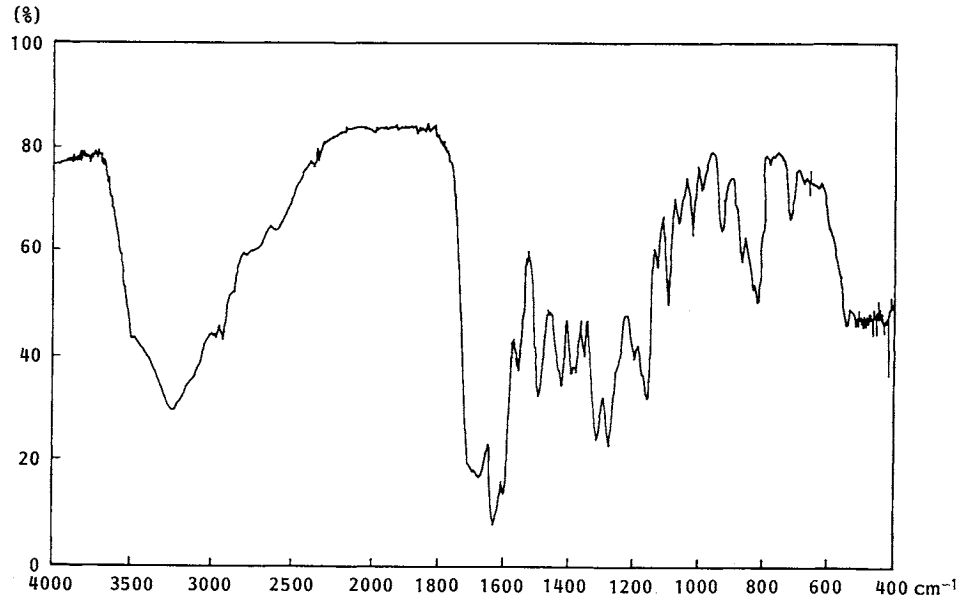


Table 2. ^{13}C and ^{1}H NMR data for pannorin (**1**).

Position	Chemical shift (δ)		Position	Chemical shift (δ)	
	^{13}C	^{1}H		^{13}C	^{1}H
C-2	161.03		C-9	102.64	6.54 (1H)
C-3	88.81	5.55 (1H)	C-10	156.46	
C-4	169.50		C-10a	106.39	
C-4a	107.46		C-10b	154.46	
C-5	132.25		5-CH_3	23.33	2.66 (3H)
C-6	124.04	7.19 (1H)	4-OH		12.18 (1H)
C-6a	138.02		8-OH		10.03 (1H)
C-7	100.88	6.56 (1H)	10-OH		9.88 (1H)
C-8	158.85				

Spectra were measured in DMSO-d_6 at 25°C. TMS was used as an internal reference (δ 0.00).

Fig. 5. UV spectra of pannorin (**1**).

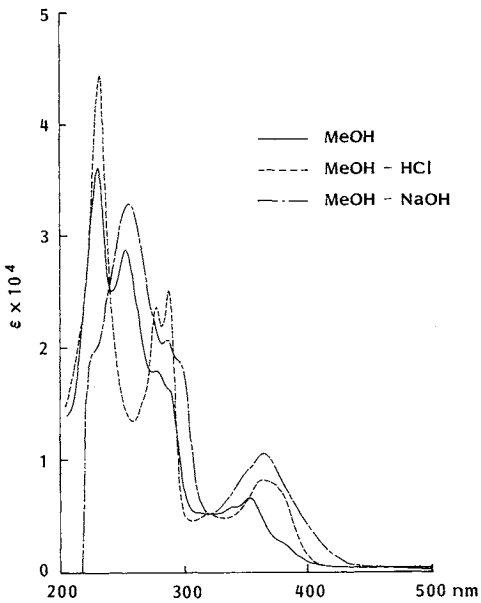
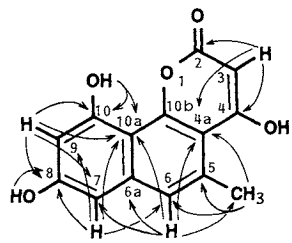


Fig. 6. Long-range ^{1}H-^{13}C coupling observed in the LSPD, HMBC and/or COLOC spectra of pannorin (**1**).



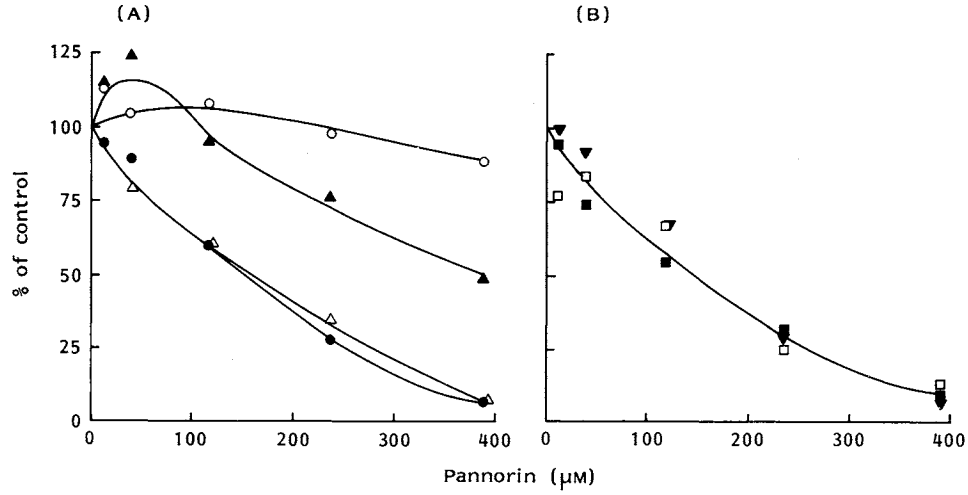
spectroscopy *via* long-range couplings (COLOC) of **1**, long-range couplings were observed between 3-H and each of C-2, C-4, C-4a; 6-H and each of C-4a, C-6a, C-7, C-10a, 5-CH_3; 7-H and each of C-6, C-8, C-9, C-10a; 9-H and each of C-7, C-8, C-10, C-10a; 5-CH_3 and each of C-4a, C-5, C-6 (Fig. 6). In the NOE experiments, irradiation of the 6-H signal at δ 7.19 gave a 17.6% enhancement of the 7-H signal at δ 6.56 and irradiation of the 5-CH_3 signal at δ 2.66 resulted in a 20.9% enhancement of the 6-H signal at δ 7.19. The phenolic resonances at δ 9.88 and 10.03 were assigned to the 10-OH and 8-OH protons, respectively, by long range selective proton decoupling (LSPD) experiments (Fig. 6). The characteristic resonances of C-3 (δ 88.81) and C-4 (δ 169.50) indicated the existence of an enol group at C-4. The enol function was further confirmed by comparison with published NMR data for 4-hydroxycoumarin.[8] Based on the above informations, the structure of pannorin was established to be 4,8,10-trihydroxy-5-methyl-2*H*-naphtho[1,2-*b*]pyran-2-one (**1**).

Biological Activity

Inhibitory effects of pannorin (**1**) on the synthesis of nonsaponifiable lipids (NSL) and sterols by rat liver enzyme system and on HMG-CoA reductase, determined as described previously,[9,10] are shown in Fig. 7. NSL and sterol synthesis from [^{14}C]acetate was inhibited by 50% at a concentration of 160 μM pannorin while incorporation of [^{14}C]mevalonate into NSL and fatty acid synthesis from [^{14}C]acetate

Fig. 7. Effects of pannorin on lipid biosynthesis from labeled precursors and on HMG-CoA reductase.

○ [^{14}C]Mevalonate into NSL, ▲ [^{14}C]acetate into fatty acids, △ HMG-CoA reductase, ● [^{14}C]acetate into NSL, □ lanosterol, ■ squalene, ▼ cholesterol.



were not affected at higher concentrations of **1**. Rat liver HMG-CoA reductase activity was inhibited 50% by **1** at 160 μM. Synthesis of squalene, lanosterol and cholesterol was inhibited similarly by pannorin. The results demonstrate that pannorin is a specific inhibitor of HMG-CoA reductase.

Discussion

Compactin analogs, like compactin (ML-236B) and monacolin K (mevinolin or lovastatin) are potent inhibitors of HMG-CoA reductase and are highly effective in lowering blood cholesterol levels.[1~3] Several of these have been on the market as hypocholesterolemic drugs.[2,3] These compounds are commonly composed of the three portions (Fig. 1): (a) 3,5-dihydroxy-δ-lactone which can be converted to the acid form, (b) hydrophobic decaline ring with or without a hydrophobic side chain ester and (c) ethyl portion bridging these two moieties. Of these portions, the lactone moiety plays a key role in inhibitory activity. Recently, a variety of compounds in which the hydrophobic portion of compactin analogs is replaced by other structures have been reported.[11,12] Some of these are even more potent than compactin analogs in inhibiting HMG-CoA reductase activity.

It should be noted that pannorin (**1**) and compactin analogs are similar in that both have a δ-lactone and hydrophobic decaline structure. Inhibitory activity of **1** was shown to be not comparable to that of compactin, and yet, the present results demonstrate a possible usefulness of its structure in synthesizing a new type of HMG-CoA reductase inhibitors.

Recently, Q-2819, a substance effective in suppression of the formation of lipid peroxides and in scavenging superoxide anion radicals, was isolated from *Chrysosporium* sp. Q-2819.[13] The spectral data of this compound suggested that it was identical to pannorin.

Acknowledgment

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References

1) ENDO, A.: Compactin (ML-236B) and related compounds as potential cholesterol-lowering agents that inhibit

HMG-CoA reductase. J. Med. Chem. 28: 401~405, 1985

2) ENDO, A.: Chemistry, biochemistry and pharmacology of HMG-CoA reductase inhibitors. Klin. Wochenschr. 66: 421~427, 1988
3) ENDO, A. & K. HASUMI: Biochemical aspects of HMG-CoA reductase inhibitors. *In* Advances in Enzyme Regulation 28. *Ed.*, G. WEBER, pp. 53~64, Pergamon Press, 1989
4) HUGHES, S. J.: Revisones hyphomycetum aliquot cum appendice de nominibus rejiciendis. Can. J. Botany 36: 727~836, 1958
5) CARMICHAEL, J. W.: *Chrysosporium* and some other areuriosporic hyphomycetes. Can. J. Botany 40: 1137~1173, 1962
6) VANHAELEN, P. M. & R. VANHAELEN-FASTÉ: Stabilite des anticoagulants dérivés de l'hydroxy-4 coumarine. 1 Tautomérie hydroxy-4 coumarine⇄chromanne dione-2,4⇄hydroxy-2 chromone. Pharm. Acta Helv. 51: 307~312, 1976
7) OBASEKI, A. O. & W. R. PORTER: 4-Hydroxycoumarin/2-hydroxychromone tautomerism: Infrared spectra of 2-^{13}C and 3-D labeled 4-hydroxycoumarin and its anion. J. Heterocycl. Chem. 19: 385~390, 1982
8) CUSSANS, N. J. & T. N. HUCKERBY: Carbon-13 NMR spectroscopy of heterocyclic compounds-IV. A 20-MHz study of chemical shifts and carbon-proton coupling constants in a series of hydroxy, methoxy and glucosyl coumarins. Tetrahedron 31: 2719~2726, 1975
9) KURODA, M. & A. ENDO: Inhibition of *in vitro* cholesterol synthesis by fatty acids. Biochim. Biophys. Acta 486: 70~81, 1977
10) ENDO, A.; M. KURODA & K. TANZAWA: Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B, fungal metabolites having hypocholesterolemic activity. FEBS Lett. 72: 323~326, 1979
11) HOFFMAN, W. F.; A. W. ALBERTS, E. J. CRAGOE, Jr., A. A. DEANA, B. E. EVANS, J. L. GILFILLAN, N. P. GOULD, J. W. HUFF, F. C. NOVELLO, J. D. PRUGH, K. E. RITTLE, R. L. SMITH, G. E. STOKKER & A. K. WILLARD: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. 2. Structural modification of 7-(substituted aryl)-3,5-dihydroxy-6-heptenoic acid and their lactone derivatives. J. Med. Chem. 29: 159~169, 1986
12) HOFFMAN, W. F.; A. W. ALBERTS, P. S. ANDERSON, J. S. CHEN, R. L. SMITH & A. K. WILLARD: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. 4. Side chain ester derivatives of mevinolin. J. Med. Chem. 29: 849~852, 1986
13) IMAI, H.; H. YAZAWA & K. NAGAI (Yamanouchi Pharm.): Substance Q-2819 and process for its preparation. PCT Int. Appl. 89 09769, Oct. 19, 1989 [CA 113: 57452e, 1990]