PF1092A, B and C, New Nonsteroidal Progesterone Receptor Ligands Produced by *Penicillium oblatum*

I. Taxonomy of Producing Strain, Fermentation, Isolation and Biological Activities

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Three new nonsteroidal progesterone receptor ligands, PF1092A, B and C, have been isolated from *Penicillium oblatum*. They were purified from the solid cultures of rice media using ethyl acetate extraction, silica gel and Sephadex LH-20 column chromatographies, and crystallization. All three ligands competitively inhibited [^{3}H]-progesterone binding to porcine uteri cytosol preparations with IC_{50} of 3.0×10 nM (PF1092A), 2.2×10^2 nM (PF1092B) and 2.2×10^3 nM (PF1092C).

Progesterone, which belongs to the group of sex steroid hormones, is suggested to play an important role in many kinds of diseases. RU486, the only clinically available progesterone antagonist, has been reported to have a potential therapeutic effect in the treatment of breast cancer[1], endometriosis[2], uterine leiomyomata[2] and meningioma[3,4], according to initial results from clinical trials. In addition, recent studies suggest that progesterone may promote bone formation in ovariectomized rats[5] and protect against bone loss in breast-feeding women[6]. Thus, agonists of progesterone could serve in the treatment of established osteoporosis. However, present clinically available progesterone agonists and antagonists all possess the steroidal moiety which results in the appearance of the side effects associated with the administration of steroids. This is especially the case for steroidal progesterone antagonists which are not suitable for long-term treatment because of their potent antiglucocorticoid activity in humans. For example, side effects thought to be caused by antiglucocorticoid activity were reported in some clinical trials of RU486[1,4]. For these reason, it seems imperative that new agonists and antagonists of progesterone be isolated that lack the steroid moiety.

In the course of screening for progesterone receptor ligands, we discovered the presence of three nonsteroidal and functional ligands, namely PF1092A, B and C, in the extracts of cell cultures of *Penicillium oblatum*.

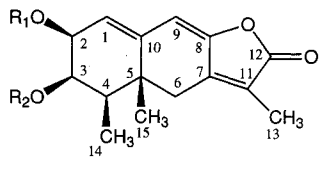
In this paper, we describe the taxonomy and fermentation of strain PF1092, and the isolation and biological activities of these new progesterone receptor ligands (Fig. 1). The physico-chemical properties, structure elucidation and total synthesis of PF1092A, B and C will be described in the following papers[7,8].

Materials and Methods

Taxonomic Studies

The fungal strain PF1092 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession number FERM P-14724. Taxonomic studies of the strain PF1092 were done according to the method of PITT[9]. The color guide of KORNERUP and WANSCHER[10] was used in the determination and standardization of colors. CYA (yeast extract 0.5%, glucose 3.0%, K_2HPO_4 0.1%, $NaNO_3$ 0.2%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%,

Fig. 1. Chemical structure of PF1092A, B and C.



	R_1	R_2
PF1092A	H	$COCH_3$
PF1092B	$COCH_3$	H
PF1092C	H	H

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agar 1.3%) and MEA media (malt extract 2.0%, peptone 0.1%, glucose 2.0%, agar 2.0%) were used for the identification of the fungus.

Fermentation

Strain PF1092 was inoculated from an agar slant into a 100-ml Erlenmeyer flask containing 20 ml of seed medium made up of 1.0% starch, 1.0% glucose, 0.6% wheat germ, 0.2% soybean meal, 0.3% yeast extract, 0.5% Polypepton (Nihon Pharmaceutical Co., Ltd.), 0.2% $CaCO_3$ and tap water (pH 7.0 before sterilization). The inoculated flask was shaken on a rotary shaker (200 rpm) at 25°C for 3 days. One milliliter of the first seed culture was transferred into 100 ml of the same medium in 500-ml Erlenmeyer flasks. After shaking at 25°C for 3 days, five milliliters of the second seed culture was added to a 500-ml Erlenmeyer flasks containing 100 g of production medium made up of rice containing water and 2.5% soybean meal (pH non-adjusted). The inoculated flasks were incubated as stationary phase cultures for 17 days at 28°C.

The progress of fermentation was monitored by high performance liquid chromatography (HPLC). The rice media sampled at day 7, 10, 12, 14, and 17 were extracted with twice their weight of 67% aq. acetone. The extract was filtered and the filtrate was injected into the injector of a Shimadzu LC-6AD HPLC. Capcell pak C_{18} (Shiseido Co., Ltd.) column was used at a flow rate of 0.8 ml/minute. The mobile phase was an aqueous solution of 50% acetonitrile. PF1092s were monitored with UV detector (Shimadzu SPD-6AV) at 320 nm. The retention time of PF1092A, B and C were 3.9 minutes, 4.9 minutes and 3.3 minutes, respectively.

Progesterone Binding Assay

(a) Receptor Preparation

Unless specified, the following procedures were carried out at 0~4°C. The uteri of porcine were homogenized in buffer consisting of 5 mM KH_2PO_4 (pH 7.4), 30% glycerol, 0.1% α-thioglycerol and 25 μg/ml leupeptin and the homogenates were then sonicated with a Branson Sonifier 450 on ice followed by centrifugation at 100,000 × *g* for 30 minutes. The resulting supernatant (cytosol) was stored at −80°C until use and used as a source of progesterone receptors for the binding assays.

(b) [^{3}H]-Progesterone Binding Assay

The reaction mixture (100 μl) containing 50 mM KH_2PO_4 (pH 7.4), 10% glycerol, 0.1% α-thioglycerol, 25 μg/ml leupeptin, 1 mM EDTA and [1,2,6,7-^{3}H(N)] progesterone (final concentration of 2.0 nM at 6.8 kBq/ml - New England Nuclear, specific activity 3.4 TBq/mmol), porcine uteri cytosol preparation (1~2 mg protein/ml) and a test sample were incubated for 1 hour at 4°C. After incubation, 100 μl of dextran coated charcoal solution consisting of 0.5% Norit A (Nacalai Tesque, Inc., Kyoto, Japan) and 0.05% Dextran T-70 (Pharmacia Fine Chemicals, Ltd., Uppsala, Sweden) were added to the incubation mixture and further incubated at 4°C for 10 minutes. The mixture was then centrifuged at 1800 rpm for 5 minutes. The radioactivity of 100 μl of the supernatant was counted in 2 ml of Aquasol-2 (Packard Instrument B.V. Chemical Operations, Groningen, Netherlands) with a liquid scintillation counter (Beckman LS6500). Non specific binding was defined as the binding observed when 10 μg/ml of cold progesterone was added to the reaction mixture.

Results

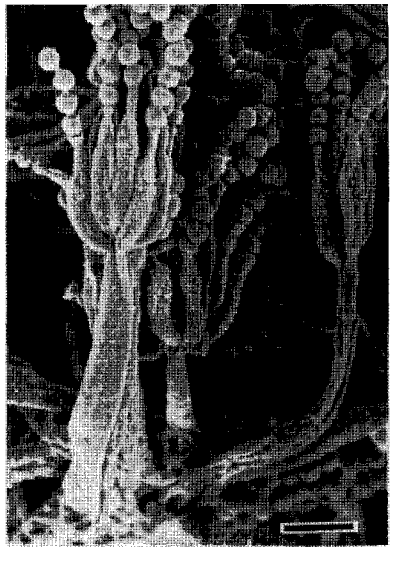
Taxonomy of Strain PF1092

The mycological characteristics of strain PF1092 were as follows. Colonies growing on CYA at 25°C attained a diameter of 20~25 mm in 7 days, and were white, floccose, and slightly funiculose. Conidiogenesis was sparse but was produced in sufficient quantities to influence colony appearance within 7 days. Exudate and soluble pigment were absent. The reverse sides of the colonies were Chamois (4C5). Colonies growing on MEA at 25°C attained a diameter of 30~40 mm in 7 days, and were white, floccose, and slightly funiculose. Conidiogenesis was sparse. A pale brown exudate were produced. The reverse side of the colonies were Light Brown (5D5). At 37°C the growth-rate was increased and conidiogenesis was abundantly produced.

Penicilli were monoverticillate. Stipes of conidiophores were verrucose walled, 30~70 × 2.5~3.5 μm. Phialides were acerose, smooth to slightly roughened, 4~7 per stipe, 8~10 × 2.5~3 μm. Conidia were globose to oblate, smooth-walled, 2~2.5 μm, borne in short columns (Fig. 2).

Fig. 2. Penicilli of *Penicillium oblatum* PF1092 on CYA, 7 days, 25°C.

Scale bar represents 5 μm.



Based on the properties listed above, strain PF1092 was identified as *Penicillium oblatum* PITT *et* HOCKING[11].

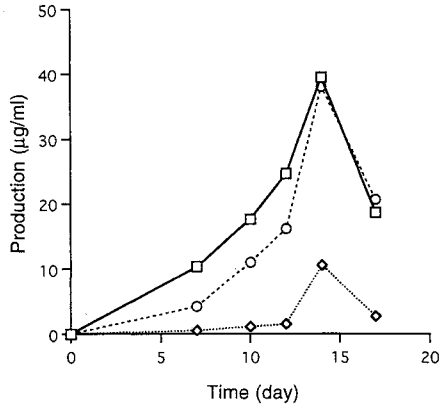
Production of PF1092s

A typical time course of the fermentation of PF1092A, B and C are shown in Fig. 3. The production of PF1092A, B and C reached a maximum after 14 days of fermentation and the yields were 39 μg/ml, 11 μg/ml and 38 μg/ml, respectively. The inhibitory effect on the [^{3}H]-progesterone binding assay (final broth extract concentration of 0.3% in the assay) reached a level of 91% inhibition after 14 days of fermentation (data not shown).

Isolation

The isolation scheme is shown in Fig. 4. The fermented rice medium (6 kg) was extracted with EtOAc (12 liters) and the extract was concentrated to dryness under reduced pressure. The residue (15.3 g) was applied to a column of silica gel (400 g). The column was washed with $CHCl_3$ and eluted with $CHCl_3$-MeOH (100:1) and (100:3). The eluted fraction was evaporated to give an oily residue (4.3 g). The residue was applied to a column of silica gel (150 g). The column was washed with Hexane-EtOAc (8:1~5:1) and eluted subsequently with Hexane-EtOAc (4:1), (3:1), (2:1) and (1:1). The eluted fraction (Hexane-EtOAc (4:1~3:1)) was evaporated to give a pale yellow powder containing PF1092B (282 mg), and the eluted fraction (Hexane-EtOAc (2:1~1:1)) was evaporated to give a pale yellow powder containing PF1092A and PF1092C (763 mg). The pale yellow powder containing PF1092B was applied to a column of Sephadex LH-20 (700 ml). The column was eluted with MeOH and the eluted fraction was evaporated to give a colorless powder of PF1092B (157 mg). The colorless powder was dissolved in $CHCl_3$-MeOH and crystallized to give colorless needles of PF1092B (49 mg). The pale yellow powder containing PF1092A and PF1092C was applied to a column of Sephadex LH-20 (700 ml). The column was eluted with MeOH and the eluted fractions was evaporated to give a colorless powder of PF1092A (298 mg) and PF1092C (159 mg). The colorless powder was dissolved in $CHCl_3$-MeOH and crystallized to give colorless needles of PF1092A (60 mg) and PF1092C (30 mg).

Fig. 3. Time course of PF1092s production.
□ PF1092A, ◇, PF1092B, ○ PF1092C.



Biological Activities

PF1092A, B and C inhibited [^{3}H]-progesterone binding to the progesterone receptor isolated from porcine uteri in a dose dependent manner (Fig. 5). The concentrations required for IC_{50} were 3.0×10 nM for

Fig. 4. Isolation procedure of PF1092A, B and C.

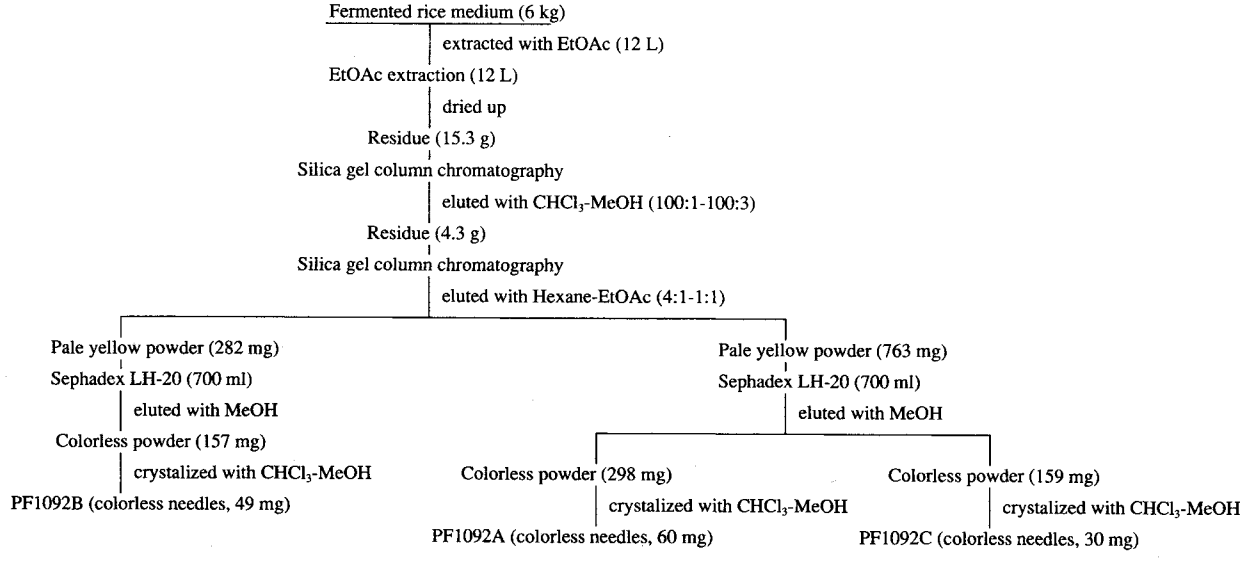
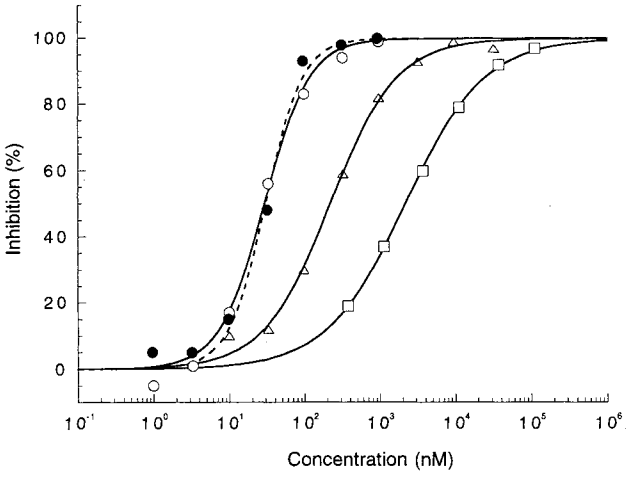


Fig. 5. Inhibitory effect of PF1092s on [^{3}H]-progesterone binding to the porcine uteri cytosol.

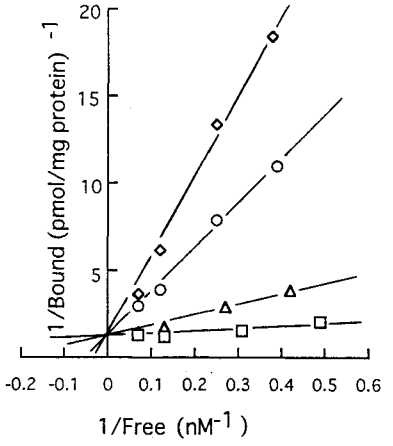
○ PF1092A, △ PF1092B, □ PF1092C, ● progesterone.



[^{3}H]-Progesterone concentration of 2.0 nM, progesterone $IC_{50}=3.1\times10$ nM, PF1092A $IC_{50}=3.0\times10$ nM, PF1092B $IC_{50}=2.2\times10^{2}$ nM, PF1092C $IC_{50}=2.2\times10^{3}$ nM.

Fig. 6. Lineweaver-Burk plot of the inhibition of [^{3}H]-progesterone binding by PF1092A.

□ control, ◇ PF1092A 3.3×10^{-7} M, ○ PF1092A 1.6×10^{-7} M, △ PF1092A 3.3×10^{-8} M.



PF1092A, 2.2×10^{2} nM for PF1092B and 2.2×10^{3} nM for PF1092C. The inhibitory effect of PF1092A was as potent as that of progesterone. Lineweaver-Burk plot showed competitive inhibition by PF1092A of progesterone binding to the progesterone receptor (Fig. 6).

The antimicrobial activities of PF1092A, B and C were evaluated by the paper disc method (20 μl of 1 mg/ml solution). They had no microbial activity against Gram-positive bacteria (*Bacillus subtilis* ATCC6633, *Micrococcus luteus* ATCC9341, *Staphylococcus aureus* 209P), Gram-negative bacteria (*Escherichia coli* NIHJ) and several kinds of fungi (*Saccharomyces cerevisiae* SHY3, *Candida albicans* M9001, *Candida pseudotropicalis* M9035, *Cryptococcus neoformans* M9010, *Debaryomyces hansenii* M9011, *Trigonopsis variabilis* M9031, *Schizosaccharomyces pombe* M9025 and *Hansenula schneggi* IAM4269).

Neither PF1092A, B or C showed cytotoxcity *in vitro* against HMV-1 (melanoma, human), KB (epidermoid carcinoma, human), MKN-1 (stomach cancer, human), PC-14 (lung adenocarcinoma, human), and T24 cells (bladder carcinoma, human) at the concentration of 10 μg/ml.

Discussion

Progesterone plays important roles not only in the reproductive organs but also in bone and the central nerve system (CNS)[12]. Pharmaceutical studies of progesterone antagonists have been performed using RU486 and other synthetic steroidal progesterone antagonists. Even though the interpretation of these studies has been complicated by the ancillary steroidal hormonal activities of these compounds, preclinical and clinical studies have indicated the therapeutic benefit of progesterone antagonists in cases of breast cancer[1], endometriosis[2], uterine leiomyomata[2] and meningioma[3,4]. In addition, recent studies have revealed that progesterone agonists could be potentially efficacious for the treatment of established osteoporosis[5,6]. All of the progesterone agonists or antagonists currently available or in clinical development are derived from steroids and consequently, many of them show cross-activity with other steroid receptors. The major aim of our research has been to find highly selective progesterone agonists or antagonists free from steroidal side-effects.

Recently, some nonsteroidal progesterone receptor ligands have been synthesized from cyclocymopol monomethyl ether derived from the natural products, cymopol[13,14] and 3-aryl-1-benzoyl-1,4,5,6-tetrahydropyridazines[15,16]. However, PF1092s are the first progesterone receptor ligands found to be produced by a microorganism.

PF1092s inhibited [^{3}H]-progesterone binding to porcine uteri in a dose dependent manner. The inhibitory effect of PF1092A is more potent than those of PF1092B and C. This result indicates that the 3-acetoxy moiety plays an important role in the structure-activity relationship.

PF1092A inhibited [^{3}H]-progesterone binding to porcine uteri progesterone receptors competitively. Therefore, PF1092A or its derivatives may modulate positively or negatively the progesterone receptor without modulating other steroidal hormone receptors. We envisage that PF1092s could be optimized for therapeutic use as an agonist or an antagonist.

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