Communications to the Editor

STEVASTELINS, NOVEL IMMUNOSUPPRESSANTS PRODUCED BY *Penicillium*

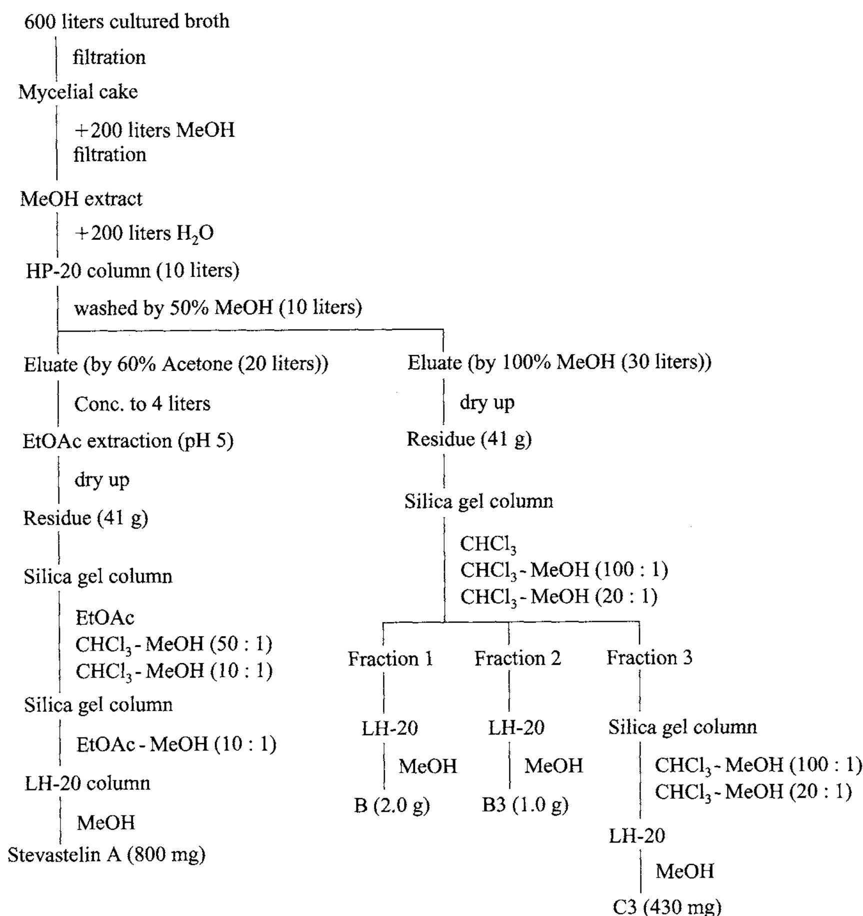
Sir:

Antigen recognition and activation of T cells is a key step in the immune system. Cyclosporin A, which is used clinically as an immunosuppressant, is known to repress T cell activation by inhibition of cytokine gene expression[1]. In our screening for novel immunosuppressants, we isolated four novel depsipeptide congenors, NK374186A, B, B3 and C3, and showed their inhibitory activities against T cell actvation[2]. In this report, we name the congenors stevastelin and show their immunosuppressive effects *in vitro* and low toxicity *in vivo*.

The activity of the immunosuppressants was monitored by the inhibition of human T cell proliferation, stimulated by the OKT3 antibody against the T cell surface antigen CD3. Human T lymphocytes were isolated by density gradient centrifugation with a commercial reagent(Nycomed). Cells were collected, washed twice with PBS, and inoculated into 96-well microplates with 10% FCS-RPMI1640 medium containing 10 mM HEPES buffer. OKT3 antibody was added to a final concentration of 2 ng/ml. Microbial samples were added 1 hour later, and T cell growth was monitored by ^{3}H-thymidine uptake at 3 days.

The microbial cultured broth which could suppress T cell proliferation was selected. Among 5000 cultures, only one culture was selected:

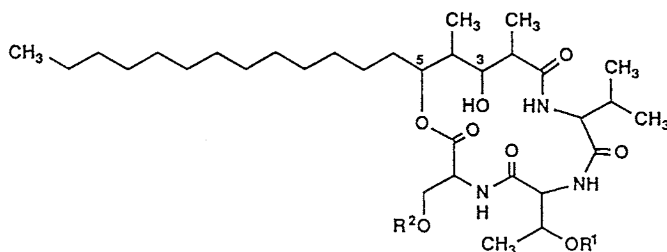
Fig. 1. Purification of the stevastelins.



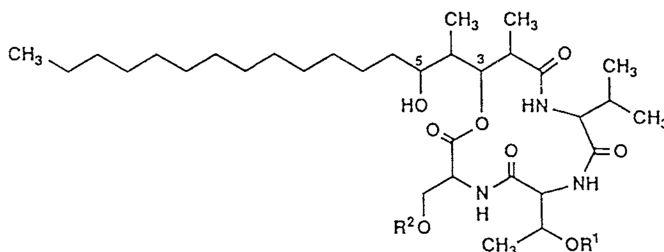
Penicillium sp. NK374186 strain, which was isolated from soil collected in Niigata Prefecture, Japan. The immunosuppressants were produced by 200 liters jar fermentation culture in a medium containing 2% glucose, 1% lactose, 1% sucrose, 1% glycerol, 2% soybean flour, 0.5% peptone, 0.2% $NaNO_3$, and 0.1% $MgSO_4$. The maximum production was attained after 4 days cultivation at 1/2 vvm, 250 rpm, 25°C. The compounds were isolated from the cultured broth as shown in Fig. 1. The compounds were extracted from mycelia by MeOH and purified by Diaion HP-20, silica gel, and Sephadex LH-20 column chromatography. From 600 liters of cultured broth, 800 mg of A, 2.0 g of B, 1.0 g of B3, and 430 mg of C3 were obtained in the form of colorless powders.

Stevastelins A, B, B3, and C3 were soluble in $CHCl_3$, EtOAc, and MeOH, but insoluble in H_2O. They gave a positive color in the phosphomolybdic acid/sulfuric acid reaction and the toluidine-chlorine reaction, and a negative color in the ninhydrin reaction. On silica gel TLC (Merck Art. No. 5715) developed with $CHCl_3$ - MeOH (20 : 1), stevastelins A, B, B3, and C3 gave single spots at Rf=0.12, 0.35, 0.49, and 0.33, respectively. The molecular formulas and molecular weights of stevastelins A, B, B3, and C3 were established as $C_{34}H_{61}N_3O_{12}S$ (735.91), $C_{34}H_{61}N_3O_9$ (655.85), $C_{34}H_{61}N_3O_9$ (655.85), and $C_{32}H_{59}N_3O_8$ (613.81), respectively based on HRFAB-MS and ^{13}C NMR spectra. The structures of these novel compounds were determined by means of chemical and spectroscopic methods including 2D NMR correlation spectroscopy. The stevastelins have a novel depsipeptide structrue, basically composed of valine, threonine, serine and 3,5-dihydroxy-2,4-dimethylstearic acid (Fig. 2). In the case of stevastelin A threonine is substituted for *O*-sulfonylthreonine, and in the case of stevastelin A, B and B3 serine is substituted for *O*-acetylserine (Fig. 2). In stevastelin B3 and C3,

Fig. 2. The structures of stevastelin A and B (upper) and stevastelin B3 and C3 (lower).



	R^1	R^2	MF/MW
A	SO_3H	CH_3CO	$C_{34}H_{61}N_3O_{12}S$/735
B	H	CH_3CO	$C_{34}H_{61}N_3O_9$/655



	R^1	T^2	MF/MW
B_3	H	CH_3CO	$C_{34}H_{61}N_3O_9$/655
C_3	H	H	$C_{32}H_{59}N_3O_8$/597

Table 1. Effect of the stevastelins on lymphocyte blastogenesis.

Compound	IC_{50} (μg/ml)		
	Human (peripheral)	Mouse (spleen)	
	OKT-3	LPS[a]	ConA[b]
A	6.1	1.3	>33
B	1.8	1.5	11.0
B3	0.42	1.2	3.8
C3	6.2	4.2	>10

[a] Mouse spleen cells were stimulated with LPS and blastogenesis inhibition was monitored by ^{3}H-thymidine uptake at 3 days.

[b] Mouse spleen cells were applied to a T cell column for T cell enrichment. Cells were stimulated by ConA and blastogenesis inhibition was monitored by ^{3}H-thymidine uptake at 3 days.

cycilization occured at C3 carbon of the stearic acid (Fig. 2). The determination of the structure of the stevastelins will be described in detail elsewhere.

The growth inhibition activity of the stevastelins against OKT-3-stimulated human T cell proliferation is shown in Table 1. Stevastelin B3 and B showed potent activity (IC_{50}=0.42 μg/ml and 1.8 μg/ml, respectively), whereas stevastlin A and C3 has weaken activity. The effects on the blastogenesis of mouse spleen lymphocytes were also examined. Activation was carried out with concanavallin A (ConA) for T cells and lipopolysaccharide (LPS) for B cells (Table 1). Mouse T cell bastogenesis (ConA) was also blocked by stevastelin B and B3 but not by A and C3. These results showed stevastelin B and B3 were more effective and suggested that the substitution of -OH funtion of threonine or serine affected stevastilin activities against T cell blastogenesis. On the other hand, the stevastelins showed the tendancy to be more active against B cells rather than T cells in mouse cells. However, an effect on human B cells has not yet been demonstrated. Thus, stevastelin B and B3 had potent immunosuppressive activity against blastogenesis.

The acute toxicity of stevastelin A and B in mice was examined. Each compound was administrated to four mice for 10 days (one dose/day) at 100mg/kg (ip). We observed no difference in the survival rate, body weight change and autopsy observation from the control animals. Thus, stevastelin A and B showed low acute toxicity *in vivo*.

T cell activation is considered to participate in several autoimmune diseases such as rheumatoid arthritis and transplantation rejection. Stevastelin B and B3 blocked human T cell activation *in vitro*, and stevastelin B showed low acute toxicity in mice. Therefore, stevastelin B and B3 may be a useful tool for the investigation of T cell activation and also to have applications as immunosuppressants. Their mode of action is also interesting. Further experiments on the biological properties of the stevastlins will be reported elsewhere.

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