Generation of Novel Rapamycin Structures by Microbial Manipulations

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A new rapamycin producing culture was isolated and designated as *Actinoplanes* sp. N902-109. The addition of a cytochrome P-450 inhibitor and precursor feeding using this culture, and biotransformation approaches generated new rapamycin analogs with modifications at C-4, C-9, C-16, C-27, C-28 and/or C-39. The immunosuppressive activity of the resulting analogs was established in the mixed lymphocyte reaction assay.

Rapamycin is a 31-membered macrocyclic lactone known to be produced by *Streptomyces hygroscopicus* ATCC 29253[1,2]. Although rapamycin was originally isolated as an antifungal agent, its immunosuppressive effect has recently attracted more attention. In the last few years this interest has been heightened, because its mechanism of action appears to be different from that of FK506, a structurally related immunosuppressive agent produced by *Streptomyces tsukubaensis* No. 9993[3]. Thus, while both rapamycin and FK506 bind to a family of proteins called FKBP, the subsequent steps of T cell activation appear to be different[4]. Whereas the target of the FK506-FKBP complex has been established to be calcineurin, a protein phosphatase, a purported effector molecule of the rapamycin-FKBP complex has been reported only very recently[5~7].

The biosynthetic pathway of rapamycin has been postulated and substantiated by labelling studies using acetate, propionate and methionine[8]. According to this scheme, the majority of the rapamycin macrolide ring (except carbons 12 and 13) is formed from 6 acetate and 7 propionate units. The C_7 unit containing the cyclohexyl ring is derived from the shikimic acid pathway, and the pipecolic acid moiety is expected to be derived from this amino acid or lysine. This biosynthetic pathway suggests that the oxygen functionalities at C-9 and C-27 are added in the succeeding steps, which in turn suggests that addition of a P-450 inhibitor can generate analogs of different oxidation states. The proposed incorporation of pipecolic acid suggests that other amino acids might be likewise incorporated. In addition, biotransformation techniques often provide specific reactions under mild conditions for conversions of complex molecules containing sensitive functionalities. FK506 has been successfully biotransformed to generate novel analogs[9].

As part of a program aimed at discovering novel immunosuppressive agents, we have identified a new rapamycin producing culture, designated *Actinoplanes* sp. N902-109. In order to generate novel analogs with potentially reduced renal and CNS toxicity associated with existing immunosuppressive agents, we have investigated microbial manipulations such as addition of a cytochrome P-450 inhibitor, precursor feeding and biotransformation approaches. In this paper, we describe taxonomy of the producing organism, fermentation, isolation, structure elucidation and biological activities of the new rapamycin analogs (Fig. 1).

Results

Taxonomy

A culture, designated N902-109, was isolated from a soil sample collected in Shizuoka Prefecture, Japan. Aerial mycelium was absent. The substrate mycelium mostly ranged from orange yellow, orange, bright orange to dark orange; it was dark brown on tyrosine agar, and pale pink to pale lavender on Czapek-sucrose agar. The soluble pigment, if present, was pale yellow to yellow brown (Table 1). When grown on M3 agar[10] for three weeks, spores were produced in compact chains and

This article is dedicated to Dr. S. Ōmura for the 60th anniversary of his birth.

Fig. 1. Structures of rapamycin analogs generated by biosynthetic manipulations.

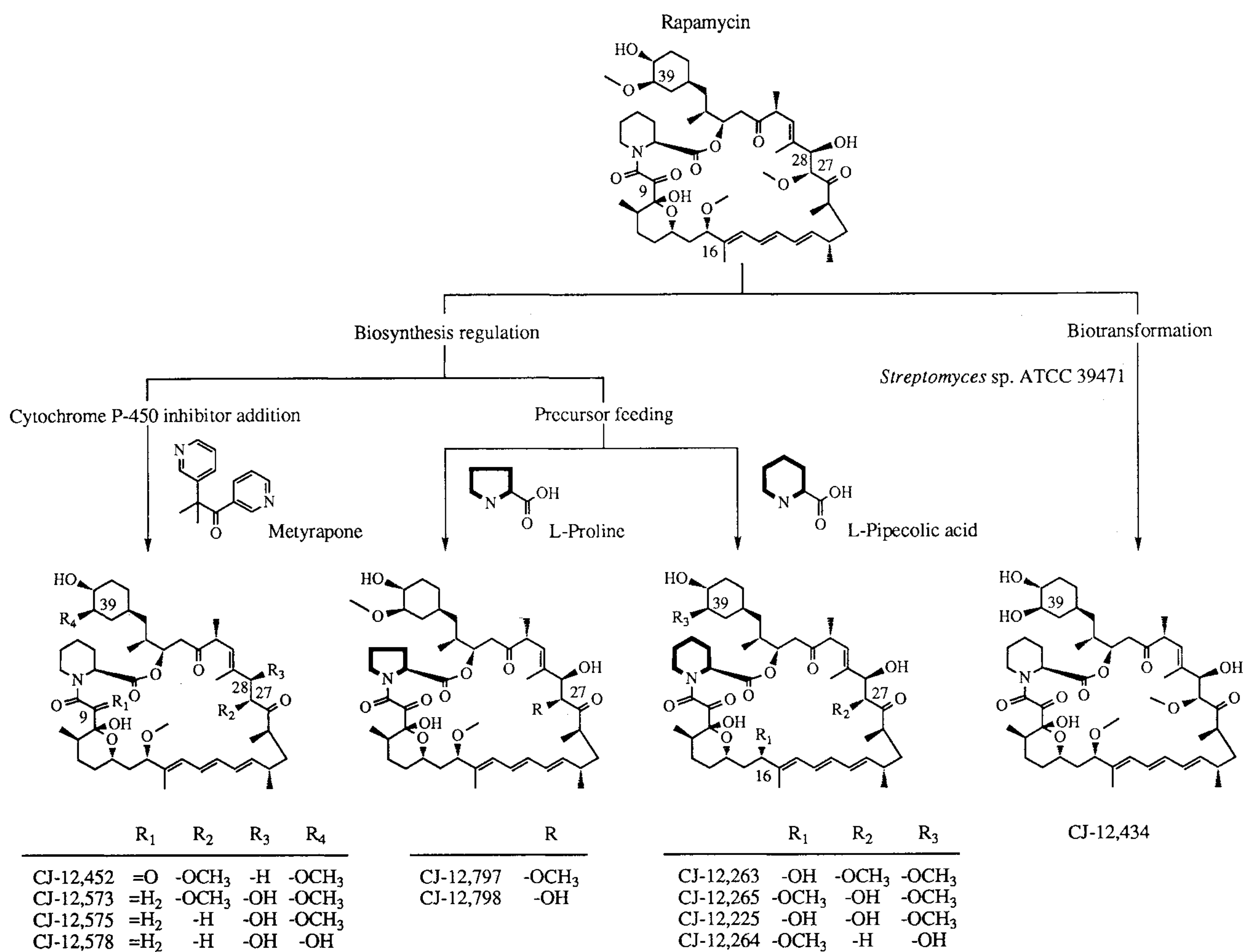


Table 1. Culture characteristics of *Actinoplanes* sp. N902-109.

Medium	Growth	Aerial mycelium	Mycerial color	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Absent	Orange (4 la, 4 lc)	Yellowish brown (3 lc)
Oatmeal agar (ISP-3)	Moderate	Absent	Pale orange yellow (3 ea, 4 ea)	Pale yellowish (2 ea)
Inorganic salts-starch agar (ISP-4)	Moderate	Absent	Bright orange (4 ia) with some red dots (6 1/2 ne)	None
Glycerol-asparagine agar (ISP-5)	Poor to moderate	Absent	Orange yellow (3 ga, 3 ia)	None
Czapek-sucrose agar	Moderate	Absent	Pale pink to pale lavender (5 ca, 5 gc) with red dots (6 le, 6 pi)	None
Glucose-asparagine agar	Moderate to good	Absent	Bright orange (4 na)	None
Gordon and Smith's Tyrosine agar	Moderate	Absent	Dark brown (5 lg)	Dark brown (5 ni)
Bennett's agar	Good	Absent	Orange to dark orange (5 la, 5 nc, 5 pc)	Yellowish brown (3 nc)
Emerson's agar	Good	Absent	Yellowish orange (4 la, 4 ia, 4 lc)	None

aggregated into globose to subglobose clusters which measured $3.5 \sim 8.0\,\mu$m diameter. The spores were globose, oval to elliptical and measured $1.0 \sim 1.2\,\mu$m diameter or $1.0 \sim 1.8 \times 0.9 \sim 1.2\,\mu$m. They were motile, especially when suspended in 1% sucrose solution. The whole-cell hydrolysates revealed the presence of *meso*-diaminopimelic acid, galactose, mannose and ribose. The culture is characterized by the orange to bright orange substrate mycelium, the irregularly shaped hyphal masses on the substrate mycelium, and the motile spores which are born in chains which aggregate into spore clusters. It is related to members of *Actinoplanes* and

Table 2. Physiological characteristics of *Actinoplanes* sp. N902-109.

Melanin production	-
H_2S production	-
Nitrate reduction	-
Gelatin liquefaction	+
Starch hydrolysis	+
Milk coagulation	+
Milk peptonization	+
Casein digestion	+
Tyrosine digestion	±*
Cellulose decomposition	-
Carbohydrate utilization	
D-Glucose	+
L-Arabinose	+
D-Fructose	+
Inositol	-
D-Mannitol	+
Raffinose	±
Sucrose	+
D-Xylose	+
Rhamnose	-

* Doubtful.

Couchioplanes[11]. Except for the absence of a sporangial wall, the culture resembles *A. kanagawaensis*[12], *A. missouriensis* Couch, *A. auranticolor* (Couch) Palleroni, and *A. teichomyceticus* Parenti, Beretta, Berti & Arioli. However, it differs from *A. kanagawaensis* and *A. auranticolor* in the failure to produce hydrogen sulfide, the ability to coagulate milk, and the failure to utilize rhamnose. It is different from *A. missouriensis* in its failure to reduce nitrate and its failure to utilize rhamnose. Although it shares the same pattern of carbohydrate utilization with *A. teichomyceticus*, it differs by the smaller sporangia (spore clusters) and spores, the failure to produce melanin and hydrogen sulfide, the failure to reduce nitrate, and the ability to coagulate milk (Table 2). In a lack of sporangial wall, the culture resembles members of the recently described genus *Couchioplanes*, from which it differs in the presence of *meso*-diaminopimelic acid. On the basis of these results, the culture N902-109 is tentatively assigned to the genus *Actinoplanes* and considered as *Actinoplanes* sp. It has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession number FERM BP-3832.

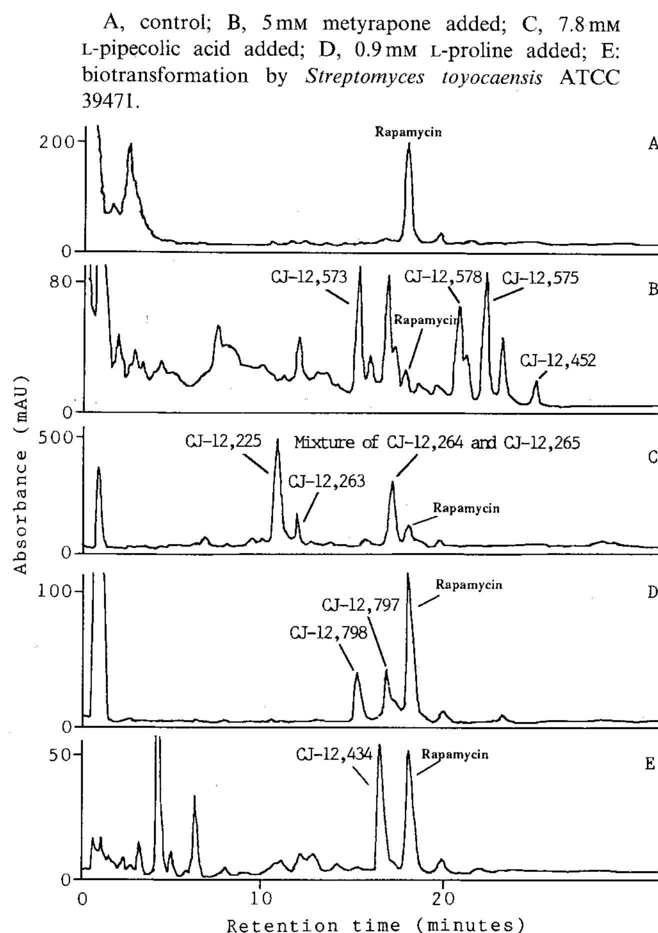
Microbial Manipulations

Cytochrome P-450 Inhibitor Addition

The new rapamycin producer, *Actinoplanes* sp. N902-109 grown in a production medium described in the experimental section produced rapamycin at 100 mg/liter level as shown in Fig. 2A. Five mM of metyrapone

Fig. 2. HPLC profiles of ethyl acetate extracts from fermentation broths.

A, control; B, 5 mM metyrapone added; C, 7.8 mM L-pipecolic acid added; D, 0.9 mM L-proline added; E: biotransformation by *Streptomyces toyocaensis* ATCC 39471.



(cytochrome P-450 inhibitor) was added to the culture to suppress rapamycin production. As a result, 4 rapamycin analogs CJ-12,452 (28-dehydroxyrapamycin, 15 mg/liter), CJ-12,573 (9-deoxorapamycin, 40 mg/liter), CJ-12,575 (9-deoxo-27-demethoxyrapamycin, 40 mg/liter) and CJ-12,578 (9-deoxo-27-demethoxy-39-*O*-demethylrapamycin, 35 mg/liter) were generated as shown in Fig. 2B.

Precursor Feeding

Precursor feeding is often used for improvement of productivity. L-Pipecolic acid feeding to the fermentation broth of *Actinoplanes* sp. N902-109 enhanced production of 4 rapamycin analogs, CJ-12,225 (16, 27-*O*-bisdemethylrapamycin, 270 mg/liter), CJ-12,263 (16-*O*-demethylrapamycin, 30 mg/liter), CJ-12,264 (27-demethoxy-39-*O*-demethylrapamycin, 75 mg/liter) and CJ-12,265 (27-*O*-demethylrapamycin, 75 mg/liter) as shown in Fig. 2C. Furthermore, in order to replace the L-pipecolic acid moiety by other amino acids, a variety of substrates were fed to the rapamycin producer. When

L-proline was fed to the fermentation broth, two rapamycin analogs, CJ-12,797 (prolylrapamycin, 25 mg/liter) and CJ-12,798 (27-*O*-demethylprolylrapamycin, 25 mg/liter) were detected by HPLC (Fig. 2D).

Biotransformation

Biotransformation of rapamycin was examined by using the 34 actinomycetes (15 macrolide and 19 polyether producers). One of them, the culture *Streptomyces toyocaensis* subsp. *humicola* ATCC 39471 was found to transform rapamycin to CJ-12,434 (39-*O*-demethylrapamycin) at the conversion ratio of 50% (Fig. 2E).

Biological Activity

The mixed lymphocyte reaction (MLR) inhibitory activity of the new rapamycin analogs is shown in Table 3. CJ-12,225, CJ-12,263, CJ-12,434 and CJ-12,265 (16-*O*-, 27-*O*- and 39-*O*-demethylrapamycin and 16, 27-*O*-bisdemethylrapamycin) showed activity comparable to rapamycin ($IC_{50}<10$ nM). The activitiy of CJ-12,797 and CJ-12,798 (prolylrapamycin analogs) decreased about 10-fold compared to demethylrapamycin analogs. CJ-12,575 and CJ-12,578 (9-deoxorapamycin analogs), CJ-12,452 (28-dehydroxyrapamycin) and CJ-12,264 (27-demethoxy-39-*O*-demethylrapamycin) remarkably lost activity ($IC_{50}>100$ nM). CJ-12,434 inhibited mitogen-stimulated proliferation of human mononuclear cells (MNCs) at similar doses of rapamycin and also suppressed delayed type hypersensitivity (Table 4).

Table 3. MLR inhibitory activity of rapamycin analogs.

Compound	IC_{50} (nM)
Rapamycin	1.3
CJ-12,263 (16-*O*-Demethylrapamycin)	2.8
CJ-12,265 (27-*O*-Demethylrapamycin)	2.8
CJ-12,434 (39-*O*-Demethylrapamycin)	3.9
CJ-12,225 (16,27-*O*-Bisdemethylrapamycin)	8.0
CJ-12,797 (Prolylrapamycin)	24.0
CJ-12,798 (27-*O*-Demethylprolylrapamycin)	26.0
CJ-12,573 (9-Deoxorapamycin)	58.0
27-Demethoxyrapamycin	90.0
CJ-12,264 (27-Demethoxy-39-*O*-demethylrapamycin)	130
CJ-12,575 (9-Deoxo-27-demethoxyrapamycin)	170
CJ-12,452 (28-Dehydroxyrapamycin)	500
CJ-12,578 (9-Deoxo-27-demethoxy-39-*O*-demethylrapamycin)	>1,800

Discussion

Using an MLR screen we have isolated a new rapamycin producing culture. From its morphology and physiological characteristics, it is considered as *Actinoplanes* sp. This culture produced rapamycin at 100 mg/liter level under optimum fermentation conditions. The titer level is comparable to that of the deposited culture *Streptomyces hygroscopicus* ATCC 29253.

In order to generate novel rapamycin analogs, we used three techniques, *i.e.*, addition of a cytochrome P-450 inhibitor, precursor feeding and biotransformation, and generated 11 new rapamycin analogs (Fig. 1). In the past, cytochrome P-450 inhibitors have been utilized to study the biosynthesis of natural products. For example, the cytochrome P-450 inhibitors S-33070 and ancymidol have been used to generate new analogs of chaetoglobosin and nigericin, respectively[13]. We used commercially available metyrapone as a cytochrome P-450 inhibitor. Addition of this agent to the fermentation medium generated 4 new analogs, which are 9-deoxo- and/or 27-demethoxy analogs (CJ-12,573, CJ-12,575 and CJ-12,578), as expected from the postulated biosynthetic pathway and a 28-dehydroxy analog (CJ-12,452). Detection of the 28-dehydroxy analog indicates that the initial oxygen atom from the acetate carbonyl group has been further removed in the presence of metyrapone.

Addition of L-pipecolic acid increased the titer of minor components (CJ-12,225, CJ-12,263, CJ-12,264 and CJ-12,265), resulting in the total titer of 450 mg/liter. This appears to substantiate the proposal for the origins of this pipecolinyl group. Addition of L-proline caused the production of a prolyl analog (CJ-12,797) and a 27-*O*-demethylprolyl analog (CJ-12,798). In this case, rapamycin was also coproduced by the normal biosynthetic pathway (Fig. 2D).

The potency of these analogs in the MLR assay is listed in Table 3. The potency of the analogs ranges from

Table 4. Biological activities of CJ-12,434.

	Human mixed lymphocytes (IC_{50}, nM)	Mitogen-stimulated mononuclear cells proliferation (IC_{50}, nM)		Delayed type hypersensitivity (ED_{50}, mg/kg)
		Con A*	IOM/PMA**	
CJ-12,434 (39-Demethylrapamycin)	3.9	0.36	1,112	4.4
Rapamycin	1.3	0.25	1,095	<3.0
FK506	0.3	0.60	0.63	10.0
Cyclosporin A	8.3	66.5	241	80.0

* Concanavalin A (10 μg/ml).
** Ionomycin (250 ng/ml)/phorbol myristate acetate (20 ng/ml).

essentially equipotent to rapamycin to those essentially inactive.

Comparison of rapamycin and its 27-*O*-demethyl analog (CJ-12,264) shows that loss of a methyl group at C-27 does not affect activity. This is also apparent in the prolylrapamycin pair CJ-12,797 and CJ-12,798. Loss of a methyl group at C-16 or a simultaneous loss of methyl groups at both C-16 and C-27, again has little effect on potency (CJ-12,263, CJ-12,265 and CJ-12,225). Loss of the methoxy functionality at C-27, however, causes fairly drastic reduction in potency, as can be seen in the rapamycin and 27-demethoxyrapamycin pair and in the 39-*O*-demethylrapamycin (CJ-12,434) and 27-demethoxy-39-*O*-demethylrapamycin (CJ-12,264) pair. Neither the C-16 nor the C-27 position is involved in binding to FKBP[14], and both positions are postulated to be involved in interacting with the effector molecule[5~7], although little is known about this interaction at present.

The C-28 hydroxy group is critical for MLR activity, since C-28 dehydroxyrapamycin (CJ-12,452) has approximately 380-fold less potency compared to rapamycin. The C-28 hydroxy group has been described to form a hydrogen bond to the amide carbonyl group of Glu-54 of FKBP[14]. 9-Deoxorapamycin (CJ-12,573) is approximately 40 times less potent than rapamycin. The interesting fact, however, is that the 9-deoxo analog retains activity. This suggests that other structures without the tricarbonyl moiety may exhibit activity. It is known that the C-9 carbonyl group does not form hydrogen bonds to FKBP[14].

Substitution of a prolyl group for the pipecolinyl group reduces potency 20-fold. According to published X-ray and NMR studies[14], this region of rapamycin is most deeply imbedded in the rapamycin-FKBP complex, and it is in contact with lipophilic residues of FKBP. The observed loss in potency can be ascribed to reduced hydrophobic interaction between prolylrapamycin and the FKBP pocket.

CJ-12,434 (39-*O*-demethylrapamycin) demonstrated inhibitory activity of mitogen-stimulated human mononuclear cells proliferation and *in vivo* activity. Chemical modifications at C-40 position have been claimed to retain the immunosuppressive activity[15,16]. This suggests that cyclohexyl ring in rapamycin can be a target for generation of further analogs with improved bioavailability. Since CJ-12,263, CJ-12,265, CJ-12,434 and CJ-12,225 retained MLR activity comparable to rapamycin, these compounds might be of interest for further biological evaluation.

Although numerous semisynthetic derivatives of rapamycin have been reported, analogs prepared by biosynthetic manipulations are relatively few. Recently, preparation of 16,39-*O*-bisdemethylrapamycin has been reported by a biotransformation approach[17]. The work described here indicates that biosynthetic manipulations are powerful techniques for the discovery of analogs which are difficult to produce by chemical modifications.

Experimental

General

UV and IR spectra were measured on a JASCO Ubest-30 spectrophotometer and a Nicolet 510 FT-IR spectrophotometer, respectively. Low and high resolution FAB mass spectra were measured on a KRATOS model 1S mass spectrometer, using a *m*-nitrobenzylalcohol matrix with and without sodium iodide. NMR spectra were recorded on a Bruker AM-500 NMR spectrometer.

Taxonomy of the Producing Organism

Cultural characteristics were studied on various media according to GORDON and SMITH[18], WAKSMAN[19], SHIRLING and GOTTLIEB[20]. The colors were described in common terminology, but exact colors were determined by comparisons with color chips from the *Color Harmony Manual*, 4th edition. Diaminopimelic acid and sugars in whole cell hydrolysate were analyzed by the methods of BECKER *et al.*[21] and LECHEVALIER[22].

Cytochrome P-450 Inhibitor Addition

The culture N902-109 was maintained on ATCC 172 medium consisting of 1.0% glucose, 2.0% soluble starch, 0.5% yeast extract, 0.5% NZ Amine type A, 0.1% $CaCO_3$ and 1.5% agar. A vegetative cell suspension of the slant culture of N902-109 was inoculated into a 500-ml flask (first seed culture) containing 100 ml of first seed medium (medium A), consisting of 2.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical), 0.3% beef extract, 0.5% wheat gluten, 0.5% yeast extract, 0.3% blood meal and 0.4% $CaCO_3$ (adjusted to pH 7.0 ~ 7.2). The flask was incubated on a rotary shaker (220 rpm) at 28°C for 4 days. The first seed culture (37.5 ml) was transferred to five 500-ml flasks (second seed culture), each containing 150 ml of the medium A and incubated on a rotary shaker at 28°C for 4 days. The second seed culture (750 ml) was transferred to five 6-liter jar fermentors with a small propeller-type impeller (7 cm diameter), each containing 3 liters of production medium (medium B), consisting of 3.0% glucose, 1.0% corn starch, 1.5% corn steep liquor, 0.5% Pharmamedia, 0.0001% $CoCl_2 \cdot 6H_2O$ and 0.3% $CaCO_3$ (adjusted to pH 7.0). The fermentation was carried out at 28°C for a day with an aeration rate of 3 liters/minute and an agitation rate at 1,700 rpm and then 5 mM of metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) were added to the cultured broth. The broth was subsequently incubated for 3 days under the same conditions.

Precursor Feeding

The culture N902-109 was maintained on ATCC 172 medium. A vegetative cell suspension of the slant culture of N902-109 was inoculated into a 500-ml flask (first seed culture) containing 100 ml of the seed medium (medium A). The flask was incubated on a rotary shaker (220 rpm) at 28°C for 4 days. The first seed culture (15 ml) was transferred to two 500-ml flasks (second seed culture),

each containing 150 ml of the medium A and incubated on a rotary shaker at 28°C for 4 days. The second seed culture (300 ml) was transferred to two 6-liter jar fermentors with a small propeller-type impeller (7 cm diameter), each containing 3 liters of the production medium (medium B). The fermentation was carried out at 28°C for a day with an aeration rate of 3 liters/minute and an agitation rate at 1,700 rpm and then precursor (L-pipecolic acid or L-proline) was added to the cultured broth at the final concentration of 1 mg/ml. The broths were subsequently incubated for 3 days under the same conditions.

Biotransformation by *Streptomyces toyocaensis* ATCC 39471

The culture *Streptomyces toyocaensis* subsp. *humicola* ATCC 39471 was maintained on ATCC 172 medium. A loopful of mycelial suspension from the slant culture was inoculated into a flask (500 ml) containing 100 ml of the seed medium (medium A). The flask was incubated on a rotary shaker (220 rpm) at 28°C for 4 days. The first seed culture (15 ml) was transferred to two 500-ml flasks (second seed culture), each containing 150 ml of the medium A and incubated on a rotary shaker at 28°C for 4 days. The second seed culture (300 ml) was transferred to two 6-liter jar fermentors with a small propeller-type impeller (7 cm diameter), each containing 3 liters of fermentation medium, consisting of 1.0% glucose, 2.0% corn starch, 0.5% NZ Amine type A, 0.5% wheat embryo, 0.5% yeast extract, 0.0001% $CoCl_2 \cdot 6H_2O$ and 0.4% $CaCO_3$ (adjusted to pH 7.2～7.4). Fermentation was carried out at 28°C for 24 hours with an aeration rate of 3 liters/minute and an agitation rate at 1,700 rpm and each of 150 mg of rapamycin was added to the vessels. Fermentation was subsequently continued for 24 hours.

HPLC Analysis

The fermentation broth (5 ml) was extracted with ethyl acetate (5 ml) followed by centrifugation on a Kokusan H-107 centrifuge at 3,000 rpm for 5 minutes. The organic layer was concentrated to dryness under reduced pressure and the obtained residue was redissolved in 500 μl of ethanol. HPLC analysis was performed on a Hewlett Packard HP1090 system. Samples (2.0 μl) were loaded on a C_{18} reversed phase column (Senshu Pack ODS, 4.6 × 150 mm) maintained at 42°C and eluted with linear gradient from methanol - water (7 : 3) to (10 : 0) at a flow rate of 0.8 ml/minute in 30 minutes. Rapamycin analogs were monitored by absorbance at 287 nm. Under these conditions, rapamycin analogs were eluted with a retention time of 19.4 minutes (rapamycin), 20.1 (27-demethoxyrapamycin), 20.3 (CJ-12,578), 22.0 (CJ-12,575), 22.4 (CJ-12,573), 23.4 (CJ-12,452), 16.6 (CJ-12,797), 18.1 (CJ-12,798), 18.0 (CJ-12,434), 13.1 (CJ-12,225), 13.5 (CJ-12,263) and 18.5 (CJ-12,264 and CJ-12,265) as shown in Fig. 2.

Physico-chemical Properties

The physico-chemical properties of the novel rapamycin analogs are summarized in Table 5. The compounds, obtained as white powders, were soluble in methanol, ethyl acetate and chloroform, and slightly soluble in *n*-hexane. They showed typical triene UV spectra identical to rapamycin. Molecular formulas were determined by HRFAB-MS, using *m*-nitrobenzylalcohol matrix with and without sodium iodide.

Structure Elucidation

Structure elucidation of the novel rapamycin analogs was mainly carried out by ^{1}H and ^{13}C NMR and FAB-MS analyses, with rapamycin and 27-demethoxyrapamycin as reference compounds. The numbering system used here is the same as that commonly used for macrolide antibiotics.

CJ-12,452, CJ-12,573, CJ-12,575 and CJ-12,578

The fermentation broth (15 liters) was extracted twice with 15 liters of ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated. The resultant oily residue (21 g) was loaded on a silica gel column (500 ml, Merck Kieselgel 60, 230～400 mesh) and a step-gradient of *n*-hexane - ethyl acetate - acetone mixture was applied. The active fractions (*n*-hexane - ethyl acetate, 1 : 2 and ethyl acetate) were applied to a Sephadex LH-20 column (50 × 750 mm) and eluted with methanol to give brown oily residue (9 g). A part (100 mg) of the residue was subjected to an ODS column (Chemcosorb 5ODS-UH, 20 × 250 mm) and eluted with methanol - water (8 : 2) at a flow rate of 5 ml/minute. Detection of rapamycin analogs was monitored by absorbance at 287 nm. The eluted peaks were collected to yield the compounds CJ-12,452 (3.7 mg), CJ-12,573 (5.0 mg), CJ-12,575 (9.1 mg) and CJ-12,578 (2.0 mg). The molecular formula of CJ-12,452, $C_{51}H_{79}NO_{12}$, corresponded to a loss of 16 mass units from rapamycin. The ^{1}H NMR spectrum indicated the absence of the oxymethine signal of H-28 (δ 4.16), together with upfield displacement of unequivalent methylene signals of H_2-28 (δ 2.34 and 2.43, see Table 6). Accordingly, the structure of CJ-12,452 was determined to be 28-dehydroxyrapamycin (Fig. 1). The molecular formula of CJ-12,573, $C_{51}H_{81}NO_{12}$, corresponded to a loss of 14 mass units from rapamycin, indicating a deoxygenated derivative. The ^{13}C NMR spectrum of CJ-12,573 (Fig. 3) showed loss of the carbonyl signal of C-9 (δ 192.1), together with upfield displacement of the C-9 methylene signal at δ 38.0 assigned by ^{13}C-^{1}H COSY experiments (Fig. 4). From these results, the structure of CJ-12,573 was determined to be 9-deoxorapamycin. The molecular formulas of CJ-12,575 and CJ-12,578 were determined to be $C_{50}H_{79}NO_{11}$ and $C_{49}H_{77}NO_{11}$, respectively. The former corresponded to a loss of 30 mass units from 9-deoxorapamycin (CJ-12,573), indicating a demethoxylated derivative. As shown in Table 6, the ^{1}H NMR spectrum of CJ-12,575 indicated a loss of the methoxy

Table 5. Physico-chemical properties of rapamycin analogs.

	CJ-12,263	CJ-12,265	CJ-12,434	CJ-12,225	CJ-12,452
Appearance	White powder	White powder	White powder	White powder	White powder
Molecular weight	899	899	899	885	897
Molecular formula	$C_{50}H_{77}NO_{13}$	$C_{50}H_{77}NO_{13}$	$C_{50}H_{77}NO_{13}$	$C_{49}H_{75}NO_{13}$	$C_{51}H_{79}NO_{12}$
HRFAB-MS (m/z)					
Found $(M+Na)^+$	922.5246	922.5290	922.5302	908.5263	920.5263
Calcd	922.5288 ($C_{50}H_{77}NO_{13}Na$)	922.5288 ($C_{50}H_{77}NO_{13}Na$)	922.5288 ($C_{50}H_{77}NO_{13}Na$)	908.5316 ($C_{49}H_{75}NO_{13}Na$)	920.5316 ($C_{51}H_{79}NO_{12}Na$)
UV λ max, MeOH (nm)	277, 287, 298	277, 287, 298	277, 287, 298	277, 287, 298	277, 287, 298
IR υ max, KBr (cm^{-1})	1640, 1718 2930, 3450	1640, 1718 2930, 3450	1640, 1718 2930, 3450	1640, 1718 2930, 3450	1640, 1718 2930, 3450

	Rapamycin	27-Desmethoxy rapamycin	CJ-12,264	CJ-12,573
Appearance	White powder	White powder	White powder	White powder
Molecular weight	913	883	869	899
Molecular formula	$C_{51}H_{79}NO_{13}$	$C_{50}H_{77}NO_{12}$	$C_{49}H_{75}NO_{12}$	$C_{51}H_{81}NO_{12}$
HRFAB-MS (m/z)				
Found $(M+Na)^+$	936.5450	906.5365	892.5212	922.5673
Calcd	936.5445 ($C_{51}H_{79}NO_{13}Na$)	906.5339 ($C_{50}H_{77}NO_{12}Na$)	892.5183 ($C_{49}H_{75}NO_{12}Na$)	922.5652 ($C_{51}H_{81}NO_{12}Na$)
UV λ max, MeOH (nm)	277, 287, 298	277, 287, 298	277, 287, 298	277, 287, 298
IR υ max, KBr (cm^{-1})	1640, 1718 2930, 3450	1640, 1718 2930, 3450	1640, 1718 2930, 3450	1640, 1718 2930, 3450

	CJ-12,575	CJ-12,578	CJ-12,797	CJ-12,798
Appearance	White powder	White powder	White powder	White powder
Molecular weight	869	855	899	885
Molecular formula	$C_{50}H_{79}NO_{11}$	$C_{49}H_{77}NO_{11}$	$C_{50}H_{77}NO_{13}$	$C_{49}H_{75}NO_{13}$
HRFAB-MS (m/z)				
Found $(M+Na)^+$	892.5581	878.5371	922.5245	908.5338
Calcd	892.5546 ($C_{50}H_{79}NO_{11}Na$)	878.5390 ($C_{49}H_{77}NO_{11}Na$)	922.5288 ($C_{51}H_{79}NO_{12}Na$)	908.5316 ($C_{49}H_{75}NO_{13}Na$)
UV λ max, MeOH (nm)	277, 287, 298	277, 287, 298	277, 287, 298	277, 287, 298
IR υ max, KBr (cm^{-1})	1640, 1718 2930, 3450	1640, 1718 2930, 3450	1640, 1718 2930, 3450	1640, 1718 2930, 3450

signal at δ 3.34, together with upfield displacement of H_2-27 (δ 2.64 and 2.70). The mass spectrum of CJ-12,578 corresponded to a loss of 44 mass units from 9-deoxorapamycin. The ^1H NMR spectrum of CJ-12,578 indicated a loss of 2 methoxy signals at δ 3.34 and 3.41, together with upfield displacement of H_2-27 (δ 2.64 and 2.70) and downfield displacement of H-39 (δ 3.30), indicating the eliminations of a methoxy group at C-27 and *O*-demethylation at C-39. Accordingly, the structures of CJ-12,575 and CJ-12,578 were determined to be 9-deoxo-27-demethoxyrapamycin and 9-deoxo-27-demethoxy-39-*O*-demethyrapamycin, respectively (Fig. 1).

CJ-12,225, CJ-12,263, CJ-12,264 and CJ-12,265

The cultured broth (6 liters) was extracted twice with 6 liters of ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated. The resultant oily residue (5.2 g) was loaded on a silica gel column (500 ml, Merck Kieselgel 60, 230~400 mesh) and a step-gradient of *n*-hexane-ethyl acetate mixture was applied. The active fractions (*n*-hexane-ethyl acetate, 1 : 2 and ethyl acetate) were applied to a Sephadex LH-20 column (50 × 750 mm) and eluted with methanol to give a brown oily residue (2.5 g). A part (20 mg) of the residue was subjected to a reversed phase HPLC column (Chemcosorb 5ODS-UH, 20 × 250 mm) and eluted with methanol-water (8 : 2) at a flow rate of 5 ml/minute. The eluted peaks were collected to yield rapamycin analogs, CJ-12,225 (3.0 mg) and CJ-12,263 (1.5 mg). Mixture of CJ-12,264 and CJ-12,265 was subjected to a silica gel

Table 6. Selected ^{1}H NMR chemical shifts of rapamycin analogs.

Compound	n	^{1}H Chemical shift (δ)*							
		H-9	16-OCH_3	H-16	27-OCH_3	H-27	H-28	39-OCH_3	H-39
Rapamycin	2	-	3.13	3.67	3.34	3.71	4.16	3.41	2.94
CJ-12,263	2	-	-	4.01	3.35	3.72	4.17	3.41	2.93
CJ-12,265	2	-	3.13	3.74	-	4.18	4.16	3.42	2.95
CJ-12,434	2	-	3.13	3.67	3.34	3.82	4.22	-	3.43
CJ-12,225	2	-	-	4.02	-	4.17	4.16	3.41	2.95
27-Demethoxyrapamycin	2	-	3.14	3.66	-	2.50 2.61	4.39	3.42	2.99
CJ-12,264	2	-	3.14	3.66	-	2.48 2.64	4.39	-	3.35
CJ-12,452	2	-	3.15	3.67	3.15	3.54	2.34 2.43	3.41	2.93
CJ-12,573	2	2.43 2.75	3.13	3.67	3.34	3.61	4.14	3.43	2.95
CJ-12,575	2	2.45 2.75	3.12	3.67	-	2.64 2.70	4.39	3.41	2.96
CJ-12,578	2	2.46 2.75	3.13	3.67	-	2.61 2.69	4.40	-	3.30
CJ-12,797	1	-	3.13	3.64	3.36	3.64	4.16	3.41	2.93
CJ-12,798	1	-	3.13	3.60	-	4.29	4.18	3.41	2.94

* Chemical shift value of major conformer.

Fig. 3. ^{13}C NMR spectra of CJ-12,573 and rapamycin.

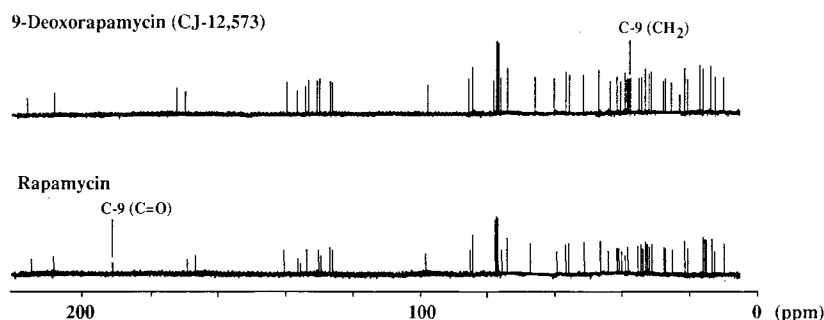
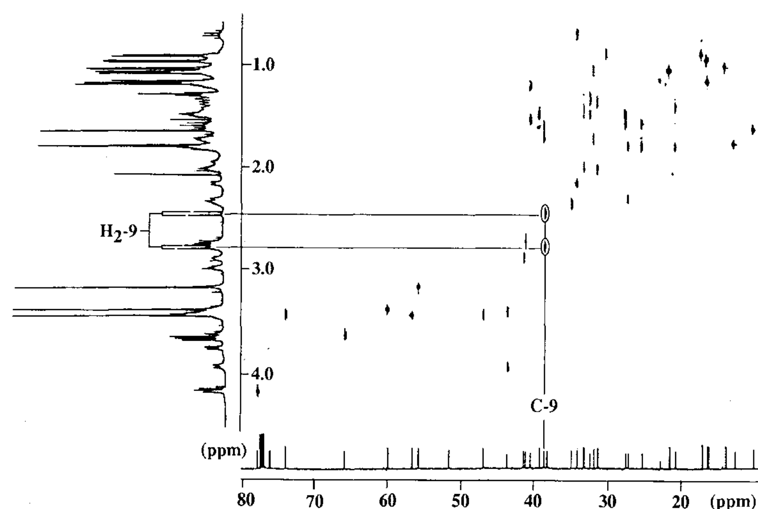


Fig. 4. ^{13}C-^{1}H COSY spectrum of CJ-12,573.



HPLC column (Chemcosorb 50-5, 20×250 mm) and eluted with chloroform-methanol (98:2), affording rapamycin analogs, CJ-12,264 (3.0 mg) and CJ-12,265 (3.0 mg). Molecular formulas of CJ-12,263 and CJ-12,265 were determined to be all $C_{50}H_{77}NO_{13}$, which corresponded to a loss of 14 mass units from rapamycin. Selected proton chemical shifts in CJ-12,263 and CJ-12,265 and rapamycin are shown in Table 6. Each of the analogs showed loss of a methoxy signal. In CJ-12,263, the methoxy signal at 3.13 ppm disappeared together with downfield displacement of H-16 (δ 4.01), indicating 16-*O*-demethylrapamycin. In the same way,

Table 7. Selected ^{13}C NMR chemical shifts of CJ-12,797, CJ-12,798 and rapamycin.

Compound	^{13}C Chemical shift (δ)						
	C-1	C-2	C-3	C-4	C-5	C-6	C-8
Rapamycin	169.23	51.20	27.01	20.58	25.24	44.16	166.73
CJ-12,797	170.85	58.82	25.08	-	26.93	47.86	164.73
CJ-12,798	170.46	58.80	24.86	-	27.00	47.78	164.80

the structure of CJ-12,265 was determined to be 27-*O*-demethylrapamycin[23] (Fig. 1). The molecular formula of CJ-12,225, $C_{49}H_{75}NO_{13}$, corresponded to a loss of 28 mass units from rapamycin. As shown in Table 6, the ^{1}H NMR spectrum of CJ-12,225 indicated the absence of 2 methoxy signals at δ 3.13 and 3.34, together with downfield displacements of H-16 (δ 4.02) and H-27 (δ 4.17), respectively. The structure of CJ-12,225 was therefore determined to be 16,27-*O*-bisdemethylrapamycin[24] (Fig. 1).

CJ-12,797 and CJ-12,798

The fermentation broth (6 liters) was diluted with 6 liters of methanol and then loaded on a Diaion HP20 (1 liter) column. After washing with 50% aqueous acetone, CJ-12,797, CJ-12,798 and rapamycin were eluted with 3 liters of 90% aqueous acetone. The eluate was concentrated to aqueous solution (1 liter) and then extracted twice with 1 liter of ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to give a brown oily residue (0.9 g). A part of the residue (50 mg) was subjected to an ODS column (Chemcosorb 5ODS-UH, 20×250 mm) and eluted with methanol-water (8:2) at a flow rate of 5 ml/minute. The eluted peaks were collected to yield rapamycin analogs, CJ-12,797 (3.7 mg) and CJ-12,798 (2.0 mg). The molecular formula of CJ-12,797, $C_{50}H_{77}NO_{11}$, corresponded to a loss of 14 mass units from rapamycin. Amino acid analysis of the hydrolysates of CJ-12,797 and CJ-12,798 was carried out after treatment with 6 N HCl at 115°C for 16 hours. Each of the hydrolysates was analyzed by HPLC, using a chiral column (SUMICHIRAL OA-5,000, 4.6×150 mm). Both compounds were found to contain L-proline. The ^{13}C NMR spectrum of CJ-12,797 indicated a loss of methylene signal of C-4 at δ 20.58, together with downfiled displacement of C-2 (δ 58.82) as shown in Table 7. These results identified CJ-12,797 to be prolylrapamycin[25]. The molecular formula of CJ-12,798, $C_{49}H_{75}NO_{11}$, corresponded to a loss of 14 mass units from prolylrapamycin. The ^{1}H NMR spectrum of CJ-12,798 indicated a loss of the methoxy signal at δ 3.34, together with downfield displacement of H-27 (δ 4.29, see Table 6). Based on the results described above, the structure of CJ-12,798 was determined to be 27-*O*-demethylprolylrapamycin (Fig. 1).

CJ-12,434

The fermentation broth (6 liters) was diluted with 6 liters of methanol and then loaded on a Diaion HP20 (1 liter) column. After washing with 50% aqueous acetone, CJ-12,434 and rapamycin were eluted with 3 liters of 90% aqueous acetone. The eluate was further purified by the same method as that of CJ-12,797 and CJ-12,798, resulting in yielding a rapamycin analog, CJ-12,434 (44.0 mg). Molecular formula of CJ-12,434 was determined to be $C_{50}H_{77}NO_{13}$, which corresponded to a loss of 14 mass units from rapamycin. Selected proton chemical shifts in CJ-12,434 and rapamycin are shown in Table 6. The methoxy signal at δ 3.41 disappeared together with downfield displacement of H-39 (δ 3.43), indicating 39-*O*-demethylrapamycin (Fig. 1).

Amino Acid Analysis of CJ-12,797 and CJ-12,798

CJ-12,797 (1 mg) was hydrolyzed with 6 N HCl (1 ml) at 115°C in a sealed tube for 16 hours. Hydrolyzate was concentrated to dryness and dissolved in water (1 ml). The solution was loaded on a Dowex 50W×4 column (5 ml) and eluted with 5% NH_4OH in water. The eluate was concentrated to dryness and redissolved in water (50 μl). The eluate (20 μl) was subjected to the HPLC analysis: SUMICHIRAL OA 5,000 column, 4.6×120 mm; absorbance at 254 nm; flow rate, 1.0 ml/minute; 2 mM $CuSO_4$ in water. Under these conditions, L-proline (retention time of 4.9 minutes) was detected. Similarly, L-proline and L-pipecolic acid (retention time of 5.2 minutes) were detected from CJ-12,798 and rapamycin, respectively.

Assessment of Immunosuppressive Activity

The immunosuppressive activity of the isolated compounds was determined in MLR. Human fresh lymphocytes were separated from the heparinized blood of normal volunteers using Ficoll-Paque (Pharmacia) density gradient centrifugation. Stimulator cells were prepared in the same way and stored in liquid nitrogen. Fresh lymphocytes (5×10^4) were cultured in the presence of thawed stimulator cells (5×10^4) with or without isolated compounds for 5 days at 37°C in the 5% CO_2-humidified air, water-jacketed incubator. After 5 days of incubation, each well was pulsed with ^{3}H-thymidine (0.5 μCi/well, 20 Ci/mmol; NEN) for an additional 18 hours. The cells were harvested onto glass filters, dried and counted in liquid scintillation cocktail.

For the mitogen-stimulated proliferation assay, murine splenic cells and human MNCs were used. Murine splenic cells were prepared from C3H/HeN mice according to standard procedure. Human MNCs were prepared as described above. Microculture was performed in round-bottomed microtiter plates (1×10^5 cells/well). Proliferation was induced by the addition of concanavalin A (10 μg/ml) or ionomycin (250 ng/ml)/phorbol myristate acetate (20 ng/ml) or lipopolysaccharide (10 μg/ml). Cell division was assessed by the addition of 0.1 μCi/well of ^{3}H-thymidine for the last 4 hours of 48-hour incubation. Cells were harvested and counted as described above.

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