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Mutational Biosynthesis of Novel Rapamycins by a Strain of *Streptomyces hygroscopicus* NRRL 5491 Disrupted in *rapL*, Encoding a Putative Lysine Cyclodeaminase

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The gene *rapL* lies within the region of the *Streptomyces hygroscopicus* chromosome which contains the biosynthetic gene cluster for the immunosuppressant rapamycin. Introduction of a frameshift mutation into *rapL* by ΦC31 phage-mediated gene replacement gave rise to a mutant which did not produce significant amounts of rapamycin. Growth of this *rapL* mutant on media containing added L-pipecolate restored wild-type levels of rapamycin production, consistent with a proposal that *rapL* encodes a specific L-lysine cyclodeaminase important for the production of the L-pipecolate precursor. In the presence of added proline derivatives, *rapL* mutants synthesized novel rapamycin analogs, indicating a relaxed substrate specificity for the enzyme catalyzing pipecolate incorporation into the macrocycle.

Rapamycin is a 31-member macrocyclic polyketide produced by *Streptomyces hygroscopicus* NRRL 5491 which, like the structurally related compounds FK506 and immunomycin (Fig. 1), has potent immunosuppressive properties (24). Such compounds are potentially valuable in the treatment of autoimmune diseases and in preventing the rejection of transplanted tissues (16). The biosynthesis of rapamycin requires a modular polyketide synthase, which uses a shikimate-derived starter unit (11, 20) and which carries out a total of fourteen successive cycles of polyketide chain elongation that resemble the steps in fatty acid biosynthesis (2, 27). L-Pipecolic acid is then incorporated (21) into the chain, followed by closure of the macrocyclic ring, and both these steps are believed to be catalyzed by a pipecolate-incorporating enzyme (PIE) (18), the product of the *rapP* gene (8, 15). Further site-specific oxidations and *O*-methylation steps (15) are then required to produce rapamycin.

The origin of the pipecolic acid inserted into rapamycin has been previously established (21) to be free L-pipecolic acid derived from L-lysine (although the possible role of D-lysine as a precursor must also be borne in mind) (9). Previous work with other systems has suggested several alternative pathways for pipecolate formation from lysine (22), but the results of the incorporation of labelled lysine into the pipecolate moiety of immunomycin (Fig. 1) clearly indicate loss of the α-nitrogen atom (3). More recently, the sequencing of the *rap* gene cluster revealed the presence of the *rapL* gene (Fig. 2), whose deduced gene product bears striking sequence similarity to two isoenzymes of ornithine deaminase from *Agrobacterium tumefaciens* (25, 26). Ornithine deaminase catalyzes the deaminative cyclization of ornithine to proline, and we have proposed (15) that the *rapL* gene product catalyzes the analogous conversion of L-lysine to L-pipecolate (Fig. 3).

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Here, we report the use of ΦC31 phage-mediated gene replacement (10) to introduce a frameshift mutation into *rapL* and the ability of the mutant to synthesize rapamycins in the absence or presence of added pipecolate or pipecolate analogs.

MATERIALS AND METHODS

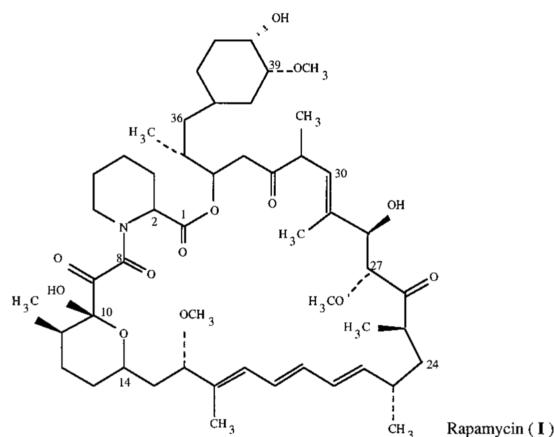
Materials. All molecular biology enzymes and reagents were from commercial sources. Viomycin was the kind gift of Pfizer, Inc. L-Pipecolic acid, L-proline, 3,4-dehydroproline, picolinic acid, pyrrole-2-carboxylic acid, *trans*-4-hydroxyproline, *cis*-4-hydroxyproline, and *cis*-3-hydroxyproline were obtained from Aldrich Chemical Co.

Bacterial strains, plasmids, and phages. The bacterial strains, plasmids, and phages used in this study are listed in Table 1.

Media and growth conditions. *Escherichia coli* DH10B (Gibco-BRL) was grown in 2× tryptone-yeast extract medium (23). *E. coli* transformants were selected with 100 μg of ampicillin per ml. The rapamycin producer *S. hygroscopicus* NRRL 5491 and its derivatives were maintained on SY agar (8) and cultivated in tryptic soy broth (Difco) with 100 mM MES [2-(*N*-morpholino)-ethanesulfonic acid]–1.0% glucose, pH 6.0 (TSBGM), supplemented with 10 μg of viomycin per ml as required. The defined medium used was as described by Cheng et al. (6). *Streptomyces lividans* JI1326 was cultivated in YEME (7) or in tap water medium (0.5% glucose, 1% sucrose, 0.5% tryptone, 0.25% yeast extract, 10 mM EDTA; pH 7.1) (8). Liquid cultures were grown at 30°C in Erlenmeyer flasks shaken at 200 to 250 rpm. Infection with the *att* mutant actinophage KC515 (4) and its derivative ΦRAPL was carried out on solid DNA medium supplemented with 10 mM $MgSO_4$–8 mM $Ca(NO_3)_2$–0.5% (wt/vol) glucose (7).

DNA techniques and plasmid construction. Procedures for electroporation, PCR, and manipulation of DNA were carried out as described in Sambrook et al. (23). Total DNA from *S. hygroscopicus* spheroplasts was isolated with DNAzol (Gibco) as described by the manufacturer. Southern hybridization was carried out with probes labelled with digoxigenin by using the DIG DNA labelling kit (Boehringer Mannheim). DNA fragments for labelling and subcloning were isolated with the Qiaex II (Qiagen) gel extraction kit. Protoplasts of *S. lividans* JI1326 were transfected with KC515 and ΦRAPL as described by Hopwood et al. (7). Recombinant phage was identified by PCR analysis.

Construction of a frameshift mutation in *rapL*. The *rapL* gene was disrupted by introducing a frameshift by KC515 phage-mediated gene replacement as follows. A 3,034-bp *Eco*RI fragment (nucleotides 93956 to 96990 of the *rap* cluster) (27) encompassing the entire *rapL* gene and flanked by *rapK* and part of the *rapM* gene (Fig. 2) was cloned into an *Eco*RI-cut pUC18-derived vector that had been modified by removal of the *Bam*HI site in the polylinker region by *Bam*HI digestion, end filling, and religation to yield pLK2 (Table 1). A unique *Bam*HI site was located in the coding region of the *rapL* gene 42 bp from the 5′ end. Plasmid pLK2 was digested with *Bam*HI, and the cohesive ends were filled in by treatment with *E. coli* DNA polymerase I (Klenow fragment) and religated.



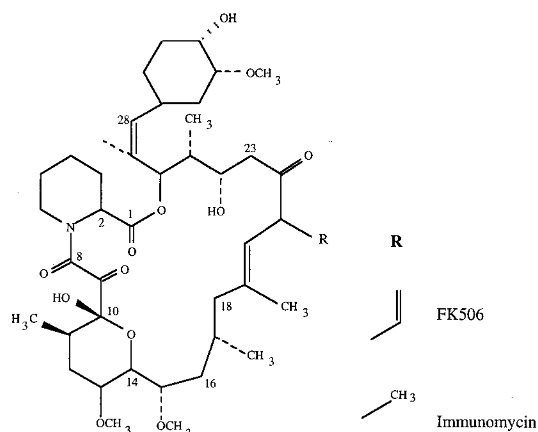


FIG. 1. Structures of rapamycin, FK506, and immunomycin.

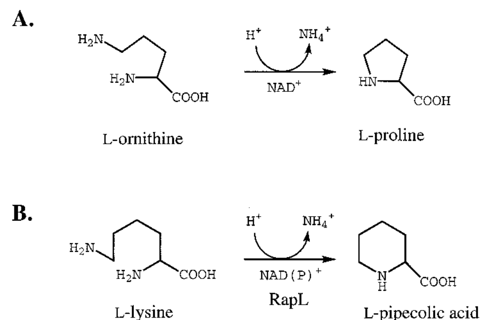


FIG. 3. (A) The conversion of L-ornithine to L-proline by ornithine cyclodeaminase (17). (B) Proposed conversion of L-lysine to L-pipecolic acid by the *rapL* gene product.

The ligated plasmid DNA was redigested with *Bam*HI and used to transform *E. coli*. Ampicillin-resistant transformants were selected, and their plasmid DNA was checked for the removal of the *Bam*HI site by restriction enzyme analysis and then DNA sequencing. The 3-kb insert was excised from the plasmid with *Eco*RI, the cohesive ends were blunt ended by treatment with *E. coli* DNA polymerase I (Klenow fragment), and the insert was cloned into *Pvu*II-cut phage KC515 vector. *S. lividans* JI1326 protoplasts were transfected with the ligation mixture, and the resultant phage plaques were screened by PCR for the presence of *rapL*-derived sequences. Positive phage ΦRAPL was selected and used to transfect *S. hygroscopicus* NRRL 5491 as described by Lomovskaya et al. (10) on DNA plates. Lysogens were selected by overlaying the plates with viomycin (50-μg ml^{-1} final concentration) 24 h after infection. Approximately 2,000 initial transductants were obtained, of which about 200 were stable integrants. Three of these were arbitrarily chosen and used to isolate viomycin-sensitive derivatives that had undergone a second recombination event deleting the integrated phage. These viomycin-sensitive isolates were obtained after three rounds of nonselective growth and sporulation on SY plates. Of the four colonies isolated, two were found to have reverted to the wild type with the *Bam*HI site restored at position 95036, while the other two harbored the desired mutation with the loss of this *Bam*HI site. The insertion and subsequent loss of the phage were confirmed by genomic Southern hybridization.

Precursor feeding and fermentation of the *rapL* mutant. *S. hygroscopicus* LEK111 (Table 1) was cultured in 500-ml Erlenmeyer flasks containing 100 ml of TSBGM. When added, proline or other amino acids were present at a final concentration of 1 mg/ml. In certain experiments, *S. hygroscopicus* LEK111 was cultivated in 2-liter flasks, each containing 400 ml of a defined medium to which no pipecolic acid was added (6). For larger-scale fermentation, 10 μl of a spore suspension of *S. hygroscopicus* LEK111 was used to inoculate a 100-ml flask containing 30 ml of TSBGM medium. The culture was incubated at 28°C for 4 days with shaking at 300 rpm. Then, 4 ml of this culture was transferred to a 2-liter flask containing 400 ml of TSBGM medium and the mixture was shaken at 300 rpm at 28°C for 4 days. This culture in turn was transferred to a 20-liter fermenter containing 15 liters of TSBGM medium supplemented with L-*trans*-4-hydroxyproline. The fermentation was carried out at 28°C for 4 days, with aeration at 15 liters of air/min and an agitation rate of 500 rpm, in the presence of Sigma A antifoam (Aldrich Chemical Co.). The cells were harvested by filtration and extracted with two volumes of methanol overnight at 4°C. Extracts were analyzed by liquid chromatography-mass spectrometry (LC-MS) with a Finnigan MAT (San Jose, Calif.) LCQ linked to a Hewlett-Packard (Böblingen, Germany) 1100 high-pressure liquid chromatograph.

For isolation of metabolites, the extracts were evaporated to dryness and then purified by flash chromatography on silica gel 60 (Merck) eluted with acetone-hexane (1:1 [vol/vol]). The fractions containing rapamycins were further purified by preparative high-pressure liquid chromatography on a reverse-phase C18 column (250 by 20 mm; 5-μm bead diameter) (HPLC Technology, Macclesfield, United Kingdom) (8). Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker DRX 500 spectrometer.

Biological activity of rapamycin analogs. Human lymphocyte 536 cells (obtained from the human genetic mutant cell repository, Camden, N.J.) were cultured in Iscove's medium supplemented with 10% (vol/vol) fetal calf serum. For the bioassay (14), cells were seeded into 96-well microtiter plates at 10,000 per well in 100 μl of growth medium. Solutions of each metabolite were prepared in dimethyl sulfoxide (DMSO) (1% [wt/vol]), and further dilutions were made to give a final concentration of 0.1% DMSO in growth medium. Each culture

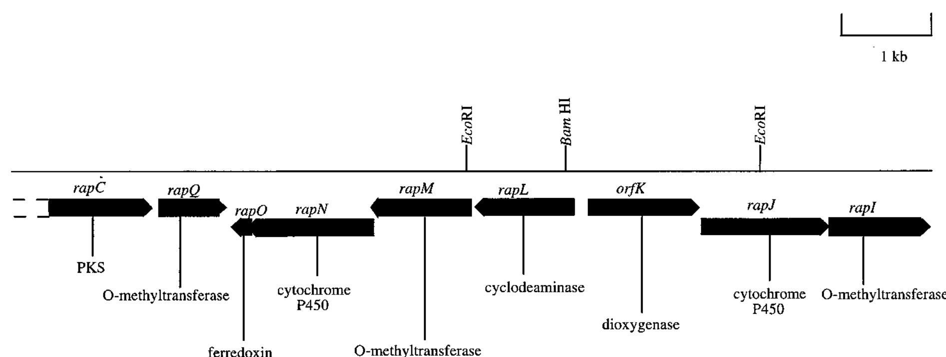


FIG. 2. A portion of the rapamycin biosynthetic gene cluster which contains ancillary (non-polyketide synthase) genes (15, 27). PKS, polyketide synthase.

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristics	Reference or source
E. coli DH10B	F^- *mcrA* Δ(*mrr hsdRMS-mcrBC*) φ80d*lacZ*ΔM15Δ*lacX74 deoR recA1 endA1*	Gibco-BRL
Streptomyces strains		
S. hygroscopicus NRRL 5491	Wild type; produces rapamycin	ATCC[a]
S. hygroscopicus LEK111	*rapL* gene disrupted	This study
S. lividans JI1326	Host for phage infection	7
Plasmids		
pUC18	Cloning vector in *E. coli*	Sigma Chemical Co.
pLK1	pUC18 with *Bam*HI site removed	This study
pLK2	pLK1 containing a 3-kb *Eco*RI fragment corresponding to nucleotides 93956 to 96990 of the *rap* cluster	This study
pLK3	pLK2 with unique *Bam*HI site removed	This study
Phages		
KC515	c^+ *attP*::*tsr*::*vph*	7
ΦRAPL	KC515 containing a 3-kb insert	This study

[a] ATCC, American Type Culture Collection, Rockville, Md.

received either a 1 μM, 100 nM, 10 nM, or 1 nM final concentration of each rapamycin or rapamycin analog or a final concentration of 0.1% DMSO in growth medium only. Each culture (in triplicate) was labelled with 1 μCi of tritiated thymidine (Amersham International; specific activity, 70 Ci/mM) per well for 3 h either immediately after addition of the rapamycin analogs (0- to 3-h label) or at 24 h (21- to 24-h label) or at 48 h (45- to 48-h label). After being labelled, the cultures were lysed with water and harvested onto glass fiber paper disks to trap the released DNA. Radioactivity incorporated into the filter disks was counted in a Packard scintillation counter.

RESULTS

Disruption of the *rapL* gene. A frameshift mutation was introduced into the *rapL* gene of *S. hygroscopicus* NRRL 5491 as described in Materials and Methods. The presence of the frameshift mutation in *S. hygroscopicus* LEK111 was confirmed by Southern hybridization (Fig. 4). Chromosomal DNA isolated from *S. hygroscopicus* NRRL 5491 and LEK111 and digested with both *Bam*HI and *Bgl*II was probed with a 3-kb *Eco*RI fragment (between nucleotides 93956 and 96990 of the *rap* gene cluster). This probe overlaps the *Bam*HI site at position 95036, which was expected to be eliminated in LEK111. Hybridizing bands of 5.9 kb (representing nucleotides 89118 to 95036) and 2.7 kb (representing nucleotides 95036 to 97710) were found for the wild-type strain as expected (Fig. 4). With chromosomal DNA from strain LEK111, only an 8.6-kb *Bam*HI-*Bgl*II fragment (representing nucleotides 89118 to 97710) was detected, indicating that the *Bam*HI site at position 95036 had been removed. This was also confirmed by PCR analysis. Chromosomal DNA was subjected to PCR with oligonucleotide primers identical to the sequences from nucleotide 93950 to nucleotide 93968 and from nucleotide 96990 to nucleotide 97010. The expected 3-kb DNA fragment was amplified from wild-type DNA, and following *Bam*HI digestion, two bands, approximately 2 and 1 kb in size, were detected. In samples containing *S. hygroscopicus* LEK111 chromosomal DNA, the 3-kb PCR product was found to be resistant to *Bam*HI digestion as predicted (data not shown).

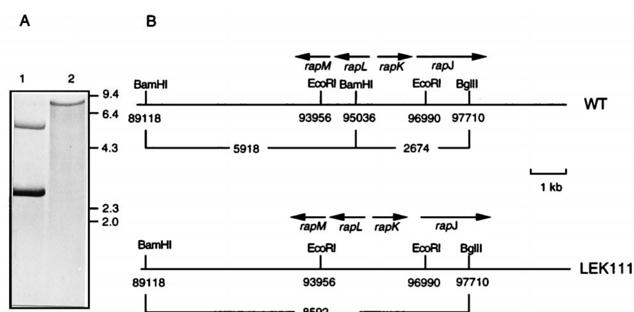


FIG. 4. Southern analysis and restriction map of the *rapL* region of *S. hygroscopicus* NRRL 5491 (WT) and *S. hygroscopicus* LEK111. (A) Genomic DNA from *S. hygroscopicus* NRRL 5491 (lane 1) and *S. hygroscopicus* LEK111 (lane 2) was digested with *Bam*HI and probed with the 3-kb *Eco*RI fragment. The sizes (in kilobases) of the λ-*Hin*dIII DNA markers are indicated on the right. (B) Restriction map and gene organization of the *rapL* region in *S. hygroscopicus* NRRL 5491 and *S. hygroscopicus* LEK111.

TABLE 2. Production of metabolites by *S. hygroscopicus* NRRL 5491 and *S. hygroscopicus* LEK111

Strain	Metabolite	Amt of metabolite produced (mg/liter) in[a]: TSBGM	TSBGM + pipecolate	TSBGM + proline	TSBGM + 4-hydroxyproline	Defined medium[b]
Wild type	I	15.2	15.9	14.4	14.0	0.12
	II	0.7	0.6	0.4	0.5	Traces
	IIIa	N.D.[c]	N.D.	N.D.	N.D.	N.D.
	IIIb	N.D.	N.D.	N.D.	N.D.	N.D.
LEK111	I	0.4	13.1	0.3	0.4	0.03
	II	1.1	0.6	1.3	1.4	0.05
	IIIa	N.D.	N.D.	N.D.	0.2	N.D.
	IIIb	N.D.	N.D.	N.D.	1.1	N.D.

[a] The values are averages of at least four fermentations.
[b] See reference 6.
[c] N.D., not detected.

LEK111 requires added pipecolate for full restoration of rapamycin production. Wild-type *S. hygroscopicus*, when grown in a rich medium (TSBGM) for 96 h, produced both rapamycin and prolylrapamycin, a rapamycin analog in which L-proline replaces pipecolate in the macrocyclic ring (Fig. 5), in a ratio of about 20:1 (Table 2). LEK111 under the same conditions produced smaller quantities of the same rapamycins in a ratio of approximately 1:2 to 1:3. In TSBGM medium supplemented with pipecolic acid, LEK111 produced rapamycins at or near wild-type levels and the ratio of rapamycin to prolylrapamycin was also very similar to that for the wild type.

TABLE 3. Metabolites produced by *S. hygroscopicus* LEK111 on medium supplemented with pipecolate or pipecolate analogs

Compound fed	Product: *m/z* (M + Na^+)	LC retention time (min)	Name
L-Pipecolic acid	936	8.84	Rapamycin
L-Proline	922	7.99	Prolylrapamycin
L-*trans*-4-Hydroxyproline	938	5.35	Compound IIIa[a]
	908	6.29	Compound IIIb[a]

[a] The compound designation is defined in Fig. 5. The structure of the compound was verified by NMR.

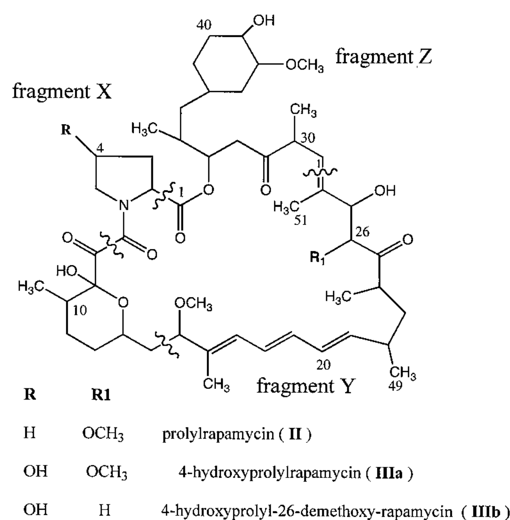


FIG. 5. Rapamycin analogs produced in this study.

Production of novel rapamycin analogs containing 4-hydroxyprolyl moieties. The production of prolylrapamycin, also previously noted by Nielsen et al. for prolylimmunomycin (18), suggested that LEK111 might incorporate alternative amino acids when added to the medium. When fermentations of *S. hygroscopicus* LEK111 were supplemented with L-*trans*-4-hydroxyproline, two new peaks were detected by LC-MS (Table 3). The major peak with the characteristic triene UV absorption at 277 nm contained a compound with an *m/z* of 908, which would correspond to a 4-hydroxyprolyl–rapamycin analog lacking a methoxy group (Fig. 5, IIIb). Smaller amounts of a compound with an *m/z* of 938 which would correspond to 4-hydroxyprolyl-rapamycin (Fig. 5, IIIa) were also detected. To obtain enough material for NMR characterization, L-*trans*-4-hydroxyproline was used to supplement a 15-liter fermentation of *S. hygroscopicus* LEK111, from which 3 mg of the compound with the *m/z* of 908 (Fig. 5A, IIIb), together with about 15 mg of pure prolylrapamycin (Fig. 5, II), was extracted as described in Materials and Methods. The NMR data (Table 4) reflected the chemical shifts and couplings expected for the 4-hydroxyproline spin system and also confirmed the absence of the methoxy group at C-26 (corresponding to C-27 in rapamycin), although the L-*trans* configuration of the 4-hydroxyprolyl moiety could not be directly established. The observed MS fragmentation data (Table 5) confirmed these findings. For the compound labeled I, the loss of 129 atomic mass units (amu) reflects the loss of the pipecolate moiety while in the compounds labeled IIIa and IIIb, the loss of an amino acid of 131 amu confirms the identity of the amino acid moiety (Fig. 5, fragment X) as hydroxyproline. The loss of fragment Y (loss of 294 amu in compound IIIa and loss of 264 amu in compound IIIb) indicated that the methoxy group in position 26 is missing in compound IIIb but not in compound IIIa. Furthermore, the loss of fragment Z (322 amu) can be seen for compounds IIIa and IIIb as well as for compound I, indicating that there is no alteration affecting this region of the molecule. Therefore, compound IIIb (Table 5) with an *m/z* of 908 is 4-hydroxyprolyl-26-demethoxy-rapamycin and compound IIIa with an *m/z* of 938 is 4-hydroxyprolyl-rapamycin. In addition to L-*trans*-4-hydroxyproline, L-*cis*-4-hydroxyproline and L-*cis*-3-hydroxyproline were also incorporated, as judged by LC-MS analysis (data not shown). No new products were observed with 3,4-dehydroproline and picolinic acid, while pyrrole-2-carboxylic acid appears to inhibit growth at the concentrations used.

TABLE 4. Selection of ^{1}H and ^{13}C NMR data for 4-hydroxyprolyl-26-demethoxy-rapamycin (compound IIIb [Fig. 5]) and rapamycin (compound I [Fig. 1])[a]

Position	^{1}H δ (ppm)	^{13}C δ (ppm)
1		171.30 (169.2)
2	5.24 (5.29)	58.17 (51.3)
3	2.65, 1.69 (2.34, 1.76)	38.48 (27.0)
4	4.38 (1.78, 1.47)	70.63 (20.6)
5	3.37, 2.94 (1.75, 1.48)	56.53 (25.3)
26	3.58 (3.71)	Not determined
27	3.89 (4.17)	70.63 (77.3)
38	2.93 (2.93)	83.90 (84.4)
39	3.37 (3.37)	73.95 (73.9)
40	1.99, 1.33 (1.99, 1.38)	31.22 (31.3)
49	3.12 (3.13)	55.68 (55.8)
51	3.39 (3.41)	56.50 (56.5)

[a] Data in parentheses are chemical shifts for equivalent positions in rapamycin (13).

Biological activities of rapamycin analogs. The immunosuppressant activities of rapamycin, prolylrapamycin, and 4-hydroxy-26-demethoxy-rapamycin were tested with human lymphoblastoid cell line 536 as described in Materials and Methods. Previous experiments have shown that rapamycin is a strong inhibitor of G_1 progression in the 536 cell line (14). For all three compounds, the 0- to 3-h drug exposure had no appreciable effect on DNA synthesis at concentrations up to 100 nM (data not shown). This implies that none of the compounds is toxic to the 536 cell line. After 24 and 48 h (Table 6), the 536 cells showed a concentration-dependent inhibition of DNA synthesis, with 50% inhibition at 10 nM rapamycin and at 30 nM prolylrapamycin. 4-Hydroxy-26-demethoxy-rapamycin was also inhibitory, but the inhibition did not reach 50%, even at 1 μM.

DISCUSSION

We have previously characterized *rapL*, a gene within the rapamycin biosynthetic gene cluster which, on the basis of a sequence comparison with authentic cyclodeaminases (25, 26), appears likely to catalyze the cyclodeamination of L-lysine to form L-pipecolate. This, in turn, is required as a specific precursor for the formation of the macrocyclic lactone in rapamycin. The clustering of such a gene for the provision of an essential precursor with the rapamycin biosynthetic genes would be an unusual but not an unprecedented arrangement

TABLE 5. MS fragmentation analysis of metabolites produced by *S. hygroscopicus* LEK111 grown in the presence of 4-hydroxyproline

Compound[b]	m/z (M + Na^{+})	Inferred mass difference[a] after loss of fragment[c]:		
		X	Y	Z
I	936	129	294	322
IIIa	938	131	294	322
IIIb	908	131	264	322

[a] Mass difference (in amu) calculated by subtracting the mass of the observed daughter ion from the mass of the observed parent ion.
[b] Compound designations are defined in Fig. 1 (compound I) and Fig. 5 (compounds IIIa and IIIb).
[c] Fragments X, Y, and Z are shown in Fig. 5.

TABLE 6. Inhibition of cell cycle progression by rapamycin and analogs[a]

Compound	Incorporation of [^{3}H]dTTP into nucleic acids (%) at indicated time and compound concentration							
	21–24 h				45–48 h			
	1 nM	10 nM	100 nM	1 μM	1 nM	10 nM	100 nM	1 μM
Rapamycin	92	48	27	20	99	46	10	7
Prolylrapamycin	98	65	26	21	103	91	22	7
4-Hydroxyprolyl-26-demethoxy-rapamycin	87	86	80	67	111	98	98	71

[a] The control experiment was the incorporation of [^{3}H-dTTP] into nucleic acids in the 536 cell line in the presence of 0.1% DMSO (100%).

(1). When in the present work the *rapL* gene was inactivated by phage-mediated gene replacement (10), the mutant strain, LEK111, was found to produce only minute amounts of rapamycin (Fig. 1, compound I), together with a derivative (Fig. 5, compound II) in which the pipecolate moiety is replaced by proline (Fig. 5; Table 2). Even smaller amounts of these rapamycins were produced when LEK111 was grown in a defined medium in the absence of added pipecolate. Since the frameshift introduced into *rapL* lies very near the 5′ end of the coding region, the low-level production of rapamycin by LEK111 cannot be due to residual activity of the *rapL* gene product. Either the media contain unsuspected traces of pipecolate or alternative pathways must exist in *S. hygroscopicus* for the endogenous production of pipecolic acid. The existence of multiple pathways which generate pipecolate via lysine catabolism has been demonstrated in *Pseudomonas* spp., *Rhizoctania leguminicola*, *Streptomyces virginiae* (3), and β-lactam antibiotic-producing strains of *Streptomyces* (12). The addition of L-pipecolate to the mutant strain completely restored to wild-type levels its ability to synthesize compound I when grown on either defined medium or TSBGM and restored the wild-type product ratio of compound I to compound II (Table 2). This is fully consistent with our previous proposal that *rapL* encodes an L-lysine cyclodeaminase, although it does not prove it (15). A complete proof of the putative role of this gene will require the future demonstration that purified RapL catalyzes the deamination together with evidence that the cloned *rapL* gene can complement the mutation in LEK111. We have no ready explanation for the observation that the mutant strain does not accumulate more of compound II than the wild type in proline-containing media (Table 2). This would have been anticipated from the known substrate specificity of PIE (8, 18). The production of some fully processed rapamycin in LEK111 shows that the effects of the disruption of *rapL* are not solely mediated by polar effects on the downstream *rapM*, *rapN*, and *rapO* genes.

It has also been demonstrated here that novel rapamycins may be generated by supplementing the fermentation medium of *S. hygroscopicus* LEK111 with proline or with amino acids that are close structural analogs of proline. In the case of the PIE from immunomycin, 4-methylproline (which is not commercially available) was found to be a better substrate than pipecolate itself for the adenylation reaction in vitro (18). A more extensive survey, therefore, might well uncover other amino acids capable of being incorporated by the PIE of the rapamycin biosynthetic pathway. The rapamycin analog, 4-hydroxyprolyl-26-demethoxy-rapamycin (Fig. 5, compound IIIb) has been characterized extensively by NMR and MS. It appears that the altered macrocyclic compound is not a good substrate for the cytochrome P450 required to initiate modification at the C-26 position. The importance of the missing methoxy group at C-26 for activity has been noted previously (5, 19), and indeed the inhibitory activity of compound IIIb in a proliferation assay was significantly less than that of rapamycin. The successful incorporation of several proline analogs into altered rapamycins indicates that the PIE of the rapamycin producer (8) has a significant tolerance for structural variation in the amino acid substrate.

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