Biosynthesis

# Mutasynthesis of Rapamycin Analogues through the Manipulation of a Gene Governing Starter Unit Biosynthesis**

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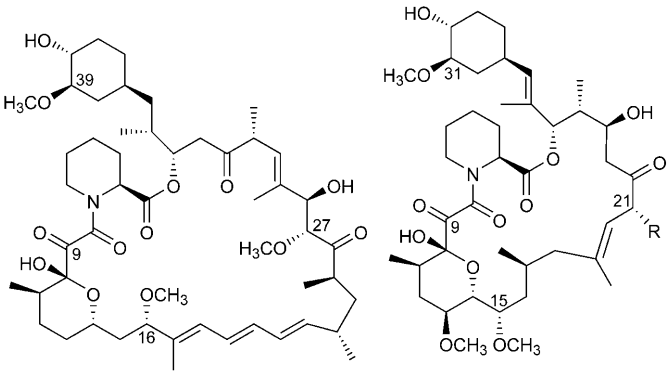
Rapamycin **1**, FK506 **2**, and FK520 **3** are biogenetically related natural products which are synthesized by mixed polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) systems and which possess potent antifungal and immunosuppressive activities. Compounds **1**–**3** possess a




**1**: Rapamycin

**2**: FK506, R =

**3**: FK520, R = Et

common structural motif which is responsible for their specific binding to FK506-binding proteins (FKBPs). This binding triggers the subsequent binding of the binary complex to a downstream protein target, either mTOR (mammalian target of rapamycin) or calcineurin (FK506, FK520) which mediate their biological effects.[1] Inhibition of mTOR by

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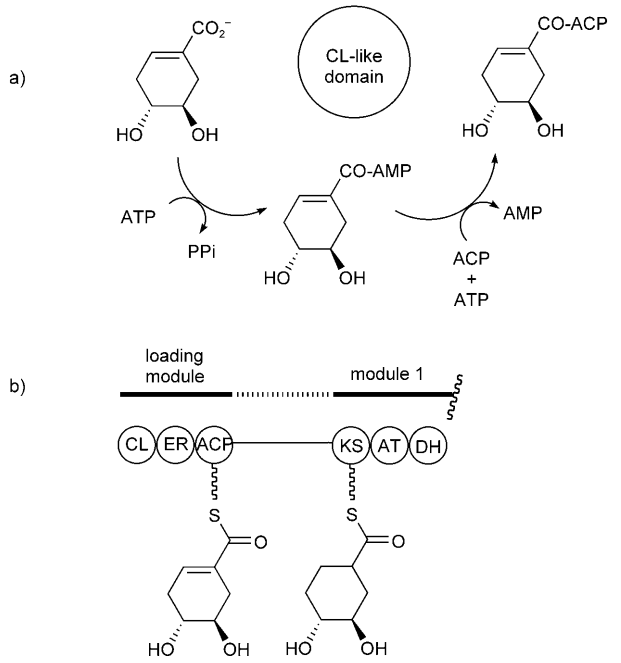
rapamycin is also the basis for current interest in such compounds as anticancer drugs.

The dihydroxycyclohexane moiety of rapamycin arises through the incorporation of a 4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC) starter unit derived from shikimic acid in an analogous manner to that of FK520.[2] Cyclohexanecarboxylic acid (CHC), the starter unit for asukamycin[3] and phoslactomycin,[4] also arises from shikimic acid. These biosynthetic pathways share a common first step, the 1,4-conjugate elimination of water from shikimic acid.

Selection and activation of the carboxylic acid group of the rapamycin PKS starter unit is carried out by a carboxylic acid ligase (CL) like domain, in a manner analogous to that of adenylation domains of NRPS systems (Scheme 1a). Following covalent tethering to the ACP domain, the double bond of




***Scheme 1.*** a) Proposed pathway for CL-catalyzed activation and subsequent attachment of the natural starter unit to the rapamycin PKS. b) Translocation of ACP-bound starter unit involves reduction of the $\Delta^1$ bond by the ER domain of the loading module of RAPS1, the N-terminus of which is shown with a linear arrangement of the predicted catalytically active domains. AMP = adenosine monophosphate, PPi = inorganic phosphate, CL = carboxylic acid ligase like domain, KS = β-ketoacyl synthase, ACP = acylcarrier protein, AT = acyltransferase, DH = dehydratase.

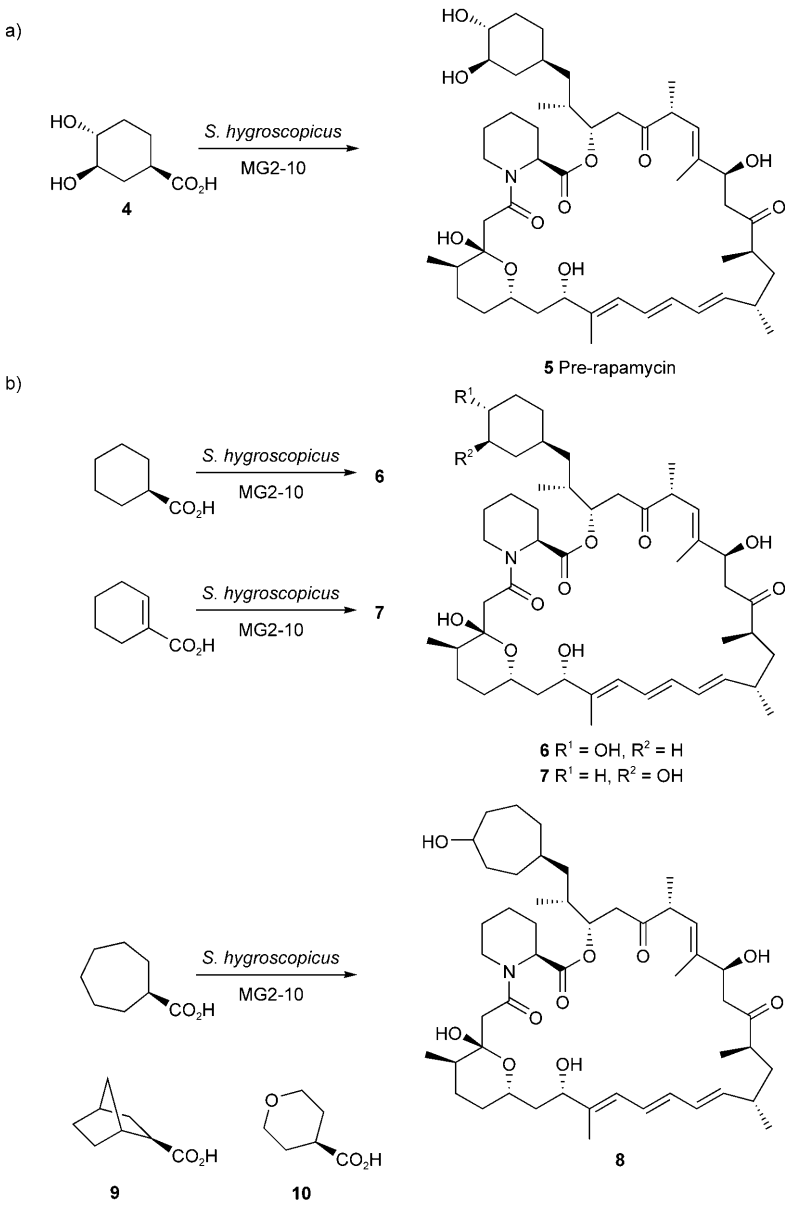
DHCHC is reduced by the action of the enoyl reductase (ER) domain of the rapamycin PKS loading module (Scheme 1b). Efficient incorporation of the pseudostarter acid 1,2-dihydro-DHCHC was also observed during feeding experiments with stable isotopes, thus leading to speculation that the domains of the PKS involved in selection of the starter unit might have broad substrate tolerance.[2,5] Indeed, a recent report has shown that novel analogues of rapamycin are biosynthesized in addition to rapamycin when simple analogues of the natural starter unit are fed to the rapamycin-producing organism.[6]

The immediate product of the rapamycin PKS is pre-rapamycin **5**, which is then modified by a series of two cytochrome P450 monooxygenases (RapJ and RapN) and three O-methyltransferases (RapI, RapM, and RapQ). When a region of DNA including the genes thought to encode these enzymes was excised (*rapKIJMNOQL*) from the rapamycin biosynthetic gene cluster, no production of rapamycins was observed from the resulting strain, *Streptomyces hygroscopicus* MG2–10. The mutant strain MG2–10 was then independently complemented with full-length copies of each of the genes which had been removed. Surprisingly, the production of **5** was only observed when *rapK* was reintroduced and expressed in the strain.[7] The gene *rapK* has previously been proposed as a candidate gene to encode a pteridine-dependent dioxygenase,[8] which (like its homologue in the FK506 cluster, *fkbO*) might catalyze the oxidation of the C9 hydroxy group after initial hydroxylation at C9 catalyzed by the cytochrome P450 RapJ.[9] However, we now have clear evidence that RapJ alone is capable of both oxygen insertion and further oxidation to give a C9 keto group.[10]

Furthermore, comparison of the sequences between RapK[11] and its homologues from the FK520 (FkbO),[12] FK506 (FkbO),[13] and hyg (ORF5)[14] biosynthetic gene clusters have revealed that the proposed binding motif for a pteridine derivative present in RapK[8] is not present in any of the other three proteins. These findings. together with the surprising effect of *rapK* on pre-rapamycin production in strain MG2–10,[7] have led us to reexamine the role of this gene, and of its product, in rapamycin biosynthesis.

We first carried out a series of experiments in an attempt to explain the lack of production of the rapamycin macrocycle by *S. hygroscopicus* MG2-10. These included feeding experiments in which exogenous pseudostarter carboxylic acid **4** was added to the fermentation medium to verify that the supply of this component was not a limiting factor. To our surprise, the addition of **4** to the fermentation led to the efficient production of **5** (Scheme 2a), which was fully characterized by mass spectrometry and NMR spectroscopy and shown to be identical to authentic pre-rapamycin.[7] Furthermore, the addition of other carboxylic acids in place of **4**, as reported for the wild-type organism,[6,15] was found to lead to the specific production of pre-rapamycin analogues in which these non-natural starter acids had been incorporated, in most cases after prior hydroxylation (Scheme 2b). CHC, cyclohex-1-enecarboxylic acid, and cycloheptanecarboxylic acid were all fed separately to *S. hygroscopicus* MG2–10, which led to the production of analogues **6**–**8**, respectively. Compounds **7** (22 mg) and **8** (77 mg) were isolated from the fermentation broth[16] by preparative chromatography[7] and their structures were confirmed by using high-resolution FT-ICR-MS$^n$.[17] Compound **6** (100 mg) was isolated and its structure confirmed by a combination of high-resolution FT-ICR-MS$^n$ and multidimensional NMR spectroscopy experiments.[18] The MS and NMR data were in complete harmony with the results of previous work on the fully processed variants of these compounds.[6] Other compounds were fed, including norbornane carboxylic acid **9**, which had previously been unsuccessfully fed to the wild-type strain.[15] LC-MS analysis showed the production of low levels of a pre-rapamycin analogue arising




***Scheme 2.*** a) Reestablishment of pre-rapamycin **5** production in *S. hygroscopicus* MG2–10 by feeding the pseudostarter unit **4**. b) Exclusive production of the analogues **6**–**8** of pre-rapamycin by feeding, respectively, cyclohexanecarboxylic acid, cyclohex-1-enecarboxylic acid, and cycloheptanecarboxylic acid to *S. hygroscopicus* MG2–10.

from incorporation (after hydroxylation) of **9**, thus demonstrating that removal of competition from the natural starter acid allows a more efficient mutasynthesis of pre-rapamycin analogues.

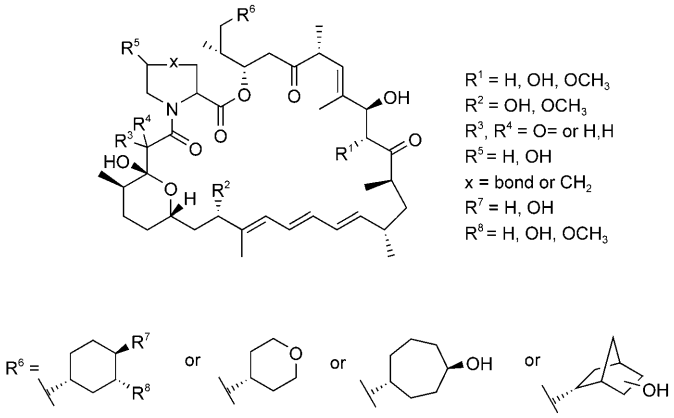
The hydroxylation of the incorporated starter acids appears to occur before synthesis of the polyketide chain.[6] Consistent with this idea, the hydroxylation seen in strain MG2–10 shows that none of the *rapKIJMNOQL* genes are involved. The enzyme or enzymes responsible remain to be identified. Interestingly, when the heterocycle tetrahydro-2*H*-pyran-4-carboxylic acid (**10**)[19] was fed to MG2-10, LC-MS studies revealed low levels of incorporation without hydroxylation. This observation reveals that hydroxylation is not essential for incorporation.

To test whether *fkbO*, the *rapK* homologue found in the FK520 cluster, has a similar function to *rapK*, *fkbO* was amplified by the polymerase chain reaction (PCR) from *S. hygroscopicus* var. *ascomyceticus* genomic DNA and ligated into the expression plasmid pSGSet1, which contains the $P_{act}$ promoter.[10] This unit was introduced into *S. hygroscopicus* MG2–10 and the strain was analyzed for production of **5**. Levels of **5** were observed similar to those produced by *S. hygroscopicus* MG2–10 when complemented by *rapK*. This result indicates that FkbO has a similar function to RapK and opens the way to analogous mutasynthesis of FK506 and FK520 analogues.

The surprising observation that feeding exogenous starter unit carboxylic acids to *S. hygroscopicus* MG2-10 complements the absence of *rapK* provides strong evidence that the product of this gene is involved directly in the biosynthesis of DHCHC or in its regulation. Further sequence comparisons (data not shown) have revealed that *rapK* and its three homologues so far identified share a C-terminal domain which is present in a broadly distributed family of over 200 proteins—members of the YjgF/YER057c/UK114 family.[20] These proteins have been tentatively implicated in a range of regulatory functions. We propose that RapK is involved in the regulation of DHCHC production from shikimate.

Rapamycin and its analogues are the only established inhibitors of mTOR and the most selective inhibitors of kinase activity known to date, and therefore represent exciting candidates for the development of anticancer therapeutics.[21] All of the rapamycin analogues presently in clinical trials (for example, CCI-779, SDZ-RAD, and ABT-578) contain semisynthetic alterations at positions derived from the starter unit.[22] We have shown here that novel starter units can be efficiently and exclusively incorporated into the rapamycin molecule by feeding them to a *rapK*-deleted mutant of *S. hygroscopicus* MG2–10. The advantages of such mutasynthesis in a strain background not capable of producing natural rapamycins are clear (compare with the production of the modified avermectin analogue doramectin).[23] By combining alternative starter units, with controlled late-stage processing, by the selective expression of the methyltransferases RapI, RapM, and RapQ as well as monooxygenases RapJ and RapN (along with the ferridoxin RapO),[10] and with potential incorporation of alternative amino acid analogues in place of the natural pipecolic acid,[24] combinatorial biosynthesis of a wide range of rapamycin analogues is now possible (Scheme 3) even before engineered alterations of the macrocycle by PKS/NRPS engineering are considered. Such analogues offer great potential as chemical genetic probes of the molecular path-




***Scheme 3.*** Illustration of selected rapamycin analogues possible by feeding complemented strains of *S. hygroscopicus* MG2–10 with natural starter and/or pipecolic acid analogues.

ways involved in rapamycin action, and as new agents with enhanced therapeutic properties.

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