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REVIEW ARTICLE

Biosynthesis of rapamycin and its regulation: past achievements and recent progress

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Rapamycin and its analogs are clinically important macrolide compounds produced by *Streptomyces hygroscopicus*. They exhibit antifungal, immunosuppressive, antitumor, neuroprotective and antiaging activities. The core macrolactone ring of rapamycin is biosynthesized by hybrid type I modular polyketide synthase (PKS)/nonribosomal peptide synthetase systems primed with 4,5-dihydrocyclohex-1-ene-carboxylic acid. The linear polyketide chain is condensed with pipecolate by peptide synthetase, followed by cyclization to form the macrolide ring and modified by a series of post-PKS tailoring steps. The aim of this review was to outline past and recent advances in the biosynthesis and regulation of rapamycin, with an emphasis on the distinguished contributions of Professor Demain to the study of rapamycin. In addition, this article describes the biological activities as well as mechanism of action of rapamycin and its derivatives. Recent attempts to improve the productivity of rapamycin and generate diverse rapamycin analogs through mutasynthesis and mutagenesis are also introduced, along with some future perspectives.
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INTRODUCTION

Rapamycin (**1**; Figure 1), produced by *Streptomyces hygroscopicus*, is a 31-membered macrocyclic natural product exhibiting various biological and pharmacological activities, including antifungal,[1] immunosuppressive,[2] antitumor,[3] neuroprotective[4] and antiaging activities.[5] Like the structurally related FK506, another *Streptomyces* metabolite with potent immunosuppressive activities,[6] rapamycin has attracted interest for the clinical treatment of organ transplant rejection[2,7] and autoimmune disease.[8] Even though rapamycin and FK506 have a similar polyketide backbone and share a common cellular receptor, the FK506-binding protein (FKBP), these immunosuppressants act through different mechanisms in cells. FK506 inhibits interleukin 2-mediated T-cell proliferation by blocking the Ca^{2+}/calcineurin-dependent transcriptional activation of the genes responsible for growth, whereas rapamycin prevents growth-promoting cytokine signaling by interacting with a mammalian target of rapamycin (mTOR) instead of calcineurin (Figure 2).[9]

In the early 1990s, a study on the biosynthesis of immunosuppressant rapamycin was begun by Demain's research group at Massachusetts Institute of Technology.[10] Classical feeding experiments with isotope-labeled precursors provided evidence that rapamycin is formed through a polyketide pathway and its macrolactone ring is derived from acetate, propionate and methionine.[11] The biosynthesis of the polyketide chain is initiated by the incorporation of a shikimate-derived 4,5-dihydroxycyclohex-1-ene carboxylic acid (DHCHC) starter unit, which in turn is elongated by 14 condensation steps with 7 acetate units and 7 propionate units (Figure 3).[11] The chain growth is then completed by the attachment of a pipecolate moiety to the linear polyketide product through a nonribosomal peptide synthetase, which also catalyzes the macrolactone ring closure to generate pre-rapamycin (Figure 3).[12–14] In addition, formation of rapamycin requires further post-polyketide synthase (PKS) modification steps, including oxidations catalyzed by cytochrome P450 monooxygenases, and *O*-methylations by *S*-adenosylmethionine-dependent methyltransferases.[12,13,15]

Since the discovery of rapamycin PKS,[12] Demain and his colleagues have studied the effect of nutrients on rapamycin production in *S. hygroscopicus*, and reported that good rapamycin production can be achieved when adding a low concentration of ammonium in the absence of phenylalanine and methionine, which are normally used as the nitrogen sources during the fermentation.[16,17] Supplementation with ferrous salt and a limitation of phosphate and magnesium salts are also required to increase rapamycin productivity.[18] Furthermore, exogenous shikimic acid was found to double rapamycin production,[19] whereas prolylrapamycin (**2**), usually produced in trace amounts, became the major product when adding proline to the fermentation, due to competition between proline and endogenous pipecolic acid.[20] Interestingly, when rapamycin analogs, such as prolylrapamycin, 32-desmethylrapamycin (WAY-125286; **3**) and 32-desmethoxyrapamycin (WAY-24688; **4**), were examined for their

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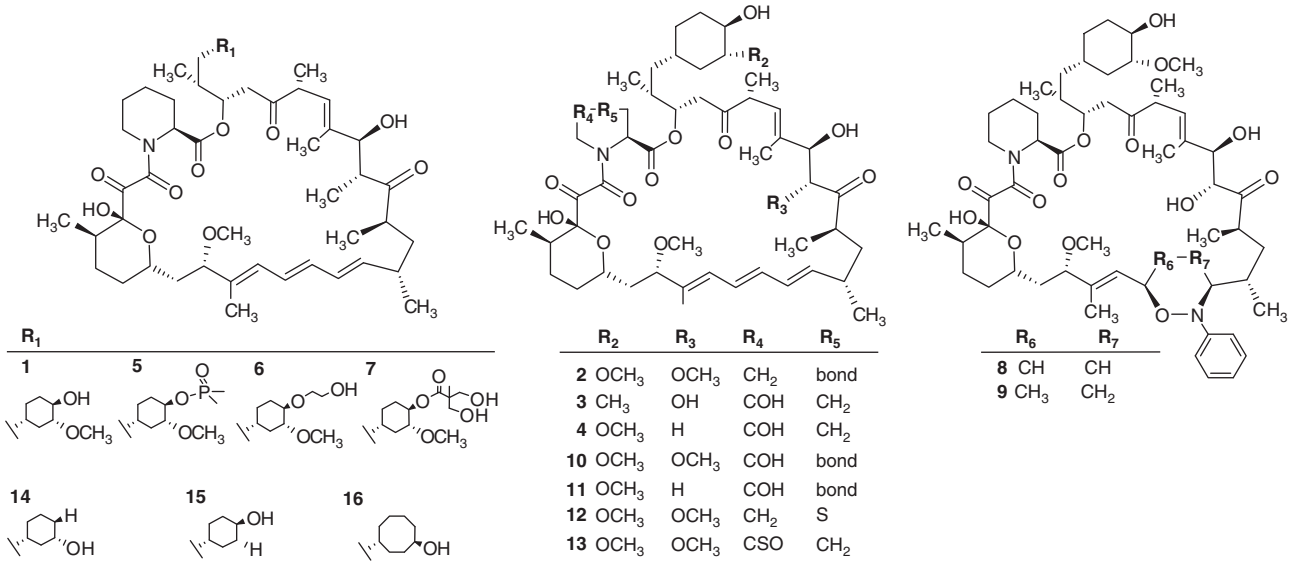

Figure 1 Structure of rapamycin and its analogs. (**1**) Rapamycin, (**2**) prolylrapamycin, (**3**) 32-desmethylrapamycin (WAY-125286), (**4**) 32-desmethoxyrapamycin (WAY-24688), (**5**) deferolimus (AP23573), (**6**) everolimus (RAD001), (**7**) temsirolimus (CCI779), (**8**) WYE-592, (**9**) ILS-920, (**10**) 4-hydroxyprolylrapamycin, (**11**) 4-hydroxyprolyl-26-demethoxyrapamycin, (**12**) 4-thiarapamycin and (**13**) 9-deoxo-5-sulfoxyrapamycin. (**14**), (**15**) and (**16**) rapamycin analogs generated by feeding pseudostarter units such as cyclohexanecarboxylic acid (CHC), cyclohex-1-ene-carboxylic acid and cycloheptanecarboxylic acid, respectively.

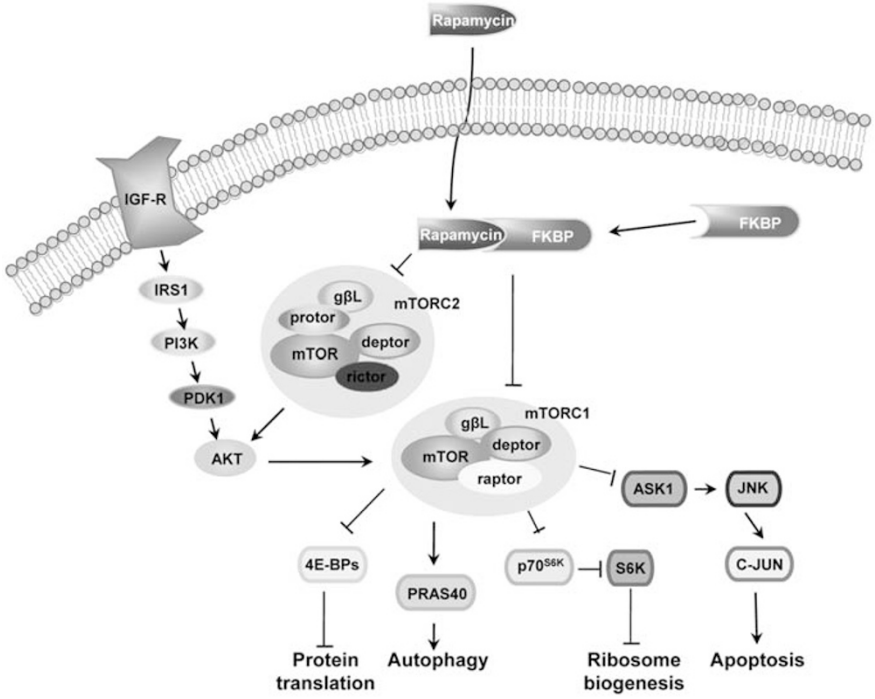

Figure 2 Mechanism of action of rapamycin. Inhibition of mTOR signaling by rapamycin regulates cell proliferation, angiogenesis and cell metabolism. mTOR, mammalian target of rapamycin; IRS1, insulin receptor substrate-1; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent protein kinase 1; AKT (PKB), protein kinase B; gβL, GTPase β-subunit-like protein; raptor, regulatory-associated protein of TOR; rictor, rapamycin-insensitive companion of TOR; deptor, disheveled, Egl-10, pleckstrin (DEP) domain containing mTOR interacting protein; protor, proline-rich protein; 4E-BPs, eukaryotic translation initiation factor 4E-binding proteins; PRAS40, proline-rich AKT substrate of 40 kDa; p70S6K, ribosomal p70 receptor; S6K, S6 kinase; ASK1, apoptosis signal-regulating kinase 1; JNK, c-JUN N-terminal kinase.

antifungal activities, all were more active than a conventionally used antifungal drug amphotericin B, although they were less active than rapamycin.[21] Fang *et al.*[22] also observed an interesting effect of the naturally coproduced elaiophylin and nigericin on the antifungal activity of rapamycin, where both of them enhanced the anti-*Candida albicans* activity of rapamycin.

On the basis of Demain's pioneering works, more recent efforts to increase rapamycin productivity and generate novel biologically active

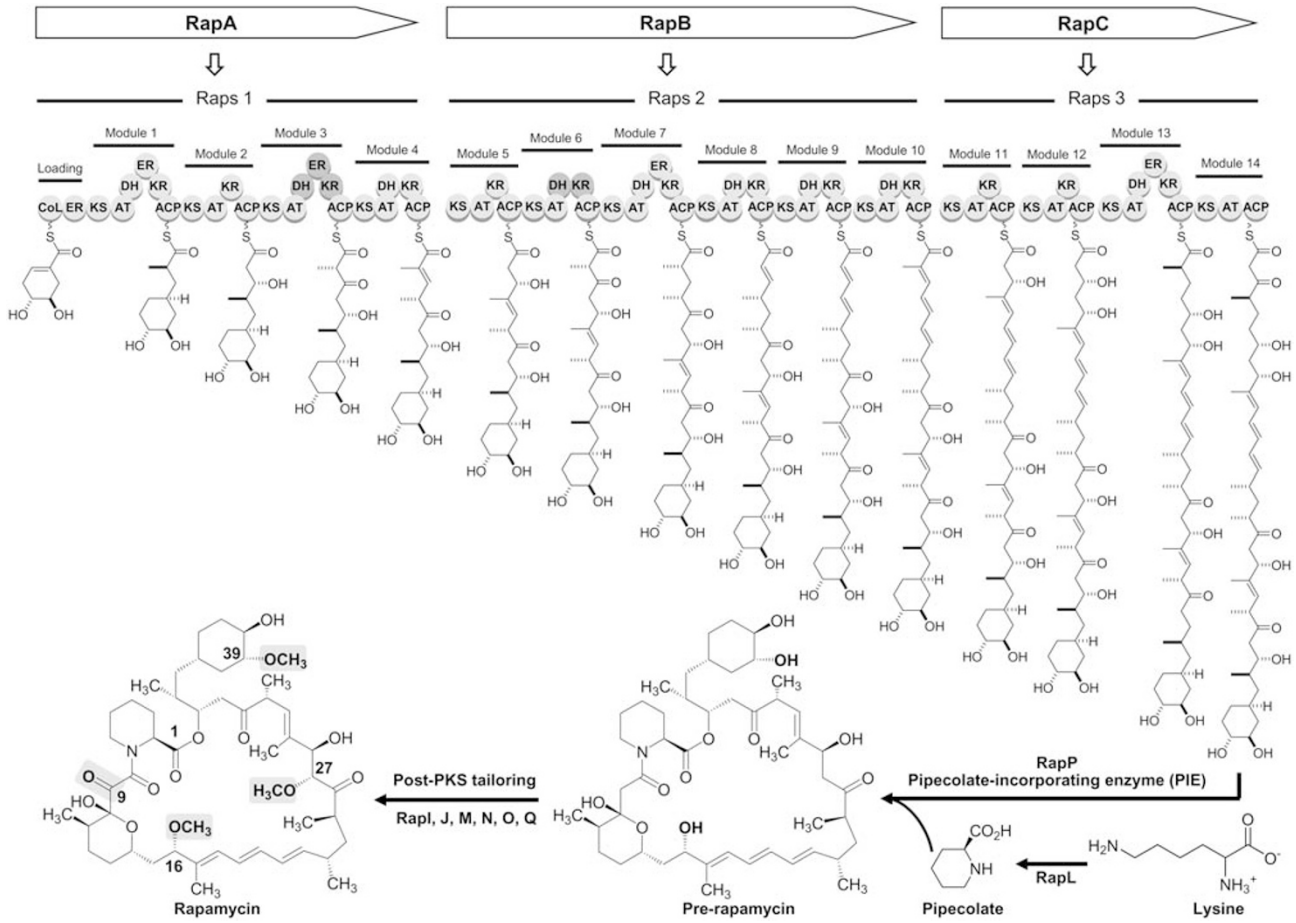

Figure 3 Rapamycin biosynthetic pathway. Rapamycin is biosynthesized by post-PKS tailoring steps through pre-rapamycin that is built up by RapA, RapB, RapC, RapP and RapL.

analogs of rapamycin have focused on precursor-directed biosynthesis, mutagenesis and mutasynthesis. For example, the Biotica group (Cambridge, UK) revealed various positive regulatory elements in rapamycin biosynthesis,[23] the Wyeth group (Madison, NJ, USA) modified the pipecolate ring of rapamycin and Leadlay and coworkers[24] synthesized several rapamycin analogs with modifications to the cyclohexyl moiety through genetic engineering and mutasynthesis.

Recently, Graziani[25] provided a review article on the advances in the field of rapamycin biosynthesis and neuroprotective activity of rapamycin analogs. There have been also a large number of thorough reviews on the potential of using rapamycin for cancer, organ transplantation and autoimmune disorders. This review summarizes the role of rapamycin and its biosynthesis, while also describing previous and current attempts to regulate rapamycin production by nutritional control, mutagenesis for improved production and mutasynthesis for the generation of novel analogs. Precursor engineering and combinatorial biosynthesis strategies are also introduced for further improving the efficiency of rapamycin production and generating diverse useful rapamycin analogs.

BIOLOGICAL ACTIVITIES OF RAPAMYCIN AND ITS ANALOGS

When rapamycin was first isolated from soil microbes collected on Easter Island, it attracted attention due to its antifungal properties against human pathogenic yeasts, *Candida* spp.[1,26] Rapamycin provided lower minimum inhibitory concentrations against eight clinical isolates of *Candida* when compared with other antifungal agents, such as candicidin, nystatin and amphotericin B.[1] Rapamycin was also found to be very active in the treatment of systemic candidosis in mice when compared with amphotericin B, and previous reports on the rapamycin–FKBP complex in yeast showed that the formation of the complex is essential for the antifungal activity of rapamycin.[27,28]

Although rapamycin was originally studied as an antifungal agent, it was soon identified as a potent immunosuppressant and is currently in use and under development as treatment in transplant medicine.[2,29] Rapamycin was approved in the United States in 1999 (and 2000 in Europe) for the prevention of acute rejection in combination with cyclosporine and steroids. A large number of groups have already shown the ability of rapamycin to diminish pathology in various animal model immune diseases.[9] Interestingly, rapamycin inhibits the chronic/acute organ rejection process that is not suppressed by cyclosporine and FK506.[30,31] It has been suggested that these immunosuppressive effects of rapamycin result from its ability to inhibit proliferation by interfering with the function of mTOR.[32] When rapamycin–FKBP complex interacts with mTOR, this blocks the cell-cycle progression of T cells in the mid-to-late G1 phase, thereby suppressing the T-cell proliferation induced by cross-linking of the T-cell receptors, antigenic peptides or cytokines such as interleukins.[33] The effects of rapamycin are not only limited to T-cell proliferation, but also include the proliferation of B lymphocytes.[34]

In addition to its antifungal and anti-immunosuppressive effects, rapamycin and its derivatives, such as deferolimus (AP23537; **5**), everolimus (RAD001; **6**) and temsirolimus (CCl779; **7**) shown in

Figure 1, have been recently identified as potent anticancer agents.[35,36] It has also been shown that rapamycin and its analogs effectively suppress cellular proliferation and angiogenesis in a number of human malignancies.[37,38] More recently, it was reported that eukaryotic translation initiation factor 4E-binding proteins activation *in vivo* by rapamycin (Figure 2) prevented dopaminergic neuron loss,[39] which is characteristic of the neurodegenerative disorder parkinsonism.[40] Along with their neuroprotective activities, rapamycin and its analogs have been also shown to stimulate neurite outgrowth.[41] A structure-based drug design approach used by Graziani *et al*.[25] showed that semisynthetic analogs of rapamycin, WYE-592 (**8**) and ILS-920 (**9**) (Figure 1), could be promising candidates for an *in vivo* neuroprotection/neurogeneration study. Furthermore, Harrison *et al*.[5] showed that rapamycin extended the median and maximal life span of both male and female mice beginning at 600 days old in separate studies simultaneously replicated at three different test sites. Feeding rapamycin to male and female mice beginning at 270 days old also increased survival, which may have resulted from the postponement of death from cancer, retarding the mechanism of aging or both.

Figure 2 shows the action mechanism of mTOR, the serine/threonine kinase that controls the cellular processes of growth, proliferation, transcription, protein biosynthesis and ribosomal biogenesis.[42] mTOR exists in two distinct protein complexes referred to as mTOR complex 1 and mTOR complex 2. Rapamycin-sensitive mTOR complex 1 consists of mTOR, a GTPase β-subunit-like protein, deptor and regulatory associated protein of TOR (raptor), whereas mTOR complex 2 includes mTOR, deptor, a GTPase β-subunit-like protein, a rapamycin-insensitive companion of TOR (rictor) and protor.[43] The inactivation of mTOR complex 1 kinase activity by rapamycin results in the inhibition of the activities of ribosomal S6 kinase and the eukaryotic translation initiation factor 4E-binding proteins, which have roles in ribosome biogenesis and protein translation, respectively. In contrast, apoptosis and autophagy are stimulated by rapamycin.[32,44]

BIOSYNTHESIS OF RAPAMYCIN

The initial precursor incorporation experiments using ^{13}C-labeled acetate and propionate showed that the macrolide ring of rapamycin is biosynthesized from seven acetate and seven propionate units. The three *O*-methyl groups of rapamycin are derived from methionine.[11] Later, several competitive incorporation studies using labeled precursors proved that the heterocyclic ring originates from pipecolic acid, which is formed from lysine (Figure 3).[45] The feeding of ^{13}C-labeled shikimic acid prepared from ^{13}C-glucose also elucidated that the cyclohexane ring in rapamycin is derived from the shikimic acid pathway.[46]

The rapamycin biosynthetic genes from *S. hygroscopicus* have been identified by hybridization with the PKS genes for erythromycin biosynthesis.[12] The rapamycin PKS consists of three multifunctional enzymes (RapA, RapB and RapC) with a total of 14 modules: modules 1–4 in RapA (900 kDa) involved in polyketide chain initiation and extension, modules 5–10 in RapB (1070 kDa) required for chain elongation up to C16 and modules 11–14 in RapC (660 kDa) responsible for the termination of the polyketide portion of the macrolactone ring. The organization of the rapamycin gene cluster is shown in Figure 3. The loading module located in the N-terminal region of RapA contains the characteristic domain, ATP-dependent carboxylic acid/CoA ligase and enoyl reductase.[15] The CoA ligase domain activates the unusual starter unit DHCHC, forming acyl-adenylate, and then transfers the activated acyl group directly to the β-ketoacyl-acyl carrier protein (ACP) synthase domain of module 1. The adjoining enoyl reductase domain is proposed to reduce the starter unit after it has linked to RapA.[15] The subsequent modules carry a set of domains for β-ketoacyl-ACP synthase, acyltransferase and ACP, which catalyze polyketide chain elongation, including auxiliary β-keto processing domains, ketoreductase, dehydratase and enoyl reductase. Most β-ketoacyl-ACP synthase, acyltransferase and ACP domains in rapamycin PKS are highly conserved, although the detailed sequences of the acyltransferase domains appear to be divided into two groups based on the substrate specificity of the extender unit, (2*S*)-malonyl-CoA or (2*S*)-methylmalonyl-CoA.[15] Except for module 14, the dehydratase and ketoreductase domains are present in all rapamycin PKS. Meantime, the enoyl reductase, ketoreductase and dehydratase domains in module 3, and dehydratase and ketoreductase domains in module 6 are presumed to be inactive, even though the sequences are indistinguishable from those of other active domains.[15]

Other genes responsible for rapamycin biosynthesis have been also identified by sequencing beyond the PKS region.[12] The chain synthesis is terminated by a specialized protein encoded by *rapP*, designated a pipecolate-incorporating enzyme, which inserts pipecolate moiety into the polyketide backbone and catalyzes subsequent cyclization.[12] The evidence that pipecolate incorporation and macrolactone ring closure are carried out by pipecolate-incorporating enzyme was obtained by a nucleotide sequence analysis and disruption of *rapP* from *S. hygroscopicus*.[47] A comparison of the deduced amino-acid sequence of *rapP* with those of authentic peptide synthetases, such as GrsT from gramicidin S,[48] TycF from tyrocidine[49] and SrfD from surfactin biosynthetic gene cluster,[50] revealed the presence of highly conserved motifs for ATP binding, aminoacyl adenylate formation, peptide bond formation and substrate transfer.[47] When *rapP* was disrupted from the chromosome of *S. hygroscopicus*, rapamycin production was significantly reduced. Furthermore, recombinant pipecolate-incorporating enzyme expressed in the heterologous host *Streptomyces coelicolor* CH999 was labeled with ^{14}C-pipecolate, indicating that the pipecolate was covalently bound to the enzyme through a thioester linkage and consequently it was phosphopantetheinylated.[47] Pipecolate, a direct precursor for the heterocyclic ring of rapamycin, is biosynthesized from lysine by *rapL* encoding a lysine cyclodeaminase. The role of RapL was shown based on the chromosomal disruption experiment in *S. hygroscopicus*.[20] In addition, heterologous expression and purification of recombinant RapL from *Escherichia coli* also characterized the *in vitro* enzymatic activity of RapL.[51]

Modifications of the 31-memebered lactone ring by oxidation and *O*-methylation are thought to be the final steps in the biosynthesis of rapamycin. Recently, the Sheridan group identified the functions of several gene products involved in post-PKS tailoring by biotransformation and gene complementation.[52] The predicted functions of the post-PKS tailoring enzymes corresponding to the conversion of pre-rapamycin into rapamycin are shown in Figure 3. RapJ and RapN encode cytochrome P450 monooxygenases that catalyze the specific oxidation step in C9 and C27, respectively, of the pre-rapamycin macrolide. RapO shares a sequence homology with known ferredoxins that are required for the activity of cytochrome P450 enzymes. The deduced gene products of *rapM*, *rapQ* and *rapI* are similar to *S*-adenosylmethionine-dependent *O*-methyltransferases, which carry out methylation at the hydroxyl groups of C16, C27 and C39, respectively, in the rapamycin molecule.[15,52]

REGULATION OF RAPAMYCIN BIOSYNTHESIS

Despite accumulated information on the rapamycin biosynthetic pathway, most of the nutritional control and regulatory factors remain unknown. Demain's group examined the effect of carbon[53] and nitrogen sources[54] on the biosynthesis of rapamycin in

S. hygroscopicus. Among 35 carbon sources, including polysaccharides, oligosaccharides, monosaccharides and organic acids, 12 carbon sources were found to support the growth of the producer strain and rapamycin production in a chemically defined medium. In particular, seven carbon sources, that is, fructose, mannose, galactose, inositol, mannitol, xylose and cellobiose, were found to stimulate rapamycin production. Although the best combination of carbon sources was 2% fructose and 0.5% mannose, a combination of 1.5 g l^{-1} aspartic acid, 0.5 g l^{-1} arginine and 0.5 g l^{-1} histidine was an effective mixture of nitrogen sources for the growth of *S. hygroscopicus* and rapamycin production.[53]

On the basis of these preliminary results, Demain's team examined additional 18 amino acids under fermentation conditions supplemented with aspartic acid, arginine and histidine.[54] As a result, rapamycin formation was significantly increased by lysine, yet decreased by phenylalanine and methionine. The lysine stimulation was presumably due to its role as a precursor of pipecolic acid, which is incorporated into the pre-rapamycin, whereas the negative impact of phenylalanine on the formation of shikimic acid, a rapamycin precursor, had an interfering effect on the rapamycin production.[16] It would appear that methionine represses the generation of rapamycin, where methionine inhibits at least one *O*-methyltransferase involved in rapamycin biosynthesis and *S*-adenosylmethionine synthetase.[55] Among the six non-amino acids (ammonium sulfate, ammonium nitrate, ammonium chloride, ammonium citrate, urea and potassium nitrate), the addition of ammonium sulfate stimulated rapamycin formation.[54] Demain's research group reported on the effects of phosphate, ammonium, magnesium and iron on rapamycin biosynthesis, and found that rapamycin production was increased when adding $FeSO_4$ up to 0.36 mM, greater than that required for growth, whereas phosphate (K_2HPO_4), ammonium (NH_4Cl) and magnesium salts ($MgSO_4 \cdot 7H_2O$) interfered with rapamycin formation at concentrations optimal for growth.[18]

The addition of exogenous shikimate resulted in an approximately twofold enhanced rapamycin production.[19] As a result, it has been predicted that shikimic acid serves as a precursor for the biosynthesis of the trisubstituted cyclohexane ring (DHCHC) outside the macrolactone from the acetate and propionate units.[11] However, the previously observed inhibition of rapamycin biosynthesis by phenylalanine treatment[16] was not recovered even by shikimate.[19]

Demain and his coworkers also evaluated the relationship between rapamycin production and a simulated microgravity environment that has only received limited attention. The growth of *S. hygroscopicus* under simulated microgravity conditions provided by a rotating-wall bioreactor led to pellet formation during cell growth, a decrease in the dry cell weight and interference in rapamycin production. An attempt to reduce the pellet formation by adding Teflon beads to the rotating-wall bioreactor proved to be unsuccessful as regards increasing the rapamycin production.[56,57]

A number of regulatory protein families, as well as physical and chemical factors including nutritional sources and microgravity, can affect a broad range of physiological processes involved in secondary metabolite production. Therefore, targeted genetic engineering of the regulatory system can be an alternative approach to improve the productivity of useful natural products. From a sequence analysis study, several open-reading frames from the rapamycin biosynthetic gene cluster have been identified having a potential regulatory function.[13] RapR and RapS, which appear to be translationally coupled proteins, have sequence similarities with bacterial response sensor and regulator proteins.[13] Meanwhile, RapH, RapG and RapY have been shown to contain a helix-turn-helix motif for DNA binding.[13] Although RapY exhibits sequence similarity to repressors of antibiotic export such as ActII from actinorhodin[58] and TcmR from tetracenomycin gene clusters,[59] the sequences of RapG are similar to those of the positive regulatory proteins SoxS and Rob from *E. coli*.[13] The N-terminal region of RapH contains a potential ATP-binding motif matching the canonical P-loop consensus.[13] A sequence analysis also revealed that both RapH and RapG contain the rare leucine codon TTA, which has been proposed to serve as part of the regulatory mechanism of secondary metabolites in *Streptomyces*.[60]

Recently, the Biotica group showed that RapH and RapG have important roles as positive regulators in rapamycin biosynthesis.[23] Overexpression of *rapH* and *rapG* under the control of the ActII-ORF4/P*actI* activator/promoter in *S. hygroscopicus* enhanced the production of rapamycin by approximately 27–55% and 20–32%, respectively. The ActII-ORF4/P*actI* expression system, which is a robust activator/promoter expression system that has been widely used for many actinomycetes, including *S. hygroscopicus*, was used to minimize the potential self-regulatory interference of RapH and/or RapG and other endogenous regulatory genes.[61] It was also observed that the introduction of an additional copy of *rapH* and *rapG* under the control of native promoter region of *S. hygroscopicus* increased rapamycin formation by 40% on average, when compared to that in the wild-type strain. Deletion experiments of both genes from the chromosome of *S. hygroscopicus* also confirmed the positive regulatory role of RapG and RapH in rapamycin biosynthesis. Subsequent complementation with *rapH* and *rapG* resulted in a restoration of antibiotic production, underlying their essential role.[23]

MUTAGENESIS AND MUTASYNTHESIS

Although rapamycin is a valuable product with various pharmacological activities, the productivity of rapamycin in wild-type *S. hygroscopicus* is low. Therefore, further improvement of the rapamycin titer and generation of diverse derivatives of rapamycin in *S. hygroscopicus* has been imperative for its commercial development and clinical applications.[62] In an attempt to improve rapamycin productivity, fermentation medium optimization as described in the previous section,[16,18,53,54] in addition to classical strain improvement,[63] protoplasts-related techniques[64] and high-throughput screening method[65] have been developed. Demain's research group obtained a higher rapamycin-producing mutant from the protoplasts of *S. hygroscopicus* FC904 mutants treated with 1 μg ml^{-1} of gentamicin, widely used aminoglycosides, where an HPLC analysis showed that the rapamycin titer for this mutant (139 mg l^{-1}) was 60% higher than that for the parent strain. An enhanced productivity of rapamycin (194 mg l^{-1}; 124% higher than that of starting strain) was also obtained when exposing the spores of the *S. hygroscopicus* FC904 strain to gentamicin and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.[63]

Another attempt to generate high-yield rapamycin-producing strains was based on the protoplast-related techniques such as protoplasts mutation, intra- and interspecies fusion. When using mutation by UV, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, diethyl sulfates treatment or fusion of different parental protoplasts of *S. hygroscopicus*, progress was not achieved. However, the combination of interspecies protoplasts fusion and one round of genome shuffling generated a high-yield rapamycin producer with an outstanding yield of 445 mg l^{-1}.[64] Furthermore, as a result of the protoplast-related mutation and fusion experiments, it was observed that nearly all the high-yield rapamycin-producing strains shared a similar phenotype: round colonies with wrinkled edges and abundant yellow aerial mycelia.

Most traditional screening techniques performed in Erlenmeyer flasks require a large amount of material and cultivation process,

thereby limiting the wide application of strain improvement strategies.[66] Yet, a high-throughput screening method for isolating high-yield rapamycin-producing strains was recently developed by Xu *et al*.,[65] where more than 7000 colonies were screened and 10 high-yielding strains were isolated based on 96-well microtiter-plates cultivation and an 'agar plug' method. As a result, one mutant among the 10 isolates yielded 420 mg l^{-1} rapamycin, which was double the productivity of the parent strain used in the submerged fermentation process.[65]

Mutasynthesis through genetic engineering is another useful approach for generating novel derivatives of rapamycin. In a previous study, the Leadlay research group at Cambridge University reported that when wild-type *S. hygroscopicus* was grown in a rich medium supplemented with proline, it produced not only rapamycin but also prolylrapamycin (**2**; Figure 1), a rapamycin analog in which pipecolate is replaced by proline in the macrolactone ring. This indicates that RapP, which is responsible for incorporating the pipecolate precursor into the macrocycle, has a relaxed substrate specificity.[20] They also found that novel rapamycins containing 4-hydroxyprolyl moieties were biosynthesized in an engineered strain of *S. hygroscopicus* mutant bearing a deletion of *rapL*, where the deduced product is able to convert lysine to pipecolic acid. Also, when fermentations of *rapL*-deleted *S. hygroscopicus* mutants constructed by Ö31-phage-mediated gene replacement were added with 4-hydroxyproline, 4-hydroxyprolyl-rapamycin (**10**) and 4-hydroxyprolyl-26-demethoxyrapamycin (**11**) were produced (Figure 1).[20]

Meantime, another group showed that the use of nipecotic acid inhibited the biosynthesis of pipecolate, thereby enhancing the incorporation of other pipecolate analogs into the rapamycin molecule. Thus, 4-thiarapamycin (**12**; Figure 1) and 9-deoxo-5-sulfoxyrapmycin (**13**; Figure 1) were obtained from cultures of *S. hygroscopicus* supplemented with sulfur-containing pipecolate analogs, (*S*)-1,4-thiazane-3-carboxylic acid and (*S*)-1,3-thiazane-4-carboxylic acid, respectively.[67] However, in an FKBP-binding assay, both of these analogs displayed a significantly weaker binding affinity than rapamycin, supporting the previous report that the pipecolate-containing region of the rapamycin molecule is critical for the binding of rapamycin to FKBP.[68,69]

The addition of exogenous unnatural starter units also provided another way of generating structurally modified rapamycin analogs. When carboxylic acids such as cyclohexanecarboxylic acid, cyclohex-1-ene-carboxylic acid and cycloheptanecarboxylic acid in place of the natural starter unit DHCHC were fed to cultures of an engineered strain of *S. hygroscopicus* MG2-10 in which *rapKIJMNOQL* is deleted from the rapamycin biosynthetic gene cluster, the resulting mutants gave pre-rapamycin analogs.[70] This study was based on the precursor-directed biosynthesis carried out by Lowden *et al*.[24] in which the feeding of alternative starter units, such as cyclohexanecarboxylic acid, cyclohex-1-ene-carboxylic acid and cycloheptanecarboxylic acid, to the fermentation of rapamycin-producing *S. hygroscopicus* led to the production of new rapamycin derivatives (compounds **14**, **15** and **16**; Figure 1).

PERSPECTIVES

Although conventional strain improvement has enhanced the production level of rapamycin without genetic information on target gene clusters, it is still laborious and time consuming. However, metabolic engineering of the precursor pathways required for rapamycin biosynthesis may be able to control the carbon flux and lead to improved production of rapamycin and its derivatives. The extender units for rapamycin biosynthesis are malonyl-CoA and methylmalonyl-CoA, which are commonly used for polyketide biosynthesis. Malonyl-CoA

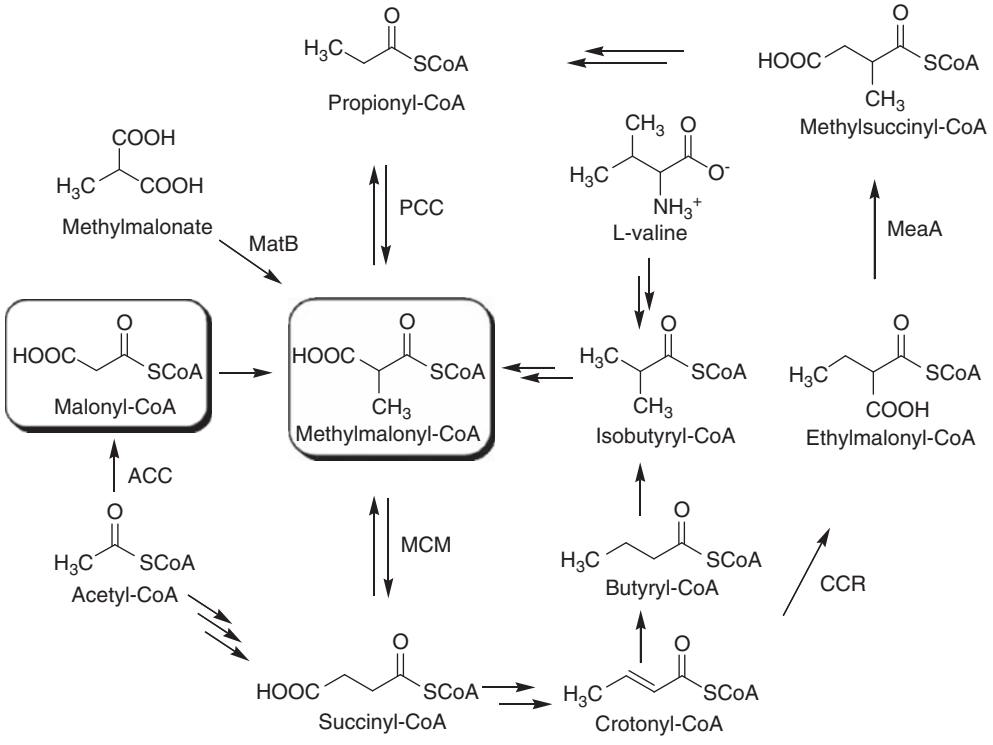

Figure 4 Proposed pathways for increasing supply of rapamycin precursors. Engineering the diverse biosynthetic pathway of malonyl-CoA and methylmalonyl-CoA enhances the rapamycin productivity. PCC, propionyl-CoA carboxylase; MatB, malonyl/methylmalonyl-CoA ligase; MCM, methylmalonyl-CoA mutase; CCR, crotonyl-CoA carboxylase/reductase; MeaA, putative coenzyme-B12-dependent mutase; ACC, acyl-CoA carboxylase.

is usually derived from the carboxylation of acetyl-CoA by an acetyl-CoA carboxylase.[71] Meanwhile, it has been suggested that methylmalonyl-CoA can be produced through several pathways, including the isomerization of succinyl-CoA, carboxylation of propionyl-CoA, catabolism of valine, CoA ligation of methylmalonate and conversion of acetyl-CoA through crotonyl-CoA-dependent pathway, as shown in Figure 4.[72] Recently, it was reported that the production of FK506 in *Streptomyces clavuligerus* CKD119 was improved by enhancing the supply of the biosynthetic precursor methylmalonyl-CoA.[73] Thus, if precursor pathway engineering is coupled with the traditional mutagenesis described herein, an efficient platform for rapamycin production could be achieved.

Combinatorial biosynthesis using the modular genetic architecture of rapamycin PKS may also offer great potential possibility for engineering enzymes to produce novel drug candidates. It has been reported that two novel tetraketides and four octaketides were biosynthesized by combining the rapamycin PKS module with another macrolide erythromycin PKS.[74] Therefore, ongoing studies of the details and mechanisms involving rapamycin biosynthesis and enzymology will be able to generate multiple rapamycin analogs with potential pharmaceutical application.

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