Natural Product Biosynthesis

Isolation and Characterization of Pre-rapamycin, the First Macrocyclic Intermediate in the Biosynthesis of the Immunosuppressant Rapamycin by *S. hygroscopicus***

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Rapamycin (**2**, sirolimus) is a lipophilic 31-membered macrocyclic polyketide produced by *Streptomyces hygroscopicus* NRRL5491. It has been shown to have immunosuppressive,[1] antifungal,[2] and antitumor activity.[3] In the commercial form rapamune, rapamycin has found clinical utility for the treatment of organ rejection following renal transplant surgery and the amelioration of some side effects associated with alternative treatments.[4]

Rapamycin biosynthesis occurs by a mixed type I polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) system.[5] The PKS uses a shikimate-derived 4,5-dihydroxycyclohex-1-enecarboxylic acid starter unit[6] and carries out a total of 14 successive cycles of polyketide chain extension with 7 propionate and 7 acetate units.[7] The NRPS component, encoded by the *rapP* gene, then incorporates the L-lysine-derived L-pipecolic acid into the chain, after which the macrocyclic ring is closed.[8] These latter steps involve transfer of the polyketide acyl chain from the PKS to the amino group of the activated pipecolic acid unit, cyclization, and release of the chain from the RapP protein through attack by a distal hydroxy group at C34 of the polyketide chain. This process yields the first putative enzyme-free intermediate, pre-rapamycin (**1**, 9-deoxo-16-*O*-desmethyl-27-desmethoxy-39-*O*-desmethylrapamycin). Pre-rapamycin (**1**) is believed to undergo further processing steps involving the products of a set of genes downstream of the PKS, including three O-methylations, two hydroxylations, and one ketone-forming oxidation, to yield **2** (Scheme 1).[9] A mutant with four of these downstream genes deleted has previously been isolated and the compound produced (16-*O*-desmethyl-27-desmethoxyrapamycin) was characterized.[10] However, the presumed immediate product of the PKS, namely, **1** has never been identified.

Herein we report the isolation and structural characterization of the key intermediate, pre-rapamycin (**1**). The region of the rapamycin cluster from *rapQ* to *rapI*, which encodes the processing genes, was removed by using a double recombination strategy (Figure 1).[11] LCMS analysis of culture extracts of this mutant (*S. hygroscopicus* MG2–10) did not

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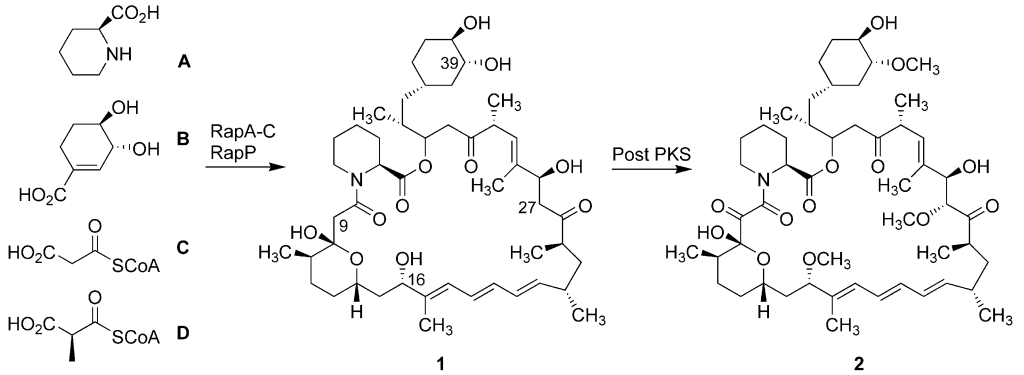


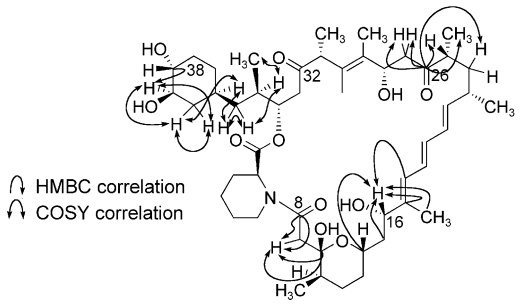
Scheme 1. Biosynthesis of pre-rapamycin (**1**) and rapamycin (**2**) from the precursors, pipecolic acid (**A**), dihydroxycyclohex-1-enecarboxylic acid (**B**), malonyl-CoA (**C**), and methylmalonyl-CoA (**D**).

show the rapamycin parent ion. In addition, exhaustive analysis of culture extracts revealed no additional compounds with a chromophore ($\lambda_{max} = 277$ nm) typical of the triene of **2** and related compounds. Complementation of this mutant, using integrative expression plasmids based on pSET152,[11] was attempted separately with each of the individual late-processing genes that had been removed: *rapK*, *rapI*, *rapJ*, *rapM*, *rapQ*, *rapN/O*, and *rapL*. Analysis of extracts where the culture was complemented by *rapK* only revealed accumulation of a new compound (*m*/*z* 841.5) that displayed a chromophore typical for the triene of **2**. High-resolution mass determination was consistent with the molecular formula

Figure 1. Region of the rapamycin cluster removed in *S. hygroscopicus* MG2–10.

$C_{48}H_{75}NO_{11}$. The fragmentation pathways of **2** and related compounds have been investigated by using electrospray ionization ion-trap mass spectrometry.[12] Compound **1** was investigated using tandem LCMS experiments and, in addition, high-resolution mass analyses of the fragmentation pathways were used to confirm the molecular formula proposed for each fragment. These data were fully consistent with the proposed fragmentation scheme (see the Supporting Information) for the putative first product of the rapamycin PKS, namely pre-rapamycin (**1**).

Compound **1** was isolated (8.5 mg) with a high degree of purity by preparative chromatography for NMR analysis.[13] The key structural differences between **1** and the fully processed **2** are the absence of a keto group at C9, the lack of a methoxy group at C27, and no *O*-methylation at the C16-OH and C39-OH groups. A range of 2D NMR experiments, namely, ^{1}H-^{1}H COSY, TOCSY, HMQC, and HMBC, were performed to aid the elucidation of the ^{1}H and ^{13}C NMR spectra of **1**. The key correlations that contributed to the assignment of the altered parts of the molecule are depicted in Scheme 2. HMBC correlations from the C10 ketal and the C8 amide carbon atoms ($\delta_C = 98.9$, 171.6 ppm) to an isolated methylene group ($\delta_H = 2.46$, 3.23 ppm) led to the identification of H9a and H9b. HMBC correlations from C14, C15, C17, C18, and C44 (C17-methyl), to a methine atom resonating at $\delta_H = 3.95$ ppm allowed the assignment of H16. The triene fragment was readily assigned from sequential COSY correlations from H44 (C17-methyl) along the spin system to H46 (C25-methyl). HMBC correlations from the C26 ketone to protons in this triene-containing fragment (H24, H25) and to a pair of doublet of doublet proton resonances ($\delta_H = 2.42$, 2.89 ppm) enabled assignment of the methylene protons H27a and H27b. The fragment H35 to H39 could be built up from COSY correlations. H39, which is geminal to a hydroxy group,



Scheme 2. Key 2D-NMR correlations for pre-rapamycin (**1**).

was assigned as resonating at $\delta_H = 3.24$ ppm. An HMBC correlation from H39 to C40 ($\delta_C = 75.9$ ppm) confirmed the presence of the C39,C40-diol. Careful examination of the complicated 2D NMR data confirmed that the assignment of the remaining proton and carbon atoms was consistent with the structure proposed for **1**.

The NMR spectra of rapamycins are complicated by the existence of rotamers.[15] Acquiring the NMR data at 10 °C made it possible to assign one of the rotamers, although the spectra were still complicated by the resonances of the second rotamer (for example, H9a, H9b). The highly complex nature of the ^{1}H NMR spectrum made the elucidation of coupling constants difficult, and for the most part impossible. It is,

however, reasonable to assume that the stereochemistry of **1** is likely to be the same as that of **2** since it is established during the biosynthesis of the polyketide chain.

The gene *rapK* was originally proposed to encode a pteridine-dependent dioxygenase[9] involved in the oxidation of a putative C9 hydroxy group to a ketone.[16] Aside from the proposed function, the evidence from the present study suggests, surprisingly, that *rapK* is required for the accumulation of **1**, the immediate product of the PKS. It is interesting to note that there are close homologues of *rapK* in the FK506(*fkbO*),[17] FK520(*fkbO*),[18] and hyg(*orf5*)[19] clusters, all of which might be assumed to fulfil similar functions. Further work is being undertaken in our laboratory to elucidate the function of the *rapK* gene and confirm that it is essential for accumulation of **1**.

Rapamycin (**2**) is a powerful immunosuppressive drug, which has been the subject of much study during the past decade. A mutant of *S. hygroscopicus* NRRL5491, which is specifically blocked in the biosynthesis of **2**, has been shown to lead to accumulation of **1**, whose structure was confirmed by NMR and MS methods. The availability of **1**, with its array of functional groups, opens the way to systematic use of both enzymatic and chemical modification to delineate more precisely the final steps of rapamycin biosynthesis and to create semisynthetic derivatives with interesting and potentially novel bioactivity.

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[13] The fermentation broth[14] was extracted three times with EtOAc. After defatting the extract with hexane the crude extract was chromatographed over silica gel (step gradient from hexane to acetone) and further fractionated over Sephadex LH-20 ($MeOH/CH_2Cl_2$, 1:1). The fractions containing **2** were rechromatographed over Sephadex LH-20 (heptane/chloroform/EtOH 10:10:1). Final purification was by reverse phase HPLC (column: Hypersil 5μ BDS C_{18}, 250 × 21.2 mm; eluent: 60 % CH_3CN/H_2O isocratic, 21 mL min^{-1}).
[14] Spores of *S. hygroscopicus* MG2–10 were used to inoculate seed medium SV2[20] and cultured at 30 °C, 48 h, 250 rpm, 2 inch throw. Production medium[21] was inoculated at 10 % v/v and allowed to ferment in a stirred bioreactor for 7 days, 26 °C, 0.75 vvm (vvm = volume of air per volume of liquid per minute), with the impellor speed 0.98 to 2.67 ms^{-1}.
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