New Rapamycin Derivatives by Precursor-Directed Biosynthesis

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Rapamycin (**1**) is a polyketide macrolide produced by *Streptomyces hygroscopicus* that displays potent immunosuppressant activity. In recent years there has been great interest in the chemistry[1] and biology[2] of rapamycin, and of the structurally similar immunosuppressants FK506 and FK520. We now describe results that promise to broaden the scope for biosynthetic engineering of new rapamycin and FK506/FK520 derivatives.

Feeding studies have confirmed the largely polyketide origin of rapamycin,[3] and have shown that the unusual trisubstituted cyclohexane ring arises from shikimate.[4] The entire biosynthetic gene cluster for rapamycin was sequenced by Leadlay, Staunton and co-workers in 1994.[5] The rapamycin polyketide synthase (PKS) is a type I mixed PKS/non-ribosomal peptide synthetase (NRPS) system in which 14 polyketide chain-extension modules are housed in three giant multimodular proteins (RAPS1 – 3). An NRPS-related multienzyme (pipecolate-incorporating enzyme)[6] inserts pipecolate and (probably) ensures closure of the macrocycle.

The genes encoding the rapamycin PKS (RAPS) have proved an excellent resource for combinatorial biosynthesis, owing in part to the diverse chemical functionality of the polyketide product. DNA from the *rap* PKS genes encoding individual and multiple enzyme activities[7] and even whole chain-extension modules,[8] has been spliced into the genes for other PKSs to generate functional, hybrid PKSs. In contrast, despite the potential clinical value of the products, this technology has not yet been extensively applied to engineer the biosynthesis of novel rapamycins.[9]

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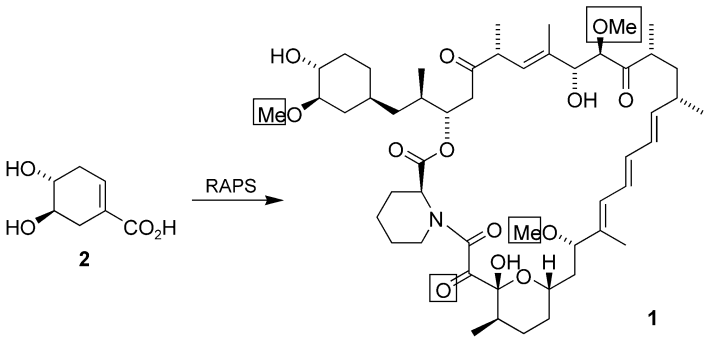
We previously identified 4,5-dihydroxycyclohex-1-enecarboxylic acid (**2**) as the most likely starter unit for the rapamycin PKS (Scheme 1).[10] We have proposed that this substrate is activated



Scheme 1. *Dihydroxycyclohex-1-enecarboxylic acid (**2**) is the starter unit for the rapamycin PKS. Post-PKS processing steps are highlighted in boxes.*

by the PKS loading module as an acyladenylate followed by acyl transfer to the 4′-phosphopantetheine cofactor of an acyl-carrier-protein domain in the loading module. Reduction of the C=C bond is proposed to occur at this stage, catalysed by the enoylreductase domain of the loading module. However, the significant incorporation of 4,5-dihydroxycyclohexanecarboxylic acid[11] hinted at a relaxed substrate specificity in the loading module. The flexibility of secondary metabolic enzymes has been exploited for the production of modified natural products of many kinds,[12] including the use of alternative PKS starter units.[13] This concept has already been applied to the generation of rapamycin analogues by feeding pipecolate analogues to *S. hygroscopicus*,[14] with their incorporation enhanced by disruption of a putative lysine cyclodeaminase gene responsible for pipecolate biosynthesis.[15]

In a preliminary survey of the substrate tolerance of the loading module, a series of 21 carboxylic acids was fed to the rapamycin-producing strain of *S. hygroscopicus*. These included monocyclic, polycyclic and branched aliphatic acids, substituted benzoic acids and heterocyclic acids. After four days' growth, the mycelia were extracted with methanol, and the extracts were analysed by reverse-phase HPLC and tandem electrospray mass spectrometry. In most cases there was no difference from the normal metabolite spectrum, consisting of rapamycin and small amounts of deoxy- and/or demethyl-rapamycins, due to incomplete post-PKS processing. However, new metabolites were present in significant amounts after feeding cyclohexanecarboxylic acid (metabolite **3**), cycloheptanecarboxylic acid (metabolite **4**) and cyclohex-1-enecarboxylic acid (metabolites **5** and **6**, isomers that interconvert in polar solvents; Figure 1). New metabolites of $M_W = 851$ and 897 were also seen at relatively low concentration after feeding cyclohex-3-enecarboxylic acid and 3-methylcyclohexanecarboxylic acid, respectively. In all cases, small amounts of related metabolites were observed due to different combinations of post-PKS processing steps.

All new metabolites possessed the characteristic rapamycin triene chromophore with λ_{max} at 277 nm. Preliminary structural

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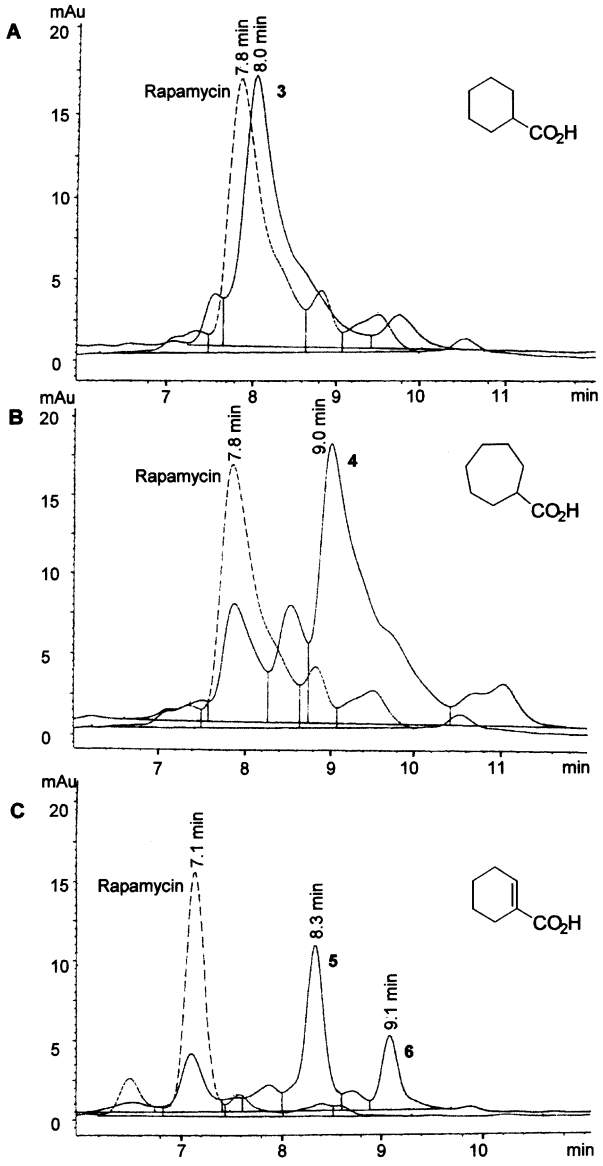
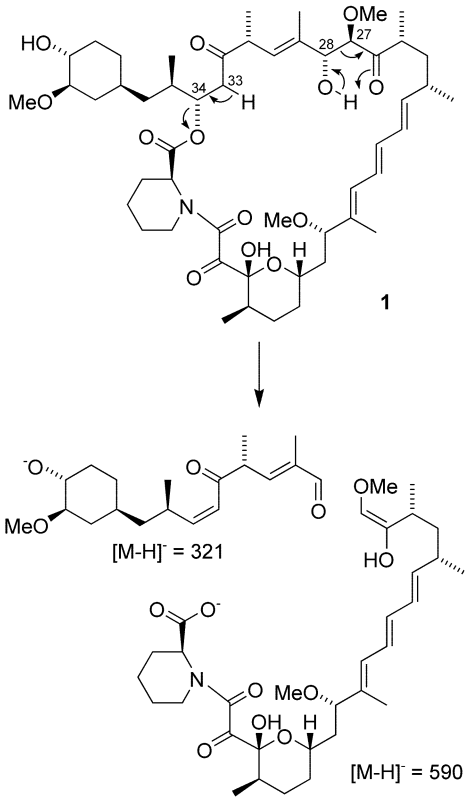


Figure 1. HPLC traces of crude methanol extracts of S. hygroscopicus *after feeding: A) cyclohexanecarboxylic acid; B) cycloheptanecarboxylic acid; C) cyclohex-1-enecarboxylic acid.*



Scheme 2. *The dominant fragmentations of rapamycin observed in the electrospray mass spectrum.*

characterisation was feasible from the fragmentation patterns in negative ion electrospray MS. The dominant fragmentations of rapamycin derivatives are retro-aldol cleavage at C27—C28 and β elimination of the pipecolate moiety across C33—C34 (Scheme 2). MS data for the new metabolites are summarised in Table 1. From these data it is clear that the C1—C27 fragment remains intact in **3** and **4**, but in **5** and **6** it probably lacks the C-27 methoxy group, which is known to be attached at a late stage in biosynthesis. In all cases, the data for the C28 – C42 fragments are inconsistent with simple incorporation of the added precursors. The data can be explained by an additional hydroxylation of each precursor ring and by reduction of the C=C bond in cyclohex-1-enecarboxylic acid.

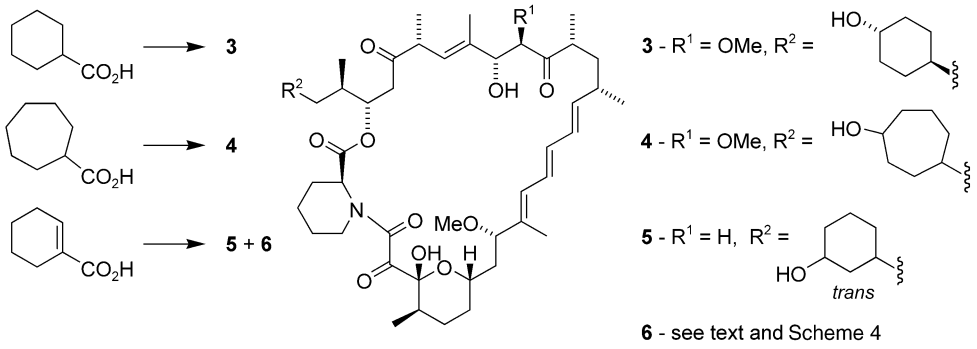
The structures of **3** – **6** were analysed in detail by ^{1}H and ^{13}C NMR (see Supporting Information for assignments), by comparison to the published NMR assignments for rapamycin[16] and through $^{1}H,^{1}H$ (COSY) and $^{1}H,^{13}C$ correlations (heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC)). Interpretation of the NMR spectra for rapamycin is complicated by the presence of a minor rotamer (~20%). We did not observe any increase in the amount of this rotamer for any of **3** – **6**, and our NMR analysis was confined to the major rotamer. For **3**, **4** and **5**, most of the signals from C1 – C36 could be assigned by direct comparison with the spectra for rapamycin. Complete assignment of the C1 – C36 signals was possible from the 2D NMR data. In **5** and **6**, the C27 carbinol and

Table 1. *Negative ion ESMS fragmentation data for rapamycin and new metabolites* ***3*** *–* ***6****.*

Compound	Yield of analogue [mg L^{-1}]	Yield of rapamycin [mg L^{-1}]	M_w	$[M-H]^-$ for C1 – C27	$[M-H]^-$ for C28 – C42/C43
rapamycin			913	590	321
3	5	0	883	590	291
4	3.6	1.4	897	590	305
5	2.7	1.2	853	560	291
6	1.2	1.2	853	560	291

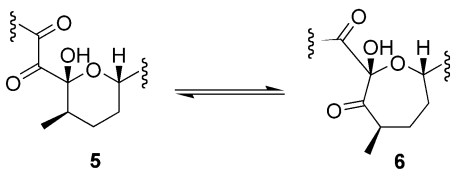
methoxy signals were absent, and a new CH_2 group was observed that exhibited cross-peaks consistent with C-27.

Significantly, in each case the carbinol signals for C39 and C40 were absent, and a single new carbinol signal was present, consistent in each case with a monohydroxylated ring in the starter unit. Assignment of the regio- and stereochemistry of this ring was complicated by severe overlap of methylene signals. By using the previously assigned C36 signals and the new carbinol signals as anchor points, it was possible to trace $^1H,^1H$ and $^1H,^{13}C$ coupling networks consistent with the structures shown in Scheme 3. Strong cross-peaks over three bonds were assumed to indicate an antiperiplanar orientation of the $^1H,^1H$ or $^1H,^{13}C$ pairs. Both regio- and stereochemistry could be confidently assigned to **3**, but only the regiochemistry of **4** could be determined due the less well defined conformation of seven-membered rings. The regiochemistry and relative stereochemistry (*trans*) of the starter unit ring could be determined for **5** and **6**, but not the absolute stereochemistry.



Scheme 3. *Structures of rapamycin analogues produced by feeding alternative starter units.*

The NMR data also revealed the nature of the isomerisation between **5** and **6**. Significant changes were observed in NMR signals around the tetrahydropyran ring. The HMBC spectrum revealed that **6** contains an oxepane ring (Scheme 4) created by hemiacetal formation at C9 instead of C10. This has precedent in the chemistry of rapamycin, whose oxepane isomer is observed at a concentration about 5% that of rapamycin.[17]



Scheme 4. *The nature of the isomerisation between **5** and **6** in polar solvents.*

The presence of the hydroxyl group on the starter unit-derived ring in **3**–**6** reveals a novel hydroxylation activity. It is unlikely that this hydroxylation occurs after polyketide chain synthesis, since cyclohexanecarboxylic acid and cyclohex-1-enecarboxylic acid do not give the same result, even though C=C reduction occurs during incorporation of the latter precursor.

Immunosuppressant activity of the rapamycin derivatives was determined by measuring, as previously described,[15, 18] their ability to induce specific cell-cycle arrest at G1 in human 536 cells, a lymphocyte cell line immortalised by Epstein–Barr virus infection. ID_{50} values for inhibition of DNA synthesis after 24 h were as follows: rapamycin, 0.1 nM; **3**, 2 nM; **4**, 3 nM; **5**, 200 nM; and **6**, 100 nM.

Our choice of starter-unit analogues was governed largely by commercial availability and cost, but these initial results will allow a more systematic exploration of the range of substrates accepted using designed, synthetic analogues. Since in vivo studies may be complicated by differences in cell permeability and alternative metabolic fates of substrates, a quantitative analysis of the substrate specificity of the rapamycin PKS will require in vitro experiments on purified enzymes.

However, our results clearly demonstrate that the loading module of the rapamycin PKS must possess a degree of flexibility in the molecular recognition of substrates, despite competition from the natural starter unit. These results suggest that transfer of the rapamycin loading module onto other modular PKS systems should allow the introduction of diverse novel functionality into other polyketide metabolites, as shown previously by fusion of the avermectin PKS loading module onto the erythromycin PKS.[19]

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Keywords: biosynthesis · loading module · polyketides · rapamycin

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