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MANIPULATION OF THE C(22)-C(27) REGION OF RAPAMYCIN: STABILITY ISSUES AND BIOLOGICAL IMPLICATIONS

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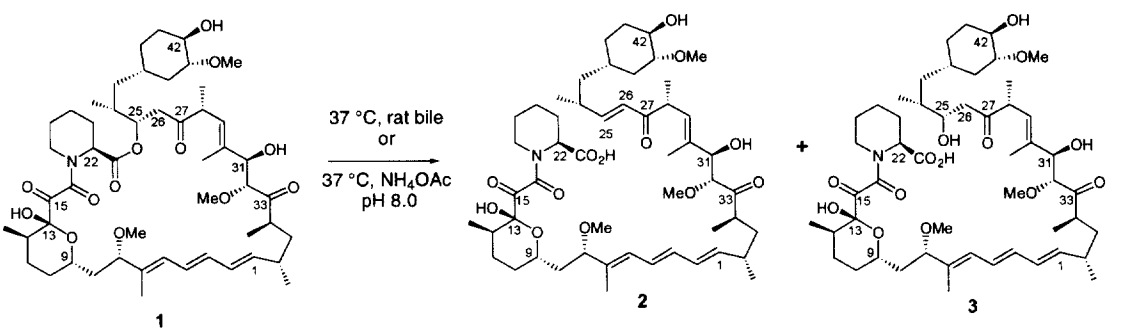
Abstract: A novel series of rapamycin derivatives with modifications in the C(22)-C(27) region has been prepared. These compounds are evaluated for their ability to prevent ring fragmentation while still retaining immunosuppressive capabilities.

Rapamycin (Rapamune®) **1** is a potent immunosuppressant natural product with a novel mechanism of action. It is currently in phase III clinical trials for the prevention of transplant rejection.[1] Rapamycin interacts with two distinct proteins to exert its immunosuppressive effects. The left hand portion, from C-8 to C-31 represents the FKBP binding domain while the remainder of the molecule binds to FRAP and has been termed the effector domain.[2] Rapamycin has been shown to be sensitive to both acid and base resulting in ring fragmentation and degradation leading to the formation of **2**.[3] Importantly, under physiologically relevant conditions, rapamycin degrades into two distinct products, the α,β-unsaturated ketone **2** and the β-hydroxy ketone **3**.[4]

Scheme 1



1 **2** **3**

These products arise via β-elimination to form the enone, or via hydrolysis of the lactone. This was a concern to us since these ring opened derivatives are less potent as immunosuppressants than rapamycin. The goal of this study was to synthesize derivatives of rapamycin that would have diminished abilities to undergo this ring cleavage and still retain immunosuppressive capabilities.

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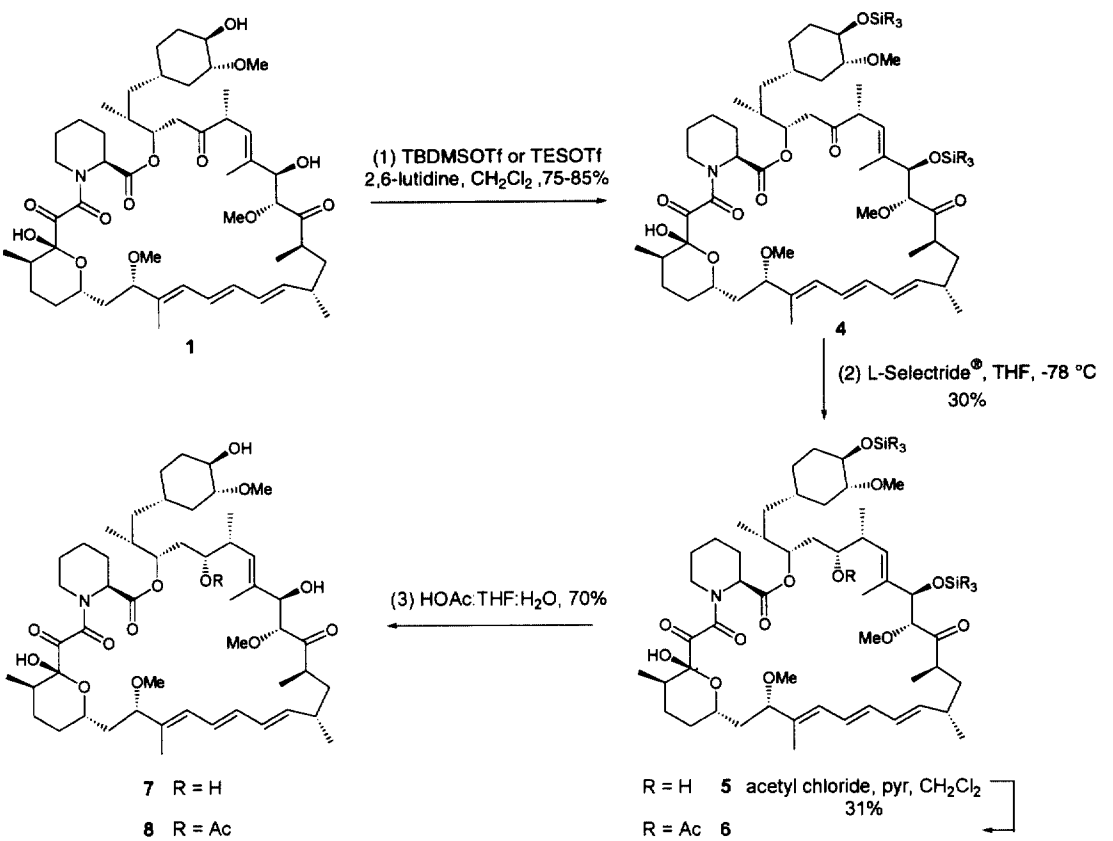
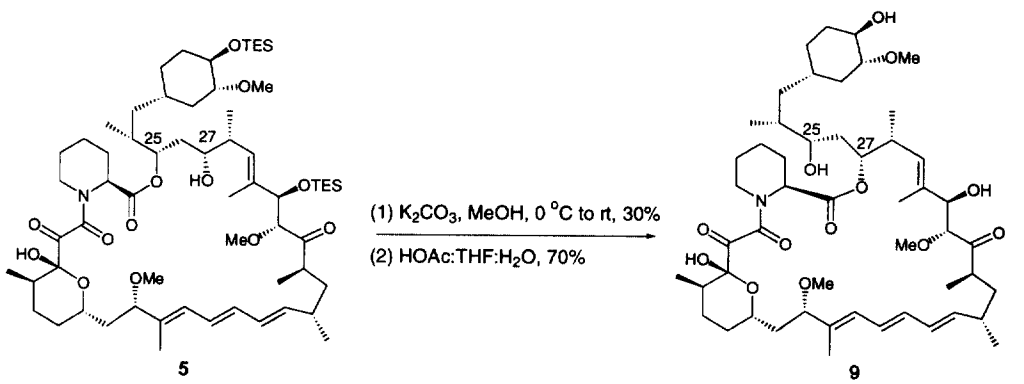
Chemistry

We postulated that suitable synthetic modification of C-22 to C-27 would provide compounds with the desired characteristics. Our initial focus was the C-27 carbonyl. Reduction of the ketone would provide the alcohol that is incapable of β-elimination but should not directly influence the hydrolysis pathway. Treatment of bis-silylated rapamycin **4** with L-Selectride® followed by removal of the protecting groups provided for C-27 hydroxy rapamycin **7** (Scheme 2).[5,6] The resultant alcohol **5** could be esterified with acetyl chloride and deprotected to give C-27 acetyl rapamycin **8**.[5] The C-27 alcohol is apparently quite hindered; attempted functionalization with a variety of other reagents failed to give useful products. Interestingly, compound **5** was found to undergo a facile ring contraction upon treatment with base to provide after deprotection, the translactonized material **9** (Scheme 3).[5,6b]

Scheme 2



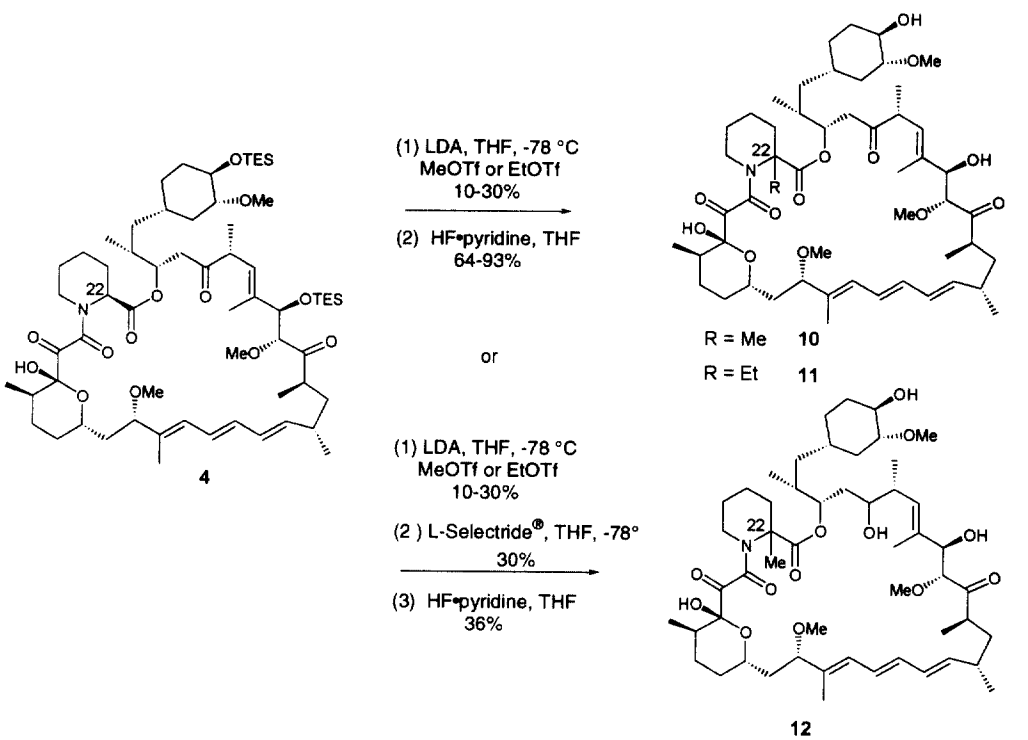
Scheme 3



5 9

A second hypothesis that we tested involved the introduction of substituents at the C-22 carbon to increase the steric encumbrance at the α-carbon and thus limit the hydrolysis pathway. Proceeding with bis-TES protected rapamycin **4**, we found that treatment with LDA at low temperature followed by quenching with a suitable electrophile lead to the formation of C-22 alkylated products **10** and **11** (Scheme 4).[5,7]

Scheme 4



4 12

The reaction works best with smaller electrophiles; use of sterically demanding reagents gave poorer yields.[8] The stereochemistry of the alkylated product was not determined, although the formation of only one diastereomer was observed. Interestingly, only one amide rotamer was seen via NMR upon alkylation of C-22.[9]

Substitution at this position presumably rigidifies this portion of the molecule to restrict rotation about the amide bond. Having the C-31 hydroxyl protected is critical to the success of this reaction. Treatment of rapamycin itself with LDA results in a retro-aldol reaction with cleavage of the C(31)-C(32) bond.[3c,6a,10] A dually functionalized molecule was also prepared by combining both methodologies to provide the C-22-alkyl, C-27-hydroxy derivative **12**.[5] We assume that the stereochemistry of the reduced product is the same as in compound **7**, although this has not been confirmed experimentally.

Results and Discussion

These analogues were tested in a series of assays to assess their stability as well as their biological activity. First, compounds were incubated in pH 7.4 phosphate buffer at 37 °C and the $T_{1/2}$ determined via an HPLC assay to assess their hydrolytic stability as compared to rapamycin. The appearance of enone **2** and β-hydroxy ketone **3** was noted. Reducing the ketone at C-27 did not significantly improve the stability, however, acetylation of the resultant alcohol (compound **8**) doubled the $T_{1/2}$. The most stable compound of the series is the alkylated derivative **10**, which displays an almost 3-fold increase in $T_{1/2}$. Interestingly, combining C-22 alkylation with reduction at C-27 did not have an additive effect but instead provides the same $T_{1/2}$ as rapamycin itself.

Compounds were also evaluated for their ability to bind to FKBP via their inhibition of the peptidyl prolyl *cis-trans* isomerase activity (PPIase) inherent to FKBP.[11,12a] Modifications to the C-27 ketone alone do not greatly disrupt the binding to FKBP. However, modifications to C-22 that directly alter the FKBP binding domain begin to show pronounced effects. The most striking case is **11**, which is over 60-fold weaker than rapamycin. Rearranged derivative **9** also shows decreased binding to FKBP. On examination of the docked model of **9** bound to FKBP clear distortions in the pipecolinate ring conformation, relative to rapamycin, are apparent.[13]

Table 1. In Vitro and In Vivo Activities of C(22) - C-(27) Modified Rapamycin Analogues.

Compound	C-22	C-27	$T_{1/2}$ (h)	PPIase IC_{50} (nM)	LAF IC_{50} (nM) (R/A)*	Skin Graft Survival (days ± SD, n = 6)
1	(rapamycin)H	=O	13	15	3 (1.0)	12 ± 1.7
7	H	OH	17.5	22	3.7 (1.3)	8.5 ± 1.2
8	H	OAc	34	NT	99 (0.08)	NT
9	H	OCOpip	21.6	310	16 (0.42)	7.0 ± 0.0
10	Me	=O	38.4	95	23 (0.42)	9.33 ± 0.5
11	Et	=O	NT	950	140 (0.07)	NT
12	Me	OH	14.6	150	9.9 (0.23)	7.0 ± 0.0
no treatment	--	--	--	--	--	7.0 ± 0.0

* The IC_{50} of rapamycin varies from 1-9 nM. The activity in the LAF assay is best expressed as a ratio of the rapamycin IC_{50}/analogue IC_{50} in the assay in which both compounds were run simultaneously.

These analogues were also evaluated in two models for their immunosuppressive effects. The initial in vitro assay (LAF) determines the ability of a compound to inhibit cellular proliferation.[12] In general, activity in the LAF assay loosely correlates with activity in the PPIase assay. Reduction of C-27 alone provides for a slight increase in activity relative to rapamycin (R/A = 1.3), whereas all of the other compounds were less active than rapamycin (R/A < 1.0). As the steric encumbrance increases at C-22, potency in LAF decreases as can be seen with compound **11**. Again, this effect can be ascribed to greater distortions in the FKBP binding domain.

The mouse skin graft model is used to determine the ability of a compound to prevent transplantation rejection in vivo.[14] Briefly, a piece of skin from male DBA/2 donors is transplanted onto the back of male Balb/c recipients. Mice are dosed with compounds at 4mg / kg / day, *ip* for 6 days and the grafts are inspected visually for signs of rejection. While all of the compounds were less active than rapamycin, methyl alkylated derivative **10** and C-27 hydroxy **7** still showed some rejection inhibition. Alkylated material **10** is particularly notable since it displays in vivo activity despite decreased LAF and PPIase activity.

In summary, a novel series of rapamycin analogues have been developed and synthesized to modulate hydrolytic stability and biological properties. These compounds display improved stability while retaining in vitro and in vivo activity despite rather dramatic modifications, in some cases, to the FKBP binding domain.

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7. Critical to determining the site of alkylation was the disappearance of the C-22 proton at 5.25 ppm and the presence of an additional methyl singlet at 1.62 ppm (400 MHz ^{1}H NMR, $CDCl_3$). Protons α to other carbonyls still retain appropriate chemical shifts.

8. Yields of the alkylation reaction decrease from 30% with MeOTf to 10-15% with EtOTf. $(PhSe)_2$ was also used as an electrophile in this reaction. Only a trace of the alkylated product was observed.

9. Rapamycin and its analogues typically exist as a mixture of two amide rotamers in solution with the *trans* amide being the major rotamer: Findlay, J. A.; Radics, L. *Can. J. Chem.* **1980**, *58*, 579.

10. The retro-aldol reaction has been effected under numerous conditions. See references 3c, 6a and Luengo, J. I.; Konialian, A. L.; Holt, D. A. *Tetrahedron Lett.* **1993**, *34*, 991.

11. Adapted from: Garcia-Echeverria, C.; Kofron, J. L.; Kuzmic, P.; Rich, D. H. *Biochem. Biophys. Res. Commun.* **1993**, *191*, 70. Inhibition of PPIase activity is necesssary but not sufficient for immunosuppressive activity. See for example ref. 11a.

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