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SYNTHESIS AND BIOACTIVITY OF PHOTOLABILE SIROLIMUS (RAPAMYCIN) ANALOGS

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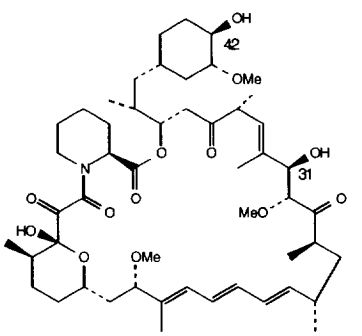
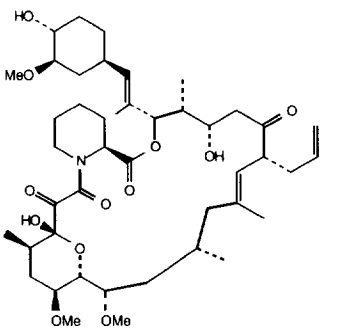
Abstract: Several photolabile sirolimus analogs have been synthesized. All ot the compounds showed an antiproliferative effect in a mitogen-induced thymocyte proliferation assay. Compounds **3** and **4** were shown to be equipotent to sirolimus in a murine skin allograft model; they should thus be useful for investigating the *in vivo* details of the signal transduction pathway(s) modulated by sirolimus. The immunosuppressive effect of analogs **3-5** is corroborated by their ability, as part of an FKBP12 complex, to bind a sirolimus effector protein (SEP).

Introduction: The structurally related macrolides, sirolimus (rapamycin, **1**) and FK506 (**2**) are important tools in the study of divergent signal transduction pathways in cells. Both macrolides bind to the same class of intracellular receptors (the FK506 binding proteins, FKBPs); yet the drug:FKBP12 complexes interfere with divergent signal transduction pathways.[1] FK506 inhibits production of cytokines, such as IL-2, from T cells activated with Ca^{+2} dependent stimuli. The FK506:FKBP12 complex binds to and inhibits the phosphatase activity of calcineurin, thereby inhibiting the upregulation of IL-2 transcription and production.[2] Sirolimus, on the other hand, modulates cytokine-induced responses with little or no effect on IL-2 production. The sirolimus:FKBP12 binary complex binds a second protein (a sirolimus effector protein, SEP) and it has been suggested that this ternary complex modulates cytokine induced signal transduction pathways.[3]



Sirolimus (Rapamycin,1)

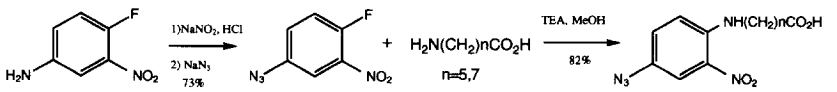


FK506 (2)

Photoaffinity labeling has been widely used, *in vitro*, to map the binding sites between proteins and

drugs, and between two proteins.[4] Several photolabile sirolimus analogs were synthesized to study the molecular details of the interaction between the sirolimus:FKBP-12 complex and the recently reported sirolimus effector protein.

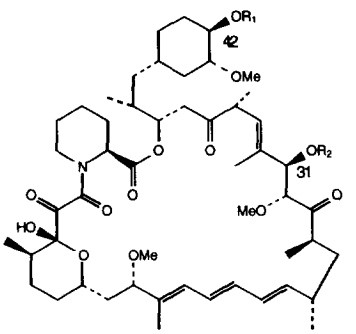
Chemistry: The photolabile groups were attached at either 31 or 42 positions, as it had been demonstrated, in some cases, that derivatization at these two positions showed little effect on the antiproliferative activity (although the 31 esters are generally less active than the 42 esters).[5] To maximize the bioactivity we designed the synthesis to allow for variations in the nature of the photoprobe, the length of the linker between the photoprobe and the drug, as well as the site of attachment on the drug of the probe. The photolabile groups in **3-5** were prepared by a previously reported method, and can be radiolabeled to increase the detection sensitivity.[6] The substituted aminocaproic and octanoic acids were prepared by the reaction of the ω-amino acid with 2-nitro-4-azido-fluorobenzene[7] as shown below. The sirolimus probes were prepared via the published dehydrative coupling methods.[5]



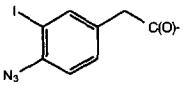
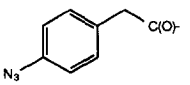
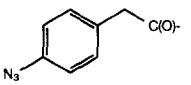
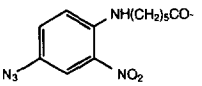
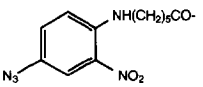
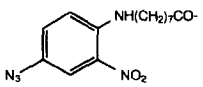
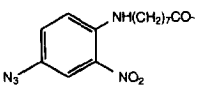
Biological Assays: The mitogen-induced thymocyte proliferation assay (LAF) was performed as previously described.[8] Briefly, C3H/HeJ thymocytes (10^6 cells/200 µl) were stimulated with 1.6 µg/ml of phytohemagglutinin and 0.25 ng/ml of IL-1b in the presence or absence of various concentrations of drug for 72 hours with ^{3}H-thymidine pulsing occurring during the last 6 hours of the incubation.

The H-2 incompatible, BALB/c to C3H combination was used in the skin allograft model.[9] The skin graft assay uses the procedure of Billingham and Medwar.[10] A void was made in the dorsal skin of the recipient mouse and a piece of skin from the donor mouse was grafted onto the void. Grafts were bandaged for six days. On day six, the bandages were removed and the graft was observed daily until rejection occurred. Rejection was defined as 95% necrosis of the graft epithelium and the detachment of the suture line between the graft and the surrounding recipient skin. Six mice were used in each treatment group and the survival times were averaged. The drugs were dissolved in a vehicle which consisted of 2% ethanol, 8% cremaphor EL and 90% water. A 0.2 ml aliquot of the solution containing the desired drug dose (4 mg/kg) was administered intraperitoneally for six consecutive days starting on the first day after grafting. Six mice were used to generate each data point.

Results and Discussion: As an initial step to assess their biological activity, the photoaffinity probes as well as sirolimus were tested in the co-mitogen stimulated proliferation assay (LAF).[8] Like sirolimus (compound **1**), the photolabile compounds **3-9** inhibited T cell proliferation in the co-mitogen stimulated proliferation assay with similar IC_{50} values (Table 1). As observed previously,[5] 42 esters were slightly more active than sirolimus, and generally more active than the 31 esters[8]. The short chain esters may be a little more active than the longer chain ones (**7** vs **8**), and the substitution on the phenyl ring is of little consequence (**3** vs **4**).



Sirolimus Photoaffinity Probes

Compound	R_1	R_2	LAF IC_{50}, nM	Skin Graft MST± SD*
1	H	H	9.8	12.01 ± 0.26
3		H	3	12.7 ± 3.2
4		H	1	10.7 ± 1.2
5	H		5.8	ND
6	H		7.8	ND
7		H	2.5	ND
8		H	10.2	ND
9	H		10.5	ND

*Mean Survival Time of the graft in days resulting from a 4 mg/kg dose i.p.
ND = not determined

Since it is well documented that sirolimus blocks allograft rejection in numerous allograft models,[1] two compounds, **3** and **4**, were tested in a murine skin allograft assay (a model of a nonvascularized transplant)[9] employing an H-2 incompatible donor-recipient combination. Compounds **3** and **4**, administered at 4 mg/kg x 6 days i.p., were equipotent to sirolimus in this allograft model (Table 1). In the murine skin allograft model,

skin from a BALB/c donor mouse is transplanted onto the back of a C3H recipient (an H-2 incompatible donor-recipient combination). The graft is observed until rejection occurs (in an untreated mouse MST is 6.94 ± 0.95 days). In this same assay , a 32 mg/kg dose of cyclosporin A gives a MST of 10.17 ± 0.41 days.

The bioactivity of these compounds were likely to be due to these analogs themselves and not through conversion to sirolimus, since the complexes of compounds **3-5,** with FKBP12, can directly bind to a sirolimus effector protein as reported previously.[3c]

Conclusions: Several sirolimus photolabile analogs have been synthesized. Upon complexing with FKBP12 these compounds directly bind to the sirolimus effector protein, and elicit the antiproliferative effect as measured in both the LAF assay and a murine skin allograft transplantation. Therefore these photolabile sirolimus analogs will be of importance in the study of the molecular details of the immunosuppressive mechanism of sirolimus.

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