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Cleavage of the Cyclohexyl-Subunit of Rapamycin results in Loss of immunosuppressive Activity

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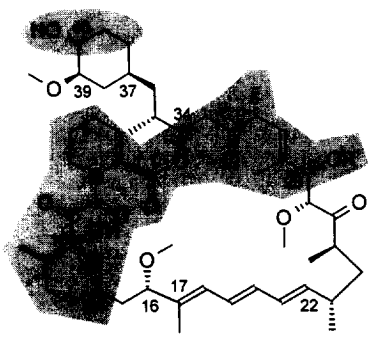
Abstract

The cyclohexyl-subunit of rapamycin was cleaved by a sequence involving a Baeyer-Villiger reaction and acid hydrolysis of the resulting lactone-acetal as key steps. Binding of this new rapamycin derivative to FKBP12 was only slightly reduced by this modification, whereas the loss of antiproliferative and immunosuppressive activity was dramatic. These findings indicate that part of the cyclohexyl-subunit of rapamycin could belong to its effector domain.

Keywords: Rapamycin; Immunosuppressant; Natural product derivation

Rapamycin **1** is a very potent immunosuppressive agent and is currently undergoing clinical trials for the prevention of allograft rejection[1]. This macrolide exerts its immunosuppressive effect primarily by inhibiting the proliferative response of T-cells to IL-2 and other cytokines[2,3].



FKBP12-binding domain

R = H Rapamycin **1**
R = Me 28-O-methylrapamycin **2**

At the molecular level, rapamycin acts as a so-called "dual-domain" inhibitor[4]. In a first step it binds to the immunophilin FKBP12 through its binding domain[5,6](shaded). The pipecolinyl ring is deeply buried in the binding pocket of FKBP12. The pyranose ring, the region extending from C34 to C28 and part of the cyclohexyl ring are in close contact with the immunophilin. The three hydroxyls at positions 10, 28 and 40 are involved in hydrogen bonds. The binding to FKBP12 is necessary, but not sufficient for immunosuppressive activity.

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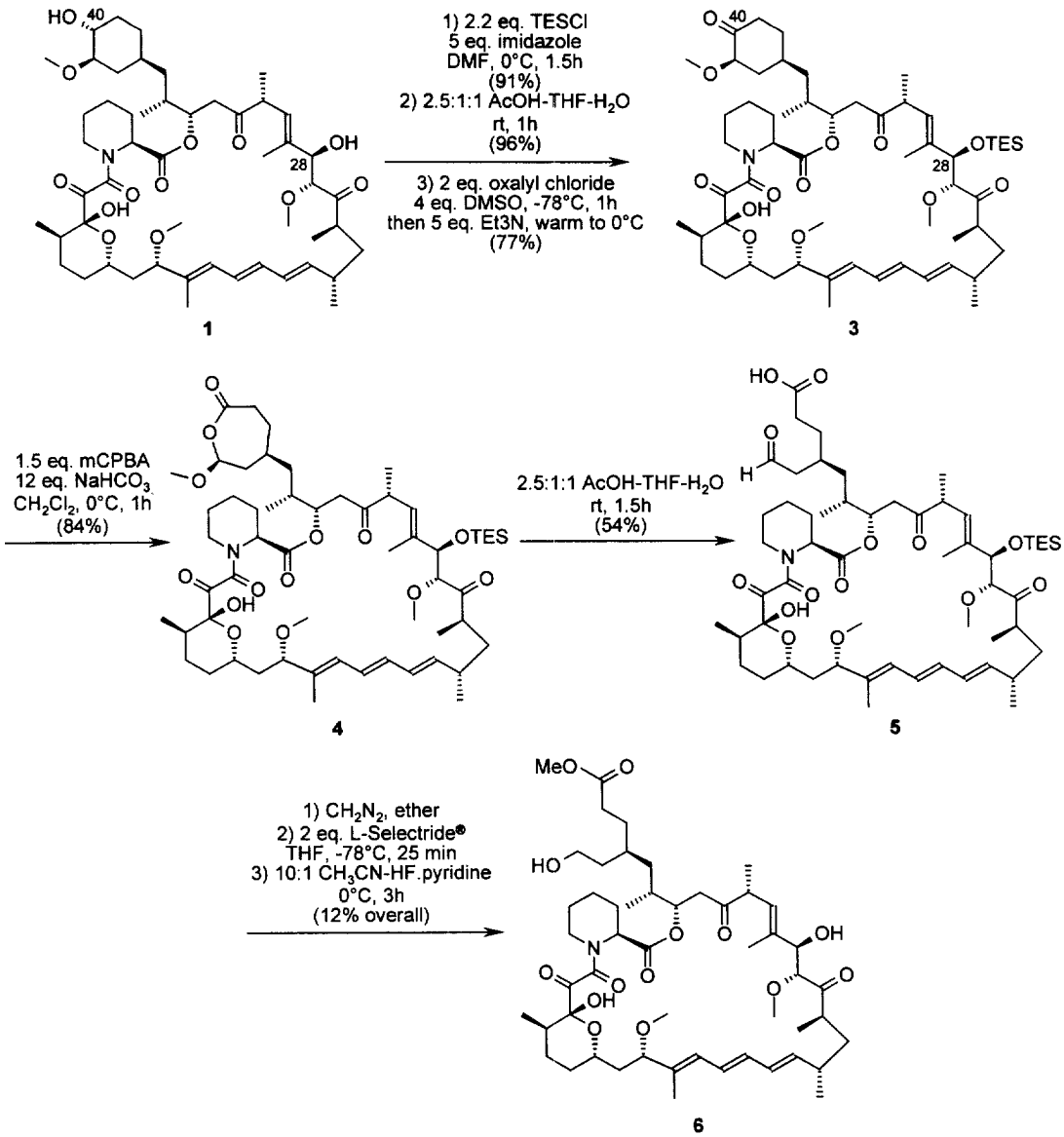
In a second step, the FKBP12/rapamycin complex binds to a protein termed FRAP[7] (RAFT[8], RAPT[9], mTOR[10]), which since its discovery has been shown to be the target for the immunosuppressive and, more generally, antiproliferative activity of rapamycin[11]. This second binding event is mediated by the effector domain of rapamycin. The X-ray crystal structure of FKBP12/rapamycin complexed to the FKBP12-rapamycin-binding (FRB) domain of human FRAP[12] shows that the most extensive contacts occur between the C17-C22 triene subunit of rapamycin and FRB.

Some of us have recently reported the X-ray crystal structure of 28-O-methylrapamycin **2** bound to FKBP12[13]. We found that, while the macrocyclic part of **2** is unchanged with respect to the FKBP12-rapamycin complex, the cyclohexyl moiety is dramatically shifted. This structural change does not affect binding of **2** to FKBP12, but results in significantly reduced immunosuppressive activity. These findings raised the question whether the loss of activity of **2** is merely due to an unfavourable steric interaction of the cyclohexyl ring, in its new position, with the target of the FKBP12-rapamycin complex FRAP, or whether the cyclohexylethyl side chain of rapamycin could exert a functional role in target recognition, thus making it part of rapamycin's effector domain. In order to attempt to answer the latter question, we sought for a modification of the cyclohexyl subunit which would profoundly alter its structure without increasing its steric demands. Cleavage of the cyclohexyl ring appeared to be an attractive option and we were indeed able to prepare such a derivative (Scheme 1).

The synthesis started with silylation of both hydroxyls at positions 28 and 40, followed by selective removal of the 40-O-triethylsilyl protecting group. The free C40-hydroxyl was then oxidized using the Swern protocol, yielding ketone **3**. Baeyer-Villiger oxidation of **3** proceeded cleanly, allowing us to obtain the rather sensitive lactone-acetal **4**. No products coming from olefin epoxidation were observed. Ring cleavage in **4** was carried out by mild acidic hydrolysis leading to acid-aldehyde **5**. The latter compound proved also to be rather unstable upon storage and was therefore not ideal for biological testing. It was further transformed into compound **6** by a three-step sequence, involving esterification of the carboxylic acid, selective reduction of the aldehyde in the presence of the other carbonyl groups present in the molecule and, finally, removal of the triethylsilyl protecting group.

The affinity of compound **6** for FKBP12, as well as its ability to inhibit the proliferation of the IL-6-dependent hybridoma clone B13-29-15 (IL-6-dep. Prol.) and the mixed lymphocyte reaction (MLR) were assessed[14] (Table 1). Cleavage of the cyclohexyl ring results in only a ca. 6-fold loss in affinity to FKBP12 in comparison to rapamycin **1**. On the other hand, the antiproliferative and immunosuppressive activities are reduced 200-fold and more than 380-fold respectively. The loss of biological activity is most likely the consequence of a decrease in affinity of the FKBP12/**6** complex towards FRAP. This reduced affinity can hardly be explained by steric factors, as cleavage of the cyclohexyl ring does not increase the steric demands of the molecule. Furthermore, the X-ray crystal structure of the FKBP12/rapamycin/FRB complex[12]



Scheme 1

suggests that the chains formed by cleavage of the cyclohexyl ring should find sufficient space to position themselves between the two proteins without perturbing ternary complex formation. An alternative explanation for the loss of activity is that part of the cyclohexyl ring, i.e. an intact C37-C38 segment, including the C39 methoxyl, is necessary for proper interaction of the FKBP12/rapamycin complex with FRAP. In other words, this subunit of rapamycin might be part of its effector domain. The proximity of that particular segment to elements of FRAP in the X-ray structure of the ternary complex[12] lends support to this conclusion (representative

Table 1: In-vitro activity profile of compound 6

	Relative IC_{50}		
Compound	FKBP12-binding[a]	IL-6-dep. Prol.[b]	MLR[b]
Rapamycin **1**	0.6	1	1
6	3.4	200	>380

[a] The ability of the compounds to compete with immobilized FK506 for binding to biotinylated FKBP12 was determined in a competitive binding assay. FK506 was included as standard in each individual experiment. Results are expressed as means of the relative IC_{50} values (i.e. IC_{50} test compound / IC_{50} FK506). The range of absolute IC_{50} values for FK506 was 0.8-1.2 nM[14].

[b] Rapamycin was included as standard in each individual experiment. Results are expressed as means of the relative IC_{50} values (i.e. IC_{50} test compound / IC_{50} rapamycin). The range of absolute IC_{50} values for RAP was 0.07-0.5 nM in the IL-6-dependent proliferation assay and 0.06-0.9 nM in the MLR[14].

interatomic distances: C38 - Cβ Phe2039 4.4Å, C39 methoxy carbon – Cα Arg2036 4.5Å). It is quite remarkable that disruption of a relatively small portion of rapamycin which is involved in the interaction with FRAP results in such a dramatic loss of activity.

In summary, we have shown that cleavage of the cyclohexyl ring of rapamycin leads to significant loss of antiproliferative and immunosuppressive activity, despite the fact that binding to FKBP12 is only slightly reduced. Our results indicate that the C37-C39 segment of the cyclohexyl ring plays a role in binding of the FKBP12/rapamycin complex to FRAP and therefore suggest that this moiety belongs to the effector domain of rapamycin.

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