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PREPARATION AND IMMUNOSUPPRESSIVE ACTIVITY OF 32-(*O*)-ACYLATED AND 32-(*O*)-THIOACYLATED ANALOGUES OF ASCOMYCIN

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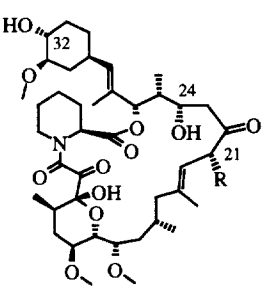
Abstract: A series of 32-(*O*)-acylated and 32-(*O*)-thioacylated derivatives of the antibiotic ascomycin (**1**) have been synthesized. These readily accessible analogues exhibit potent immunosuppressive activity *in vitro*, as measured by an interleukin-2 reporter gene assay and the mixed lymphocyte reaction. Such molecules are expected to have a therapeutic potential in chronic inflammatory diseases of the airways such as asthma.

The antifungal antibiotic ascomycin (**1**) has been known for more than 30 years[1]. Both ascomycin and the better known allyl analogue, the immunosuppressant FK506 (**2**), are powerful inhibitors of antigen-stimulated T-cell activation and proliferation[2]. Similar to the chemically different cyclosporin A (CsA), ascomycin and its analogues when bound to the intracellular binding protein FKBP-12, block the signal transduction pathway in T-cells through inhibiting the Ca-dependent protein phosphatase calcineurin[3]. As a consequence, translocation of the transcription factor NF-AT from the cytoplasm to the nucleus is blocked, resulting in a failure to activate genes necessary for T-cell proliferation (e.g. interleukin-2). Both FK506 and CsA are successfully used in transplantation for prevention of organ graft rejection[2,4]. Therapeutic effects with CsA have also been observed in rheumatoid arthritis[5] and psoriasis[6]. Furthermore, recent preclinical data indicate that selective T-cell inhibitors such as CsA and ascomycin analogues could be of interest in treating asthma, a chronic inflammatory disease of the airways[7]. Indeed, oral CsA has been shown to be of clinical benefit in severe asthmatic patients[8]. These findings are in line with the current view that the T-cell plays a pivotal role in the pathology of asthma[9]. However, due to their inherent, mechanistically related side effects there might be a concern of using current immunosuppressants such as FK506 and CsA as an oral therapy for non-life threatening asthma[10]. Systemic exposure and hence the likelihood of untoward side effects could be reduced by giving such compounds locally by inhalation[11]. Furthermore, in contrast to FK506 and CsA, the ideal immunosuppressant should have no oral activity because current inhalation devices cannot prevent the majority of the inhaled drug (80-90%) from being deposited in the upper airways, and finally being swallowed. We therefore initiated a program with the objective of finding simple, potent immunosuppressive and anti-inflammatory ascomycin analogues which would be suited for inhalation therapy of respiratory diseases such as



1 ascomycin R=ethyl
2 FK506 R=allyl

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asthma. This paper reports the synthesis and the biological characterization *in vitro* of a series of easily accessible 32-(*O*)-acylated and 32-(*O*)-thioacylated analogues of ascomycin (**1**).

Chemistry: The synthesis of benzoyl carbamic acid 32-(*O*)-ascomycinyl ester **3** and of the acyl thiocarbamic acid 32-(*O*)-ascomycinyl esters **4-9** is illustrated in Scheme 1 and Table 1. Protection of the competing 24-hydroxyl group as the TBS-ether turned out to be advantageous and was accomplished in quantitative yield by a bis-silylation/ monodesilylation protocol. Reaction of the mono-protected ascomycin with acyl isothiocyanates (Table 2) in toluene or benzoyl isocyanate in dichloromethane, followed by deprotection using aqueous HF in acetonitrile afforded compounds **3-8** in moderate yields. A special deprotection protocol was applied for the synthesis of compound **9**. In order to prevent dioxolanone ring-opening under aqueous acidic conditions, the silyl ether was selectively cleaved with trimethylsilyl triflate in dichloromethane at -78°C[12]. The synthesis of non-commercially available acyl isothiocyanates is illustrated in Table 2. They were made from the corresponding acid chlorides by reaction with sodium thiocyanate in ethyl acetate[13]. No purification step was necessary and, after removal of sodium chloride by filtration, the products were obtained in moderate to good yields and sufficiently pure for direct use.

Scheme 1

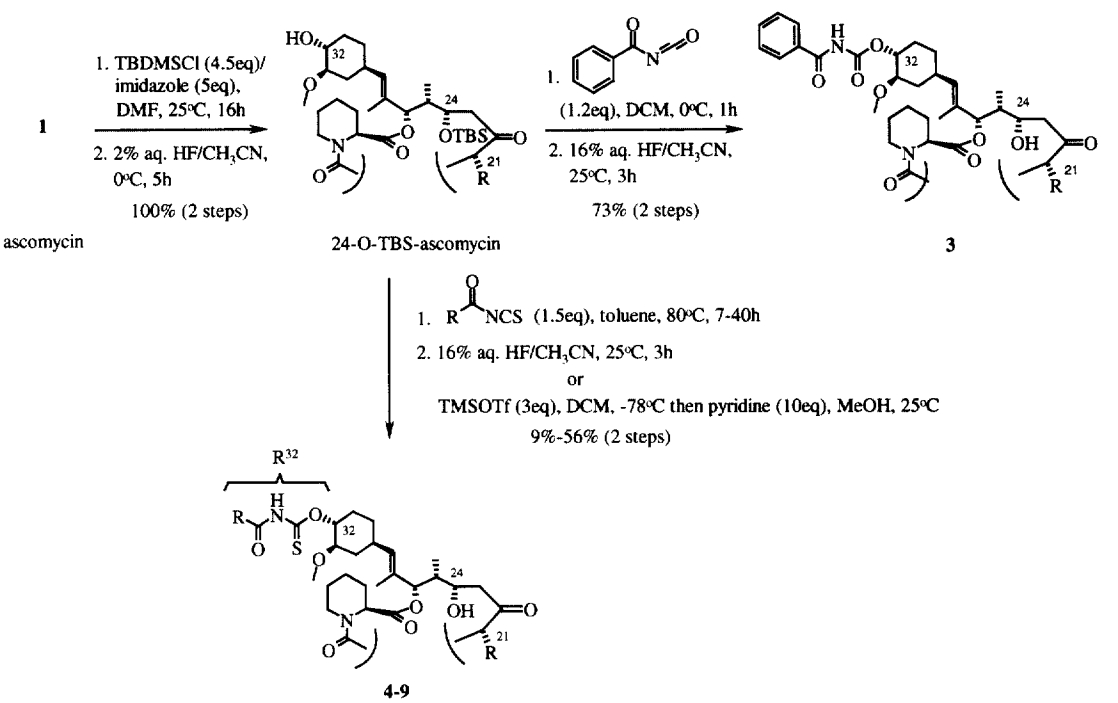
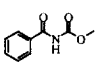
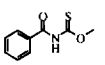
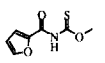
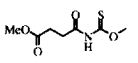
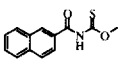
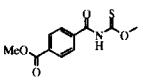
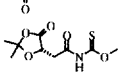
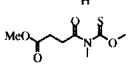
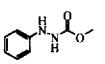
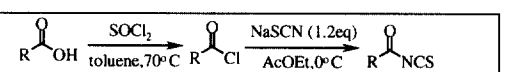


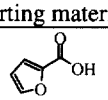
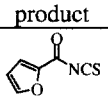
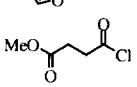
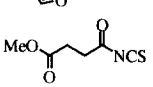
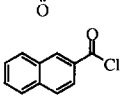
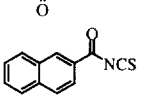
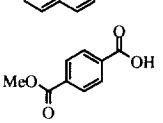
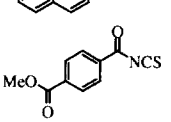
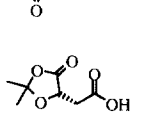
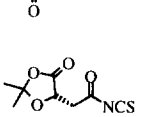
Table 1: Structures and *in vitro* immunosuppressive activities of compounds **1-11**

compound	R32	R21	IL-2 RGA rIC_{50}[a]	MLR rIC_{50}[a]	FKBP-12 rIC_{50}[a]
1 ascomycin	OH	ethyl	1.7	1.9	0.9
2 FK506	OH	allyl	1	1	1
3		ethyl	1.2	2.9	1.3
4		ethyl	3.4	11	6.3
5		ethyl	3.5	7.6	3.4
6		ethyl	4.6	19	1.6
7		ethyl	6.5	15	6.6
8		ethyl	9.0	8.4	7.3
9		ethyl	12	12	0.9
10		ethyl	6.9	36	2.7
11		ethyl	0.7	2.5	1.5

[a] Mean IC_{50} [nM] values (n=2-8) of the test compounds are compared to that of FK506 and expressed as relative IC_{50}: rIC_{50}=IC_{50} substance / IC_{50} FK506. The absolute IC_{50} values for FK506 in the IL-2 RGA, the MLR and the FKBP-12 binding assay are 0.2nM, 0.3nM and 1.2nM, respectively.

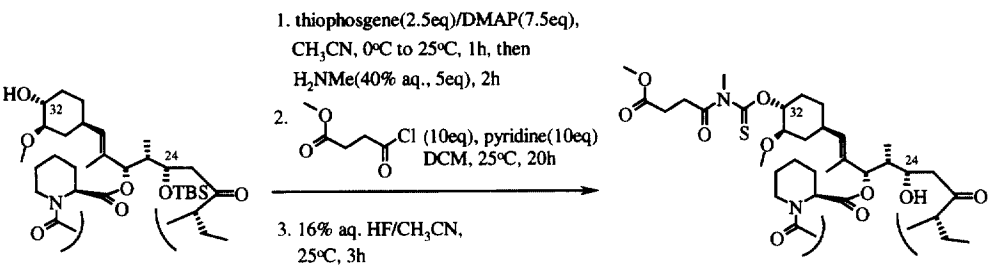
Table 2: General synthesis of acyl isothiocyanates



starting material	product	yield
		21% (2steps)
		95%
		95%
		73% (2steps)
		64% (2steps)

The synthesis of compound **10** is illustrated in Scheme 2. 24-(*O*)-TBS-ascomycin was first converted to the corresponding *N*-methyl-thiocarbamic acid 32-(*O*)-ascomycinyl ester by activation with thiophosgene/4-dimethylaminopyridine in acetonitrile and *in situ* reaction with aqueous methylamine. Acylation of the *N*-methyl-thiocarbamate intermediate with 3-chlorocarbonyl propionic acid methyl ester followed by the usual deprotection with aqueous HF led to product **10**.

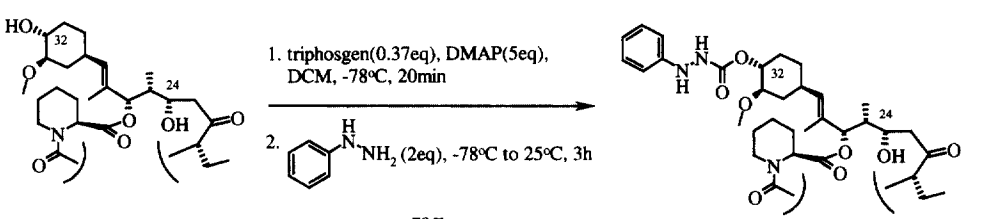
Scheme 2



24-O-TBS-ascomycin 12% (3 steps) **10**

Finally, Scheme 3 illustrates the synthesis of compound **11**. Remarkably, no 24-hydroxyl protection was necessary in this case and selective 32-hydroxyl mono-acylation was achieved by activation of ascomycin (**1**) at -78°C with triphosgen/4-dimethylaminopyridine followed by *in situ* reaction with phenylhydrazine.

Scheme 3



ascomycin **1** 79% **11**

Results and Discussion: Table 1 illustrates the *in vitro* immunosuppressive activity of **1-11** as measured in the interleukin-2 reporter gene assay (IL-2 RGA) and the mouse mixed lymphocyte reaction (MLR)[14]. Both assays are models for *in vitro* T-cell activation. In the case of the IL-2 RGA the activity of the IL-2 promoter in a

mitogen-stimulated human T-cell line is determined. The MLR is a model for T-cell activation by alloantigen. Since binding to FKBP-12 is a prerequisite for the immunosuppressive activity of ascomycin analogues the results for binding to FKBP-12 are also shown. All values are expressed as relative activities to FK506 (**2**) which is about twice as potent as the parent ascomycin (**1**)[14]. The benzoyl carbamic acid ester derivative **3** was equipotent to ascomycin (**1**) in both cellular assays the IL-2 RGA and the MLR. Consistent with this, analogue **3** and ascomycin (**1**) showed similar activity in the binding to FKBP-12. Replacement of the oxygen atom by sulfur in the R^{32} linker led to a decrease of immunosuppressive activity as illustrated by direct comparison of compounds **3** and **4**. In general, increasing the size of the R^{32} substituent in the acyl thiocarbamic acid ester series **4-10** gave less potent compounds although the loss of activity was only moderate (≤10). This indicates that there is some degree of steric tolerance at position 32 of ascomycin which allows chemical modification of the compound. This has also been observed by others[15] and may be explained by the structure of the active drug/FKBP-12/calcineurin complex[16]. It is noteworthy that compound **10** was only about twice less potent than the non *N*-methylated analogue **6**. Thus, the NH bond of the carbamic acid ester linker seems not to be involved in any significant hydrogen-bond interaction with the target proteins. The most potent compound synthesized in this series was derivative **11**. Replacing the 32-hydroxy group of ascomycin (**1**) by the larger *N'*-phenyl-hydrazinecarboxylic acid ester moiety doubled the potency of the parent compound in the IL-2 RGA and retained the activity in the MLR. It is noteworthy, that the IC_{50} values determined with human T-cells (IL-2 RGA) were comparable with the IC_{50} values determined with mouse T-cells (MLR) differing only by a factor of 1 to 5. As reported previously[17], and confirmed in this study, binding to FKBP-12 is a prerequisite for immunosuppressive activity of ascomycin analogues but not necessarily predictive for their immunosuppressive efficacy *in vitro*. For example, compound **9** was equipotent to compound **3** and parent ascomycin (**1**) with respect to binding to FKBP-12 but was about 10 times weaker in the IL-2 RGA and the MLR. A possible explanation for this finding is that the dioxolanone-substituent strengthens the macrolide/FKBP-12 interaction as indicated by the low rIC_{50}, but weakens the affinity of the macrolide/FKBP-12 binary complex for its cellular target, the protein phosphatase calcineurin thereby reducing the immunosuppressive activity of **9**.

In summary, we have synthesized a series of easily accessible 32-(*O*)-acylated and 32-(*O*)- thioacylated ascomycin derivatives and evaluated their immunosuppressive properties *in vitro* in the IL-2 RGA, the MLR and the FKBP-12 binding assay. Modification of the 32-hydroxy group of ascomycin (**1**) led to the identification of analogues (e.g. **3** and **11**) whose immunosuppressive activity was equal or better than that of the parent natural product. Further SAR of 32-carbamic and thiocarbamic acid ascomycinyl ester analogues as well as their characterization in *in vivo* models of allergic asthma are ongoing and will be the subject of a future publication.

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In all assays the mean IC_{50} [nM] value of the test compound (n=2-8) was compared to that of FK506 and expressed as relative IC_{50}: $rIC_{50}=IC_{50}$ substance / IC_{50} FK506. The absolute IC_{50} values for FK506 in the IL-2 RGA, the MLR and the MBA are 0.2nM, 0.3nM and 1.2nM, respectively.
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