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Chemistry of the Immunomodulatory Macrolide Ascomycin and Related Analogues

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1. Introduction

This chapter, after giving a short overview of the natural product ascomycin and related analogues, provides a summary of the biological properties and the clinical use of the ascomycin derivative pimecrolimus, a calcineurin inhibitor, developed and registered for topical treatment of the inflammatory skin disease atopic dermatitis. This is followed by an in-depth description of the structural features of ascomycin and synthesis aspects including specific modifications in the binding region, cleavage reactions, semisynthetic approaches, and modifications in the effector and cyclohexyl regions. The literature covers journal articles up to July 2010 with emphasis on chemistry. Structure-activity relationships are mentioned only qualitatively. No attempt has been made to review the patent literature in depth.

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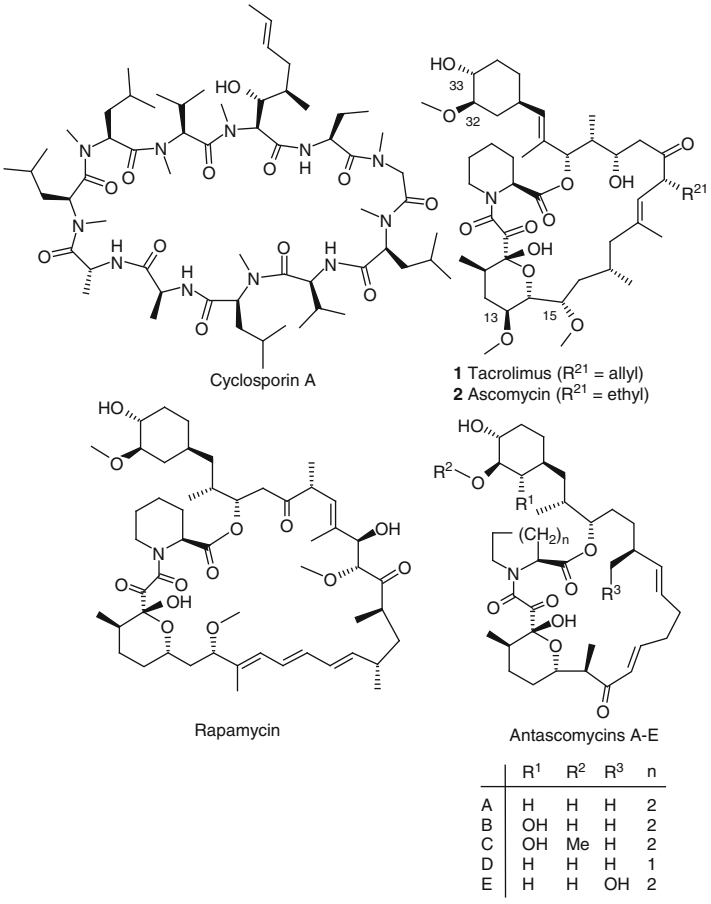
1.1. Ascomycin and Related Natural Products

The successful application of cyclosporin A (CyA, Sandimmune®) in the prevention of transplantation rejection stimulated a worldwide search for new selective T-cell inhibitors resulting in the discovery of the macrolactam tacrolimus (**1**, FK506, Prograf®, Scheme 1) (*1–3*). Ascomycin (**2**), a compound initially referred to as FR-900520 and isolated earlier from *Streptomyces hygroscopicus* var. *ascomyceticus*, (Fig. 1) due to its antifungal activities, and for which the structure was not elucidated originally, was later shown to be a close structural analogue of tacrolimus (ethyl instead of allyl on C-21) (*4–7*). Analogues bearing other substituents, such as methyl (*5*), or propyl (*8*), on C-21, or, homologues in which several methyl groups on the macrocycle are replaced by a higher alkyl (*i.e.* ethyl) have been isolated as well (*9, 10*).



	R1	R2	R3	n
A	H	H	H	2
B	OH	H	H	2
C	OH	Me	H	2
D	H	H	H	1
E	H	H	OH	2

Scheme 1. Structures of cyclosporin A, tacrolimus (**1**), ascomycin (**2**), and related macrolactams

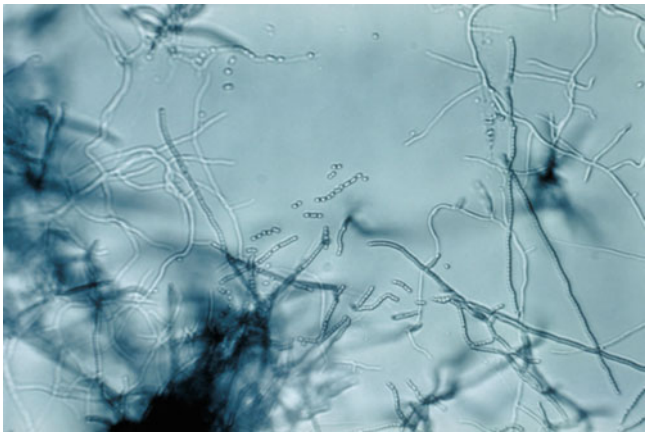
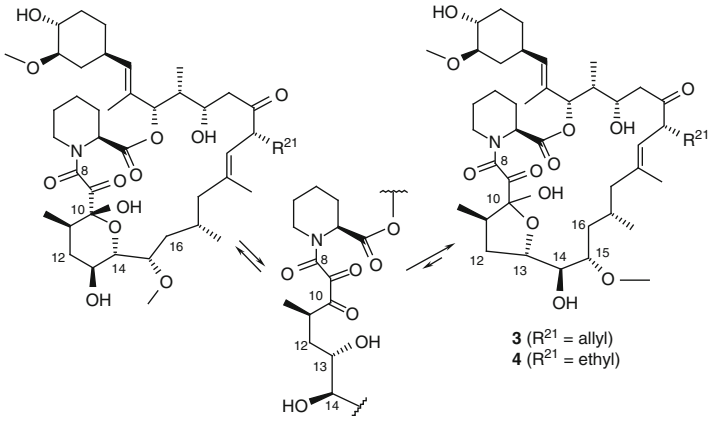


Fig. 1. Photograph of *Streptomyces* sp. grown in tap water agar. Branching filaments, abundant aerial mycelia, and long chains of small spores are visible, all of which are characteristic of the organism. Taken from en.wikipedia.org



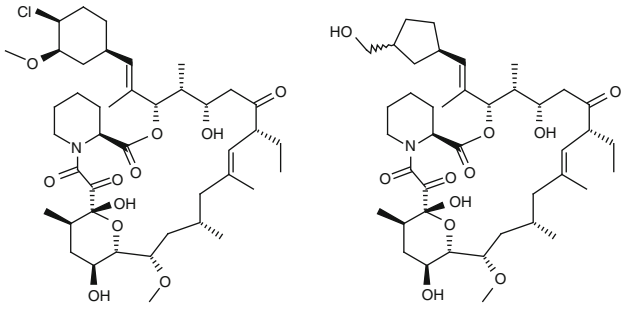
Scheme 2. Equilibria of 13-*O*-desmethyl derivatives **3** and **4** of tacrolimus (**1**) and ascomycin (**2**)

Macrolactams featuring a proline unit instead of the pipecolic acid moiety have been found as by-products in the fermentation broth of tacrolimus producer strains (*11*). Numerous 32-, 13-, and/or 15-*O*-desmethyl derivatives of ascomycin (**2**) and tacrolimus (**1**) are readily available through biotransformation (*11*–*15*). Interestingly, the 13-*O*-desmethyl-derivatives **3** and **4** exist in solution as highly complex mixtures of isomers (Scheme 2). Thus, seven different isomers of 13-*O*-desmethyl-FK506 (**3**) could be differentiated in COSY, HMBC, and HMQC experiments. The formation of these isomers could be explained by an epimerization at C-10, formation of hemiketal rings between C-10 and C-13, or, C-9 and C-13, and the occurrence of (*Z*)/(*E*)-isomers at the amide bond (*16*).

Other analogues of ascomycin that contain hydrogen, methyl, or ethyl instead of a methoxy at one or both of C-13 and C-15 have been prepared through fermentation employing a genetically modified ascomycin gene cluster (*17*). Related natural products containing an α,β-diketo-pipecolate subunit are rapamycin (Sirolimus) and the antascomycins (Scheme 1). Rapamycin, another macrolactam, had also been discovered like CyA and ascomycin in an antifungal screening of fermentation broths (*18, 19*). Although it was discovered quite early that rapamycin inhibits immune responses in rats (*20*), its therapeutic potential, however, was initially not fully recognized. It was the discovery of tacrolimus (**1**) that led to a renewed interest in rapamycin as an immunosuppressant (*21, 22*). The antascomycins A, B, C, D, and E have been isolated from a strain of *Micromonospora* (*23*). They bind strongly to macrophilin (FK506 binding protein, FKBP12) and antagonize the effects of **1** and **2** on T-cells. Their biological properties have not yet been fully explored.

1.2. Ascomycin Derivatives, a Novel Class of Anti-inflammatory Compounds

The discovery of this novel class of anti-inflammatory compounds started with reports of the high efficacy of oral cyclosporin A in the treatment of psoriasis, atopic dermatitis, and other inflammatory skin diseases. However, efforts failed to render this calcineurin inhibitor effective topically, in order to minimize its systemic immunosuppressive side effects. Using a newly developed model of allergic contact dermatitis (ACD) in pigs, calcineurin inhibitors of the tacrolimus- and ascomycin-type were shown to be highly effective after topical application. These findings provided the first pharmacological evidence of the potential of this novel class of topical agents for the treatment of inflammatory skin diseases (*24*). Topical application of the first representative of this class, SDZ 281–240 (Scheme 3), in



Scheme 3. Structures of the 33-*epi*-chloro-derivative of ascomycin (**2**) pimecrolimus (**2a**), and SDZ 281–240

chronic plaque psoriasis under *Finn*-chamber occlusion confirmed the validity of this concept in man (*25*). Intensive studies on structure-activity relationships and comparative pharmacological evaluations among a large number of newly synthesized derivatives to identify a compound combining high anti-inflammatory activity with minimal side effects finally resulted in the discovery and development of pimecrolimus (**2a**, SDZ ASM 981, Scheme 3) (*26, 27*). A detailed review of the discovery and development of the then new class of topical calcineurin inhibitors has been published (*28*).

1.2.1. Pimecrolimus

1.2.1.1. Pharmacology *In Vitro* and *In Vivo*

Pimecrolimus (**2a**) binds with high affinity to the cytosolic receptor macrophilin-12 and inhibits the phosphatase calcineurin, an enzyme required for the dephosphorylation of the cytosolic form of the nuclear factor of activated T-cells (NF-AT). As a consequence, it prevents in T-cells the transcription and release of both T-helper type 1 cell (TH1) and T-helper type 2 cell (TH2) inflammatory cytokines such as interleukin-2 (IL-2), interferon-γ (IFN-γ), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α), and granulocyte macrophage colony-stimulating factor (GM-CSF) as well as T-cell proliferation (*29*). A graphical representation of the biological mechanism of action is shown in Fig. 2. The inhibitory effect has been shown using the Jurkat human T-cell

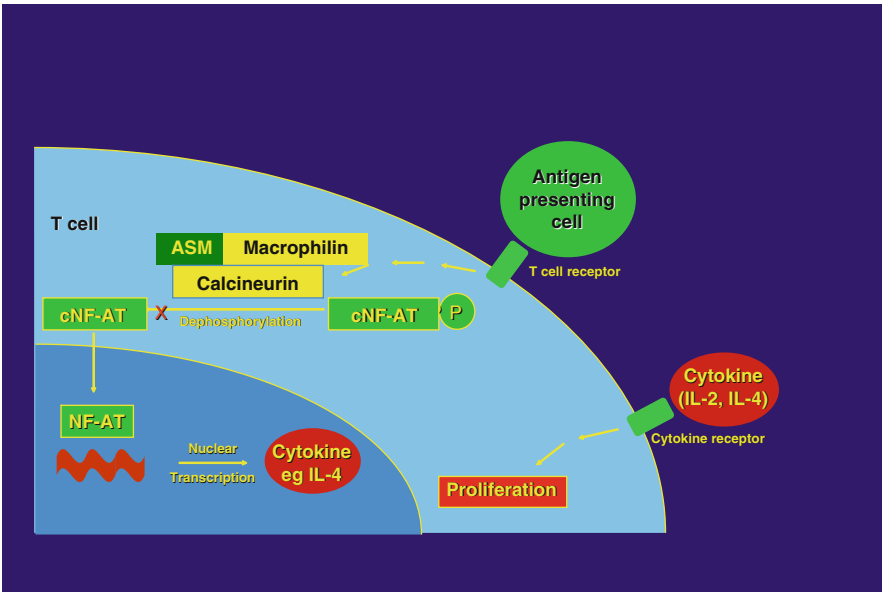


Fig. 2. Mechanism of action of pimecrolimus (ASM) on T-cells

line, peripheral blood mononuclear cells from healthy subjects, as well as human T-helper cell clones isolated from the skin of an atopic dermatitis patient. In these T-cell clones, pimecrolimus (**2a**) inhibits cytokine production at (sub)nanomolar concentrations and as potently as tacrolimus. Furthermore, pimecrolimus shows selectivity for antigen-primed memory T-cells, an effect not seen with tacrolimus (*30*). Pimecrolimus (**2a**) also prevents the production of TNF-α and the release of pro-inflammatory mediators like histamine, hexosaminidase, and tryptase in activated primary human skin mast cells and rodent mast cell lines (*31*). Pimecrolimus does not affect the proliferation of keratinocyte, endothelial, and fibroblast cell lines and has, in contrast to corticosteroids, no effect on the differentiation, maturation, functions, and viability of human dendritic cells (*32*). A recent study revealed that pimecrolimus (**2a**) increases at low nanomolar concentrations innate immune functions of human keratinocytes, such as expression of Toll-like receptors 2 and 6, as well as the production of antimicrobial peptides (cathelicidin, human beta defensin-2 and 3). Pimecrolimus (**2a**) also enhances the functional capacity of keratinocytes to inhibit the growth of *Staphylococcus aureus*. These data suggest that **2a** can amplify the cutaneous innate host defense (*33*).

Topical pimecrolimus penetrates similarly into, but permeates less through the skin *in vitro*, when compared to corticosteroids or tacrolimus. In comparison with bethamethasone, clobetasol, and difluorcortolone used as 1% solutions, pimecrolimus (**2a**) permeates less through the human skin by factors of 60–110. When comparing pimecrolimus (**2a**) and tacrolimus (**1**) in the same vehicle at the same concentration, the same skin concentrations were found with both compounds, but permeation rates of pimecrolimus through human and pig skin were lower by a factor of 9–10 (*34*). When comparing pimecrolimus cream 1% (Elidel®) and tacrolimus ointment 0.1% and 0.03% (Protopic®), similar skin concentrations were determined with pimecrolimus and tacrolimus. However, the permeation rates through the skin were found to be lower with pimecrolimus (**2a**) than those of tacrolimus (**1**), with both ointment preparations by factors of about 6 and 4, despite higher drug concentrations in Elidel® cream. In agreement with the results obtained with human skin, the permeation rate of pimecrolimus (**2a**) through normal and inflamed pig skin was found to be lower than that of tacrolimus (**1**) as well (*35*). These data indicate a lower systemic exposure to pimecrolimus after topical application as compared to tacrolimus and corticosteroids.

Pimecrolimus (**2a**) exhibits a high level of anti-inflammatory activity in animal models of skin inflammation after both topical and systemic applications (*36*). In the pig model of ACD, topical pimecrolimus is as effective as potent corticosteroids and tacrolimus ointment 0.1% (Protopic®). Unlike clobetasol, topical pimecrolimus does not cause skin atrophy nor affects blanching or skin texture in pigs. As shown in mice, topical pimecrolimus does not affect epidermal *Langerhans'* cells, antigen-presenting cells that play a critical role in the local immunosurveillance (*37, 38*). While the treatment with standard topical corticosteroids, including hydrocortisone, resulted in a reduction in MHC class II-positive *Langerhans'* cells by 96–100% in the treated skin, no effect on *Langerhans'* cells was noted. In contrast, corticosteroids greatly impair the integrity, function, and induce apoptosis of *Langerhans´*

cells (LC) in mice. A recent analysis of skin biopsies of atopic dermatitis patients has confirmed that treatment for 3 weeks with the corticosteroid ß-methasone 0.1%, but not Elidel® cream 1%, resulted in depletion of *Langerhans*' cells, while both drugs significantly reduced T-cells (*39*). These results indicate that topically applied pimecrolimus is unlikely to interfere with the function of *Langerhans*'/dendritic cells to differentiate naïve T-cells into effector T-cells, which is key for the developing immune system and maintenance of specific immunocompetence.

Pimecrolimus (**2a**) proved to be highly anti-inflammatory effective also after systemic administration to rodents. Oral and subcutaneous treatment of mice reveals pimecrolimus to be as potent as tacrolimus and more potent than cyclosporin A in inhibiting the elicitation phase, which is the clinically apparent inflammatory phase of ACD (*40*). In contrast to cyclosporin A and tacrolimus (**1**), oral treatment of mice with **2a** neither impairs the induction phase of ACD (sensitization) nor decreases weight and cellularity of draining lymph nodes, indicating that the primary immune response in ACD is not impaired by pimecrolimus (**2a**). In rat ACD, oral pimecrolimus is more potent than cyclosporin A by a factor of 4 and more potent than tacrolimus by a factor of 2 in inhibiting the elicitation phase of ACD. In contrast to tacrolimus, pimecrolimus has no effect on ongoing immune responses in the lymph nodes draining the application site of the hapten (*41*).

In comparison to cyclosporin A and tacrolimus, pimecrolimus (**2a**) has a lower potential to affect systemic immune responses. In rats, subcutaneous injections of cyclosporin A and tacrolimus suppress the localized graft-versus-host reaction 8-fold and 66-fold more potently than pimecrolimus. In the same species, the potency of tacrolimus to inhibit antibody formation against sheep red blood cells is 48-fold higher than that of pimecrolimus. Oral cyclosporin A and tacrolimus are immunosuppressive at lower doses than pimecrolimus in the rat kidney transplantation model by factors of 3 and 15, which correlates with exposure to lymph nodes (*42*).

Pimecrolimus (**2a**) may have therapeutic potential in inflammatory conditions beyond dermatological disorders as well. Results from ophthalmic studies in dogs with chronic keratokonjunctivitis sicca treated locally with experimental pimecrolimus eye drops indicate that pimecrolimus has therapeutic potential in inflammatory eye diseases in man (*43*). Studies in standard rat models of arthritis show that oral pimecrolimus (**2a**) exerts dose-dependent anti-inflammatory and disease-modifying efficacy indicating therapeutic potential for the treatment of human rheumatoid arthritis (*44*). Studies in a SCID model of inflammatory bowel disease indicate that oral pimecrolimus has therapeutic potential, superior to those of cyclosporin A and tacrolimus (*45*).

Taken together, the data suggest that pimecrolimus (**2a**) has favorable pharmacological profiles *in vitro* and *in vivo*:

- When applied topically, it has a high and selective anti-inflammatory activity in the skin, minimal percutaneous resorption, and a low potential to affect local and systemic immunosurveillance. It differs from corticosteroids by its selective action on T cells and mast cells, by a lack of effects on *Langerhans*' cells/dendritic cells, by the lack of induction of skin atrophy, and by much less permeation through the skin. It differs from tacrolimus by less permeation

through skin and by a lower potential to affect systemic immune responses, thus specifically targeting skin inflammation.
- When applied systemically, it exerts a high anti-inflammatory activity, but has a lower potential for immunosuppression and/or is better tolerated than tacrolimus or cyclosporin A (*28*).

1.2.1.2. Clinical Profile

Therapeutic efficacy and safety of topical pimecrolimus (**2a**) has been established in short-term and long-term management of atopic dermatitis in extensive double-blind, randomized, vehicle-controlled studies with patients including adults, children, and infants. In short-term studies with children, considerable efficacy was already evident at the first evaluation on day 8; significant relief from pruritus was observed also within the first week of treatment. In infants, results were similar to those obtained in the studies with children. Long-term studies were performed in children and infants (1-year treatment) and in adults (6 months). In summary, clinical trials have shown Elidel® to be highly effective in relieving the signs and symptoms of atopic dermatitis in adults, children, and infants. Clinically significant improvement was seen within 3 days of the first application. In long-term studies, Elidel® has demonstrated a unique ability to prevent disease progression if applied at the first signs or symptoms of disease.

In addition to its therapeutic efficacy, pimecrolimus (**2a**) has proven to be safe and well tolerated, as derived from animal and human studies. Topical application led to consistently low systemic exposure, irrespective of age, disease severity, or body surface treatment. Pimecrolimus cream 1% (Elidel®) was approved in the USA at the end of 2001 and in European and other countries in the autumn of 2002. Experience with more than 10 million patients treated so far in clinical practice has confirmed the high efficacy and safety elaborated in the controlled studies. Detailed reviews on clinical studies with pimecrolimus 1% cream (Elidel®) in patients with atopic dermatitis and other inflammatory skin diseases have been published (*46–51*).

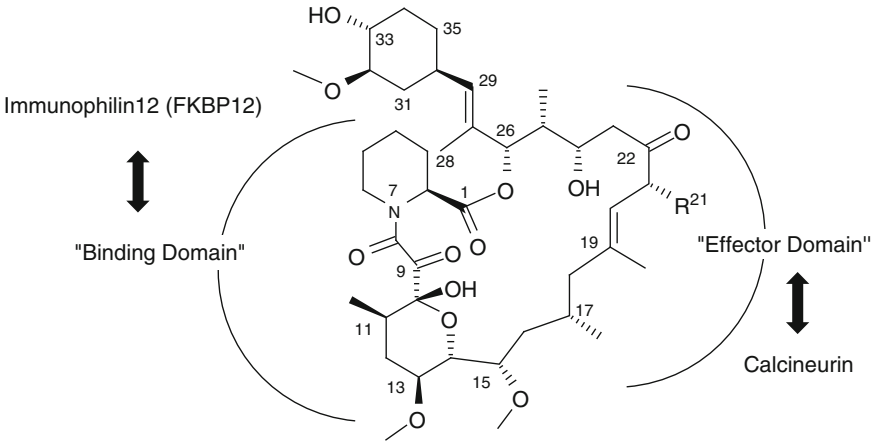
In addition to topical application, pimecrolimus (**2a**) was shown to be highly effective and safe after oral treatment. In psoriasis patients, pimecrolimus down-regulated the expression of genes associated with leukocyte activation/proliferation, lymphocyte chemotaxis, and trafficking as well as inflammation. No changes in gene expression were observed that might be linked with drug-related side effects (*52*). Multicenter studies with 3-month treatments of psoriasis and atopic dermatitis patients have proven the efficacy and safety of this compound, thus confirming preclinical studies.

1.3. Structural Features of Ascomycin

As confirmed by X-ray crystal structure analysis and NMR-studies the left hand parts of ascomycin (**2**) and tacrolimus (**1**) mediate binding to their common

immunophilin macrophilin (FK506-binding protein, FKBP-12) and have therefore been termed "binding domains" (Scheme 4). The right hand parts of the macrolactams, together with elements of the immunophilin, interact with the protein-phosphatase calcineurin, which plays a key role in the Ca^{2+} dependent activation of lymphocytes, and are called "effector domains" (*53*–*58*). The X-ray crystal structure of pimecrolimus (**2a**) is shown in Fig. 3. A model of the complex of pimecrolimus and macrophilin derived from the binding complex of L-685,818 and macrophlin is presented in Fig. 4 and also as cover picture of this volume.

Ascomycin (**2**), tacrolimus (**1**), and related analogues represent highly functionalized 23-membered macrocycles, containing a pipecolate residue in an amide (C-8) and an allylic ester linkage (C-26−C-29) with a polyketide backbone. Both macrocycles feature fourteen chiral centers, an endocyclic trisubstituted double bond (C-19−C-20) with (*E*)-geometry and located in an allylic position to a ketone functionality (C-22), three methoxy groups (at C-13, C-15, and C-32), and three secondary hydroxy groups at C-33, C-14, and C-24, with one of these part of a β-hydroxy ketone unit (C-22−C-24). Most notably, within the binding domain the macrolactams feature a unique pattern of three adjacent carbonyl groups (C-8−C-10, tricarbonyl portion, α,β-diketo-amide moiety), of which one carbonyl group (C-10) is masked as a hemiketal with the secondary C-14−OH, resulting in a tetrahydropyran unit (C-10−C-14). Biosynthetically, the C-9 carbonyl is introduced at a late stage *via* C-9−hydroxylation of the corresponding 9-deoxo precursor followed by its oxidation (*59*). In $CDCl_3$-solution, ascomycin (**2**) and tacrolimus (**1**) exist as mixtures of amide bond rotamers ((*E*):(*Z*)=approx. 2:1) (*60*). In the crystalline state, tacrolimus and **2** adopt an (*E*)-amide configuration, whereas the (*Z*)-diastereomer is observed in the tacrolimus/macrophilin complex (*54*, *61*). Interestingly, due to electronic repulsion, the planes of the conjugated carbonyls (C-8/C-9) are almost orthogonally oriented. The cyclohexyl side chain and the pyran unit adopt chair conformations, whereas all substituents except the C-10−OH are oriented equatorially.



Scheme 4. Dual domain model of tacrolimus (**1**) and ascomycin (**2**)

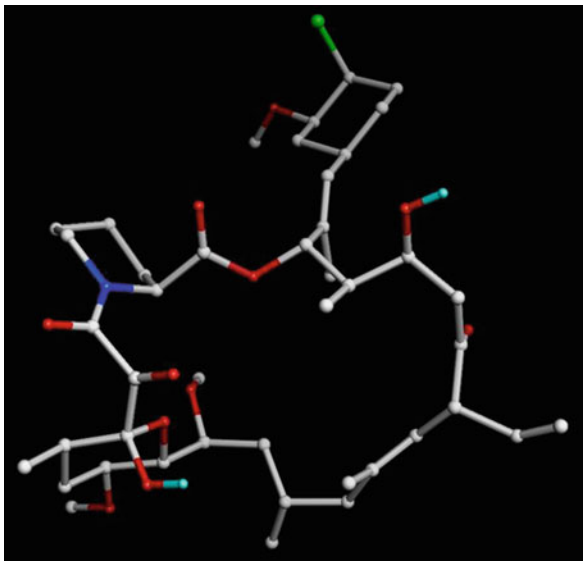


Fig. 3. View of the X-ray crystal structure of pimecrolimus (Weber HP, Sandoz, unpublished results): The ring of pimecrolimus adopts a *cis*-amide conformation and the structure is identical to the X-ray structure of ascomycin (*113*)

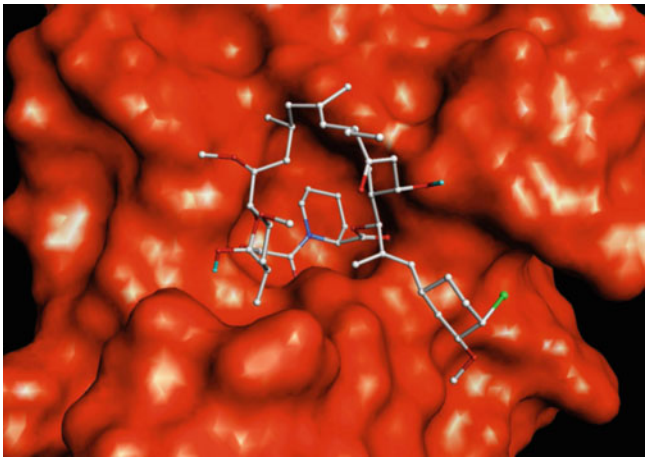
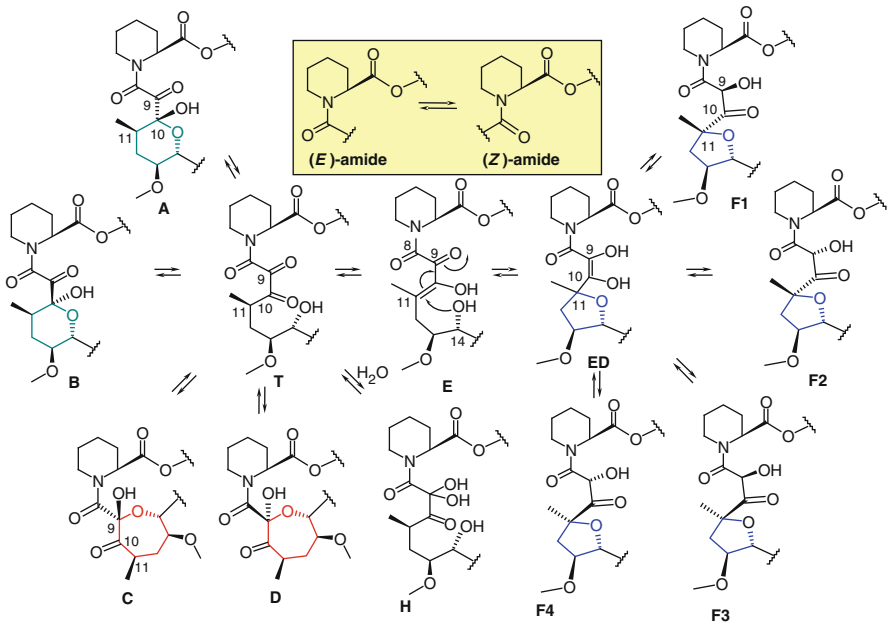


Fig. 4. Model of the complex of pimecrolimus (**2a**) and macrophilin – derived based on the X-ray structure of the native macrophilin (Burkhard P, Taylor P, Walkinshaw MD (2000) J Mol Biol **295**: 953; PDB: 1d60), and the conformation of L-685,818 (18-hydroxyascomycin) as observed in the binding complex with macrophilin (Becker JW, Rotonda J, McKeever BM, Chan HK, Marcy AI, Wiederrecht G, Hemes JD, Springer JP (1993) FK-506-binding protein: three-dimensional structure of the complex with the antagonist L-685,818 J Biol Chem **268**: 11335; PDB: 1fkd) in the trans-amide conformation. The surface of macrophilin is marked in red and pimecrolimus is represented by sticks. Contacts with macrophilin are formed by the pipecolinyl and pyranose rings and the dicarbonyl groups. The pipecolinyl ring is embedded in a deep cavity. The chlorine atom does not form strong contacts to the protein

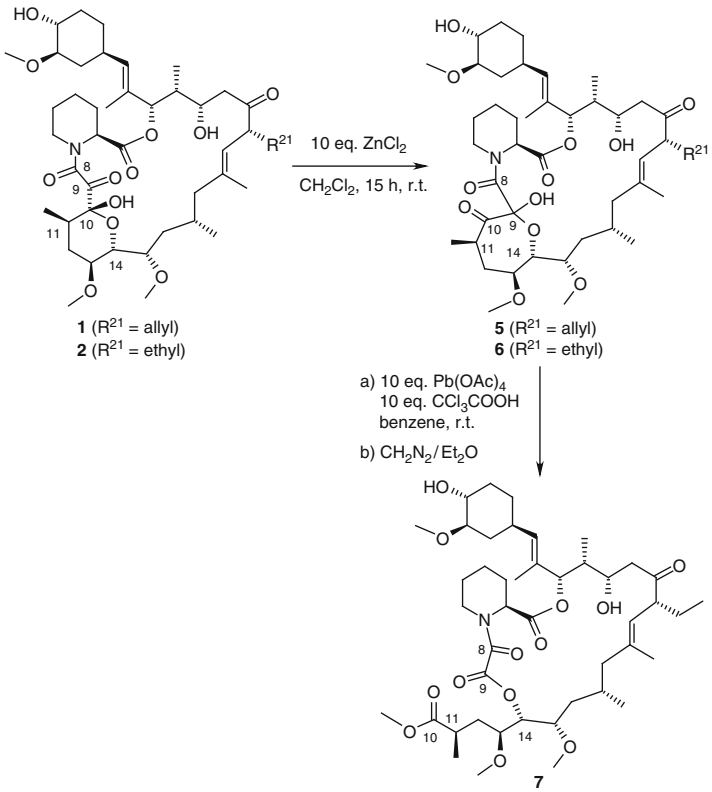
1.3.1. Structural Flexibility of Ascomycin

Although the structure of ascomycin (**2**) shown in Scheme 4 is the main isomeric form adopted in organic solution, the close proximity of the tricarbonyl unit to C-14−OH potentially allows the formation of numerous alternative isomers (Scheme 5). Thus, liberation and enolization of the tricarbonyl portion followed by re-hemiketalizations could give rise to the four six- or seven-membered hemiketal forms **A**-**D** and their C-11−isomers 11-*epi*-**A**-**D** (not shown in the Scheme). Furthermore, anticipating a 1,4-addition of C-14−OH to the enolized tricarbonyl form **E** allows the generation of a set of isomeric "furano-ascomycins", **F1**-**F4**. In addition, in an aqueous environment, the formation of a hydrate form, **H**, could also be anticipated. Finally, each of the above potential equilibrium products could exist as a mixture of the amide bond rotamers.

Despite the numerous equilibrium products that could be formed, only the isomeric forms **B** and **C** (Scheme 5) of ascomycin (**2**) and tacrolimus (**1**) have been identified and characterized so far (*62–64*). The existence of the tricarbonyl form **T** and its hydrate form **H** has not yet been established, but is suggested by the following findings. Addition of minor amounts of water to a colorless solution of **2** in acetonitrile causes a yellow coloration indicating the formation of the free tricarbonyl form. Further addition of water to the yellow solution leads back to a colorless solution, indicating the conversion of **T** into its hydrate form **H**. An equilibrium among the tricarbonyl form **T**, the hemiketal form **A,** and an alternative hemiketal form (most probably **C**), has also been suggested by the results from reversed-phase LC/MS experiments. The major equilibrium product of ascomycin



Scheme 5. (Hypothetical) structural flexibility of ascomycin (**2**) and tacrolimus (**1**)



Scheme 6. Conversion of tacrolimus (**1**) and ascomycin (**2**) to their hemiketal forms **5** and **6**, and the chemical degradation of **6** to **7**

(**C**) has been isolated *via* selective crystallization and reversed-phase chromatography (*63*). Its structure has been confirmed by NMR spectroscopy and its synthesis followed by oxidative degradation (Scheme 6) (*64*). Thus, the action of *Lewis* acids (*i.e.* zinc halides) in non-protic organic solvents converts ascomycin (**2**) and tacrolimus (**1**) into the corresponding seven-membered C-9−hemiketal forms, **6** and **5**, almost quantitatively. Lead tetraacetate-mediated chemoselective α-ketol cleavage of **6**, followed by esterification of the crude product and chromatography provided the ester **7** in a 80% yield, thus confirming the C-14−O−C-9 linkage.

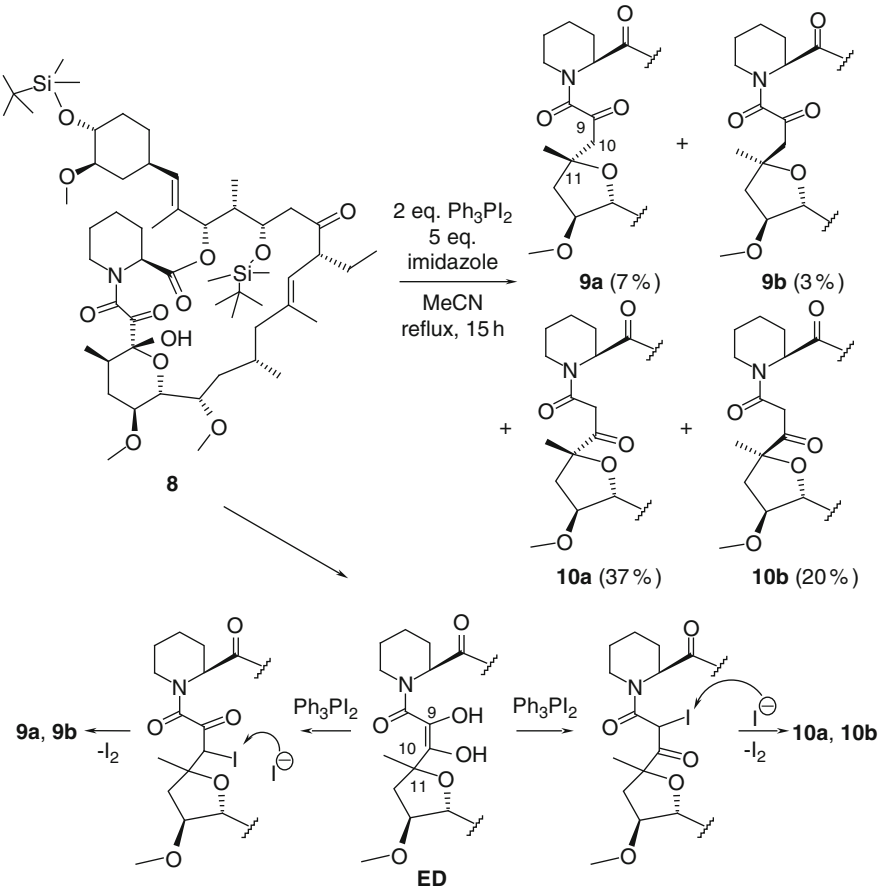
2. Synthesis Aspects

2.1. Synthesis of the Four Diastereomeric "Furano-Ascomycins"

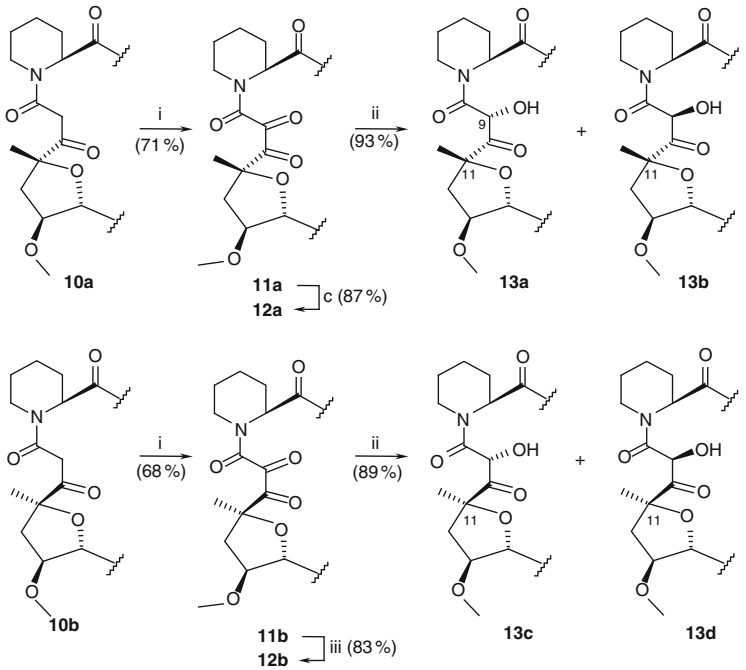
An unexpected and interesting reaction occurring in the binding domain of ascomycin (**2**) could be used to synthesize the "furano-ascomycins" **F1**-**F4** (Scheme 5) from **2** (*65*). Thus, bis-silylation of **2** gave **8**, which upon action of

diiodo-triphenyl-phosphorane in the presence of imidazole in refluxing acetonitrile, gave a mixture of the silyl protected 9- and 10-deoxo-furano-ascomycins, **9a**, **9b**, **10a**, and **10b** (Scheme 7). The reaction probably proceeds through equilibration to the ene-diol form **ED**, replacement of either of the OH groups by iodide, followed by iodide ion-mediated deiodination. Starting from **10a** and **10b**, simple functional group manipulations afforded the (11*S*)-furano-ascomycins **13a** and **13b** and their (11*R*)-isomers **13c** and **13d** (Scheme 8). Thus, oxidation of the activated methylene groups in **10a** and **10b** with *Dess-Martin* periodinane in the presence of pyridine yielded the yellow tricarbonyl derivatives **11a** and **11b**, which, after desilylation, provided the derivatives **12a** and **12b**, in high yields. Chemoselective reduction of the highly activated C-9−carbonyl group of **12a** and **12b** with zinc/glacial acetic acid, followed by chromatography afforded the individual isomers **13a**-**13d** in high yields.

The furano-ascomycins **13a** and **13b** differ only at the configuration of C-9, as could be shown by equilibration under basic conditions. Analogous results



Scheme 7. Transformation of **8** to the 9-, and 10-deoxo-furano-ascomycins **9a**, **9b**, **10a**, and **10b** and a possible mechanism



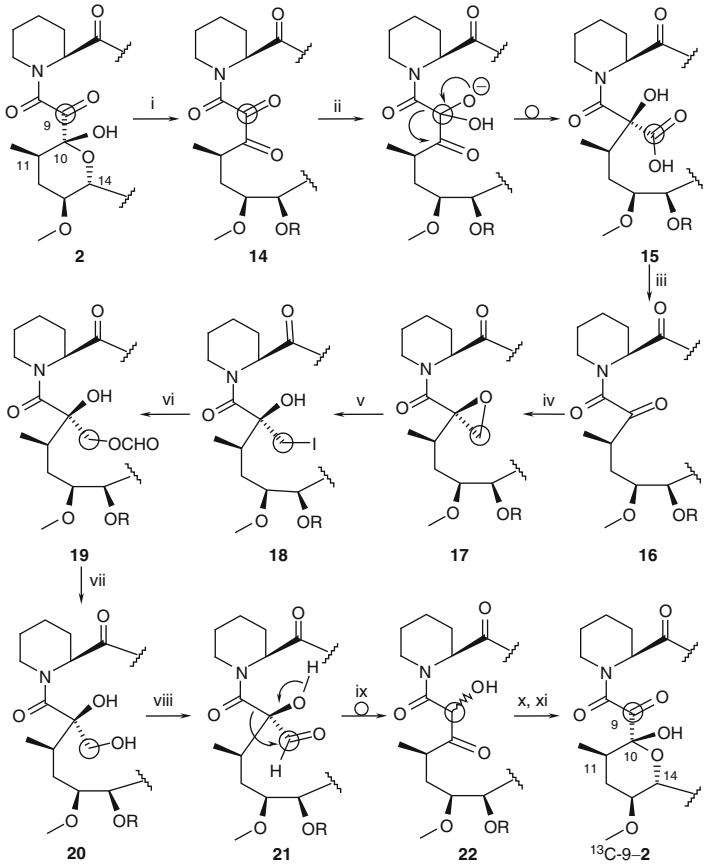
Scheme 8. Transformation of the deoxo-furano-ascomycins **10a** and **10b** to the furano-ascomycins **13a**, **13b**, **13c**, and **13d**

were obtained starting from the 11-*epi*-isomers **13c** and **13d**. The isolated pure furano-ascomycins are remarkably stable at room temperature for several months. Furthermore, no reconversion into ascomycin (**2**) in protic or aprotic solutions under neutral, basic, or acidic conditions could be seen. Also, starting from ascomycin (**2**) no formation of **13a-13d** could be demonstrated in solution. Thus, there is no evidence for equilibrium between the furano-ascomycins and the parent compound ascomycin. No biological activities of these compounds have been reported.

2.2. Synthesis of ^{13}C Labeled Ascomycin

It is essential to have appropriate tools to establish the purity of a drug substance (DS) unambiguously. In the case of an ascomycin-derived DS it is important to be able to distinguish between the "real by-products" and DS-related inherent equilibrium compounds. Researchers at Novartis succeeded in labeling **2** at the diagnostically most relevant C-9 or C-10−carbons in the binding domain (*66*). ^{13}C-Labeled ascomycin (^{13}C-n−**2**), or drug substances derived thereof, serve as versatile tools for studying equilibrium phenomena in more complex mixtures, such

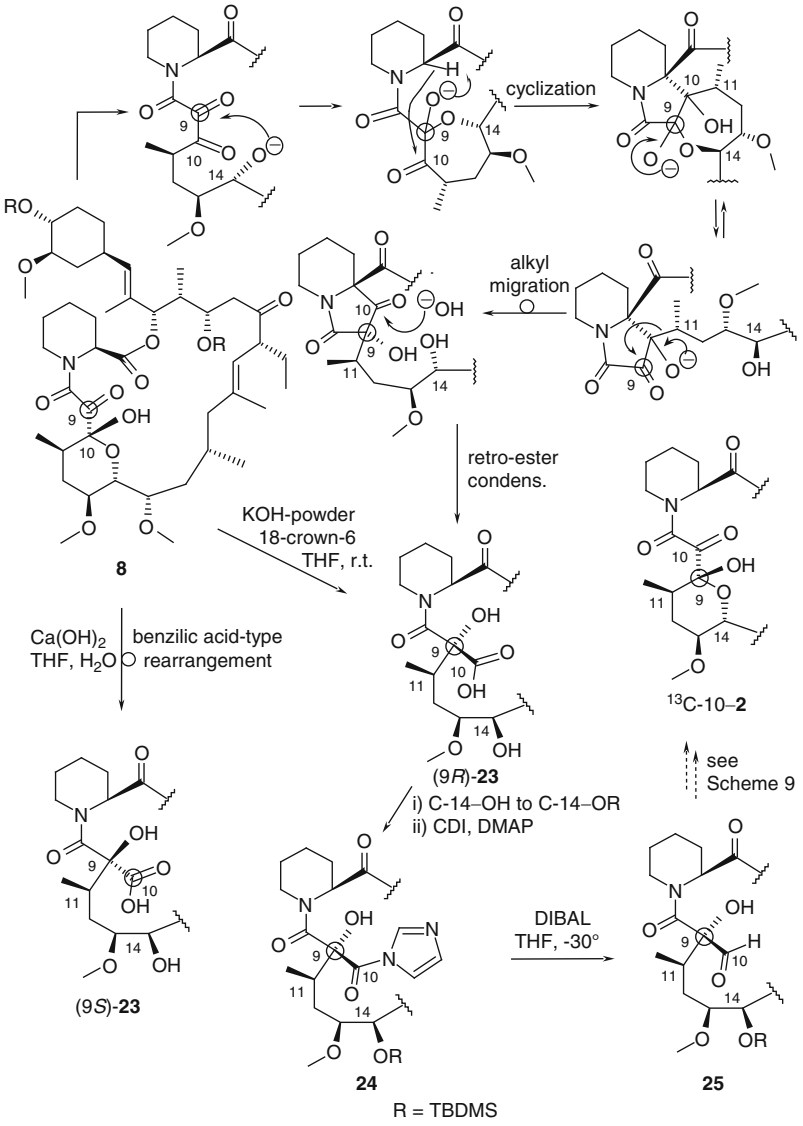
as galenical formulations. For the synthesis of ^{13}C-9–**2**, a ring contraction/ring expansion strategy has been applied (Scheme 9). Thus, ascomycin (**2**) was silylated to furnish the yellow 14,24,33-tris-*O*-TBDMS- derivative **14**, bearing the unmasked tricarbonyl unit. Treatment of **14** with excess calcium hydroxide in THF-water afforded, *via* an irreversible benzilic acid-type rearrangement reaction, the ring-contracted α-hydroxy acid **15** as a >95:5 mixture of diastereoisomers in favor of the (10*S*)-enantiomer. Oxidative decarboxylation of the latter with lead tetraacetate furnished the ring contracted ketoamide **16** quantitatively, setting the stage for ring expansion. The ^{13}C-label was introduced through reaction of **16** with ^{13}C-methylene iodide and butyllithium. Further functional group manipulations



i) TBDMSOTf, 2, 6-lutidine, CH_2Cl_2, r.t.; ii) $Ca(OH)_2$, THF, water, r.t.;
iii) $Pb(OAc)_4$, C_6H_6, r.t.; iv) (^{13}C)-CH_2I_2, BuLi, THF, –78°; v) MgI_2, CH_2Cl_2, r.t.;
vi) $AgBF_4$, DMF, r.t.; vii) $ZnCl_2$, MeOH, r.t.; viii) Oxalyl chloride, DMSO, Et_3N;
ix) $ZnCl_2$,CH_2Cl_2 , r.t., or Florisil, THF, reflux; x) Oxalyl chloride, DMSO, Et_3N;
xi) aq. HF, MeCN, r.t.

Scheme 9. Synthesis of ^{13}C-labeled ascomycin (^{13}C-9–**2**)

provided the α-hydroxyaldehyde **21** as the key intermediate for the subsequent ring expansion protocol. Thus, exposure of **21** to zinc chloride in methanol at room temperature, or to magnesium silicate on silica gel (Forisil®) in refluxing THF solution, provided the ^{13}C-9−labelled 9(*R*/*S*)-dihydroascomycin **22** regioselectively. Oxidation of the secondary hydroxy group in **22**, followed by deprotection, completed the synthesis of ^{13}C-9−**2**.



Scheme 10. Synthesis of ^{13}C-9–**25**

Potentially, ^{13}C-9−ascomycin (^{13}C-9−**2**) could be converted into ^{13}C-10−ascomycin (^{13}C-10−**2**) applying a cascade of diastereoselective rearrangement reactions (Scheme 10). As described above, treatment of 24,33-bis-*O*-TBDMS-ascomycin **8** with calcium hydroxide in THF-water solution results in a facile benzilic acid-type rearrangement to give diastereoselectively the (9*S*)-α-hydroxy acid, (9*S*)-**23**, in a quantitative yield. Remarkably, changing to anhydrous reaction conditions (powdered KOH, 18-crown-6, THF, room temperature) afforded almost exclusively (>97:3) the (9*R*)-isomer, (9*R*)-**23**, in a high yield. Quenching and trapping experiments revealed that its formation proceeds not *via* a benzilic acid-type rearrangement, but *via* a cyclization followed by an α-ketol-rearrangement involving the migration of C-11, which is then followed by a diastereoselective (retention at C-2) *retro*-ester condensation. Thus, the newly created quaternary carbon atom of the α-hydroxy-acid (9*R*)-**23** is the former C-9 carbon of ascomycin (**2**). Silylation of the C-14−OH, followed by reduction of the acid *via* its imidazolide **24** provided the (9*R*)-α-hydroxy-aldehyde **25**, which potentially could be transformed to **2** as described in Scheme 9 for the (9*S*)-isomer. Thus, provided that C-8 migrates in the ring enlargement reaction (as observed for the (9*S*)-isomer), this sequence would allow the conversion of ^{13}C-9−ascomycin (^{13}C-9−**2**) into ^{13}C-10−ascomycin (^{13}C-10−**2**).

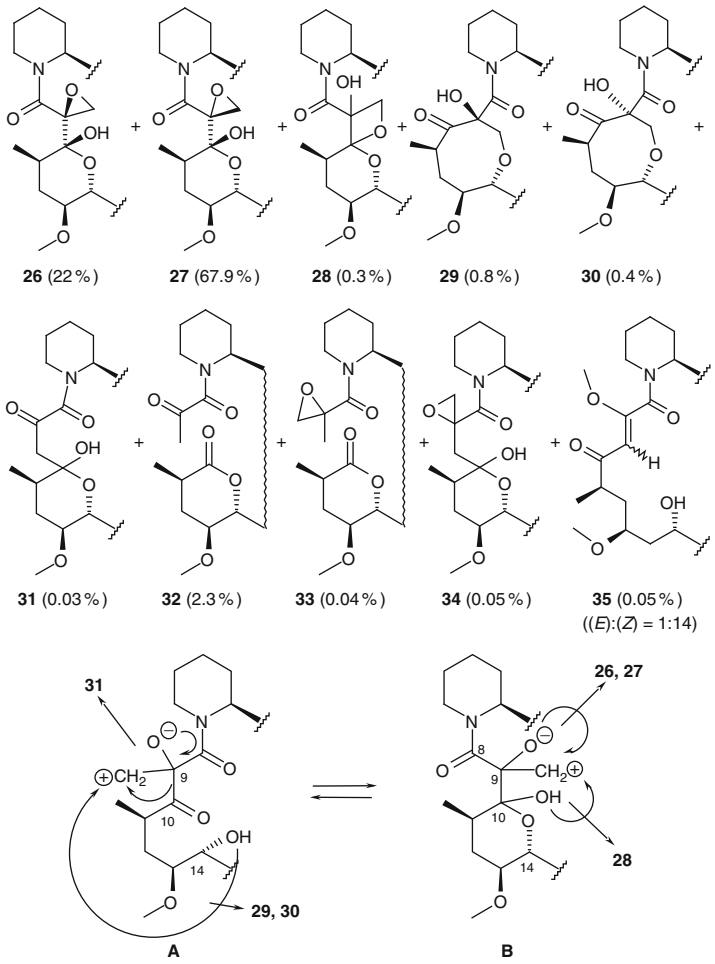
Tritium labeled tacrolimus was prepared by a metal-catalyzed hydrogen isotope exchange procedure (*67*). The radiochemical purity was 98% and ^{3}H-NMR spectroscopy was used to identify the tritium incorporation in about 10–15 different positions. When administered to rats, HTO-formation turned out to be less than 6% of the dose, thus indicating a reasonable biological stability.

2.3. Reactivity of the Binding Domain

The structural flexibility of ascomycin (**2**) and related macrolactams in the binding domain imparts a high reactivity to this region towards a broad variety of reaction conditions. Thus, selective transformations on other parts of the molecule are often difficult to achieve without provoking concomitant changes in this unit.

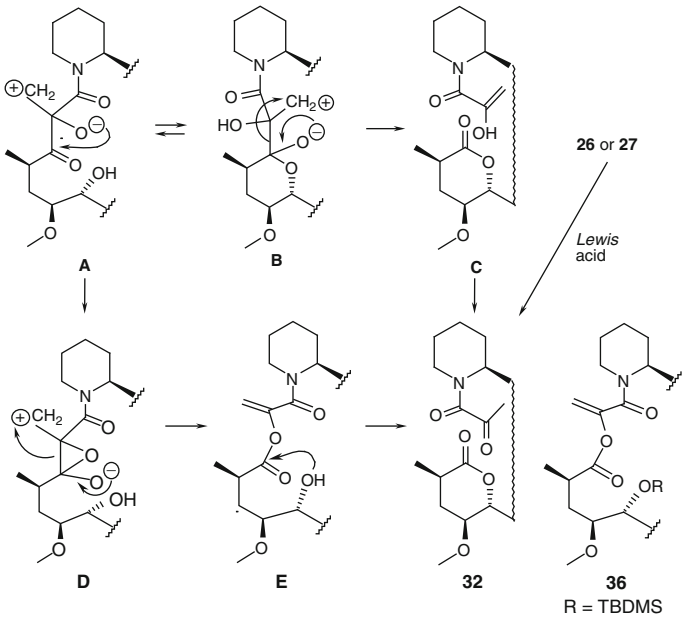
2.3.1. Reactivity of Ascomycin and Tacrolimus Towards Diazomethane

Reaction of ascomycin (**2**) with excess ethereal diazomethane led to the diastereoisomeric 9-epoxides **26** and **27**, as expected, together with eight minor by-products **28**–**35** (Scheme 11; for clarity only the relevant portions of the macrocycle are drawn) (*68*–*70*). Compounds **26**–**32** result through insertion of a methylene unit, while compounds **33**–**35** arise from **31** and **32** through a second methylene insertion reaction. The formation of **26**–**31** could be rationalized through the intermediacy of the betaines **A** (keto-form) and **B** (hemiketal form) (Scheme 11). Thus, ring closure with C-9−OH leads to the formation of the oxiranes **26** and **27**. Alternatively, ring

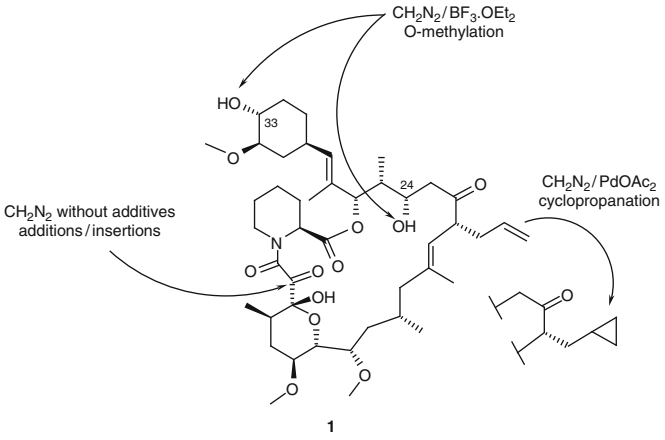


Scheme 11. Products from the reaction of ascomycin (**2**) with diazomethane and a possible mechanistic picture for the formation of the products **26**–**31**. The *long wavy lines* indicate partial structures

closure with C-10−OH or C-14−OH, leads, respectively, to the unusual *spiro*-oxetane **28**, or the oxocanone isomers **29** and **30**. The ring-enlarged derivative **31** could be formed through migration of C-10 to the positively charged carbon of the betaines **A** or **B**. Two distinct pathways could be proposed for the formation of the unusual *seco*-compound **32** (Scheme 12). Thus, starting from the betaine **B**, a *Grob*-type fragmentation would provide the enol form **C** of **32**. Alternatively, starting from the betaine **A**, ring closure to the hydroxy-epoxide **D** followed by a similar fragmentation could lead to the enol ester **E**, which after an intramolecular transesterification, leads to the *seco*-compound **32**. In fact, the enol ester **36** was isolated as a by-product from the reaction of 14,24,33-tris-*O*-TBDMS-ascomycin with diazomethane. This supports the intermediacy of compounds of type **E** in these



Scheme 12. Possible pathways for the formation of the product **32** in the reaction of ascomycin (**2**) with diazomethane, and the structure of the isolated intermediate **36**. The *long wavy lines* indicate partial structures



Scheme 13. Selective reactions of diazomethane with tacrolimus (**1**)

reactions. Interestingly, treatment of the oxiranes **26** and **27** with *Lewis* acids leads to the *seco*- compound **32** in an almost quantitative yield (*69*, *71*).

Although the tricarbonyl portions of ascomycin (**2**) and tacrolimus (**1**) are highly sensitive towards diazomethane, selective O-methylations on C-24−OH and C-33−OH could be achieved in the presence of catalytic amounts of $BF_3.OEt_2$ (Scheme 13). On the other hand, reaction with excess of diazomethane in the

presence of $Pd(OAc)_2$ led selectively to cyclopropanation of the double bond on the allylic side chain of tacrolimus (**1**), leading to 37,38-cyclopropano-FK506, which showed excellent macrophilin binding but somewhat weaker T-cell inhibitory activities (*26, 72–74*).

2.3.2. Rearrangement Reactions in the Binding Domain

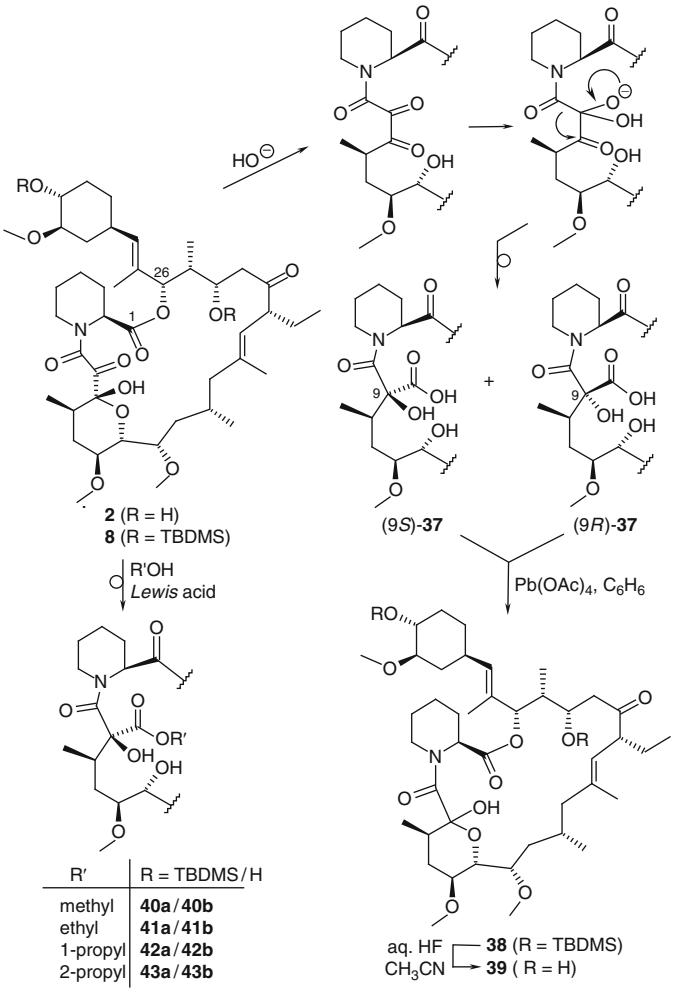
2.3.2.1. Benzilic Acid-Type Rearrangement Reactions

Early attempts to cleave the endocyclic pipecolic ester bond by a base-catalyzed saponification procedure did not lead to the expected 1,26-*seco*- derivative, but instead to a 22-membered lactone with a rearranged C-9−C-10 region (Scheme 14). Based on labeling studies, a benzilic acid-type rearrangement occurring on the free tricarbonyl form was proposed as a mechanism for this ring-contraction reaction. More detailed studies performed at Novartis showed that the reaction conditions originally applied (*i.e.* 1.1 eq. LiOH, THF-H_2O) (*73, 75, 76*) provided not only the hydroxy acid (9*R*)-**37** but also its 9-*epi*-derivative (9*S*)-**37** (*77, 78*). Oxidative degradation of both isomers led to **38,** which after deprotection, led to the common ring-contracted *nor*-C-9 ascomycin derivative **39**, thus showing that all other chiral positions remained unchanged. Further studies showed that the derivative (9*S*)-**37** was formed *via* a distinct reaction pathway and could be prepared selectively by applying anhydrous reaction conditions. An almost exclusive formation of the 24,33-bis-*O*-TBDMS protected (9*S*)-hydroxy esters **40a** (R′=methyl) and **41a**, **42a** and **43a** (R′=ethyl, propyl, *iso*-propyl), or their unprotected congeners **40b**, **41b**, **42b** and **43b,** could be accomplished by reacting 24,33-bis-*O*-TBDMS-ascomycin (**8**) or unprotected ascomycin (**2**) in the appropriate alcohol with *Lewis* acids (*i.e.* ZnX_2, MgX_2, X=Cl, Br, I; $Ti(O\text{-}^iPr)_4$) (*71*).

2.3.2.2. Rearrangement Reactions in Aprotic Media

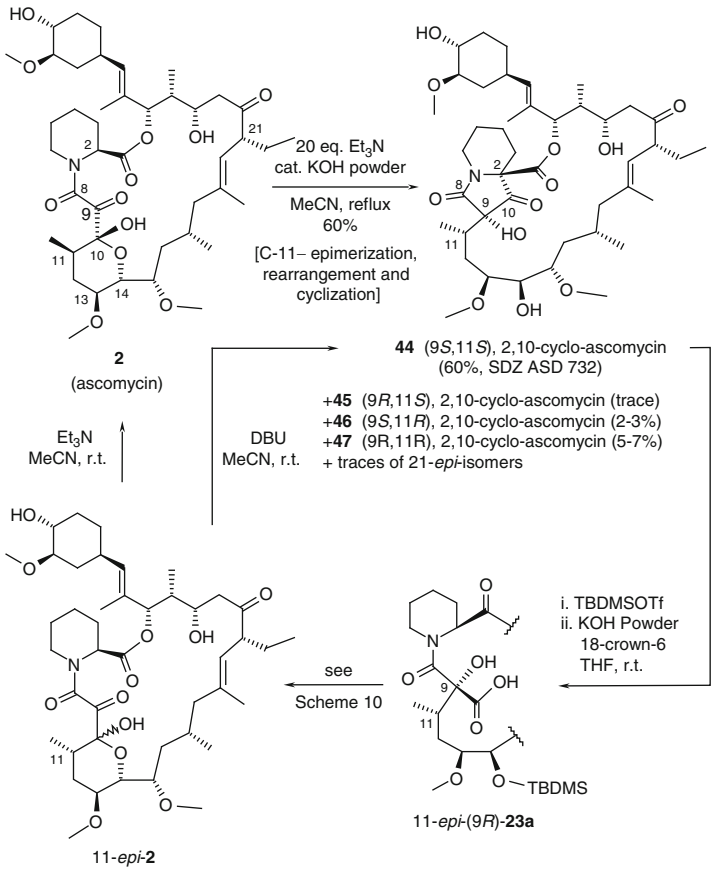
Treatment of unprotected **2** or 24,33-bis-*O*-TBDMS protected ascomycin (**8**) with various non-nucleophilic bases in aprotic solvents led to a set of unusual rearranged derivatives, which have been termed "cyclo-ascomycins" (Scheme 15). Thus, refluxing ascomycin with excess triethylamine and catalytic amounts of powdered KOH in acetonitrile yielded (9*S*,11*S*),2,10-cyclo-ascomycin **44** (SDZ ASD 732), together with traces of the stereoisomers **45**–**47** (*79, 80*). Separation of the diastereomers **44**–**47** was accomplished using stimulated moving bed technology or centrifugal counter-current chromatography (*81, 82*).

The isomer **44** could be transformed to 11-*epi*-ascomycin in a series of steps (Scheme 15) (*83*). Thus, silylation of **44** afforded 14,24,33-tris-*O*-TBDMS-protected **44** that on treatment with powdered KOH and 18-crown-6 under aprotic



Scheme 14. Benzilic acid-type rearrangement reactions of ascomycin (**2**)

conditions led to 11-*epi*-(9*R*)-**23a**. This compound, upon further transformations analogous to those described in Scheme 10, led to 11-*epi*-ascomycin (11-*epi*-**2**). In contrast to ascomycin (**2**), which exists in $CDCl_3$ solution as a mixture of six- and seven-membered hemiketal forms (ratio 15:1), 11-*epi*-**2** adopts exclusively two diastereomeric (10*S*)- and (10*R*)-six-membered hemiketal forms in the ratio 4:1. Notably, 11-*epi*-**2** is stable to acidic conditions, but is converted completely to **2** under weakly basic conditions, whereas no conversion of **2** to 11-*epi*-**2** was observed under the same conditions or on storage under protic, aprotic, organic, or aqueous solutions. Interestingly, treatment with DBU converts 11-*epi*-**2** to **44** as the sole product. Therefore, it is likely that the formation of **44** from **2** involves

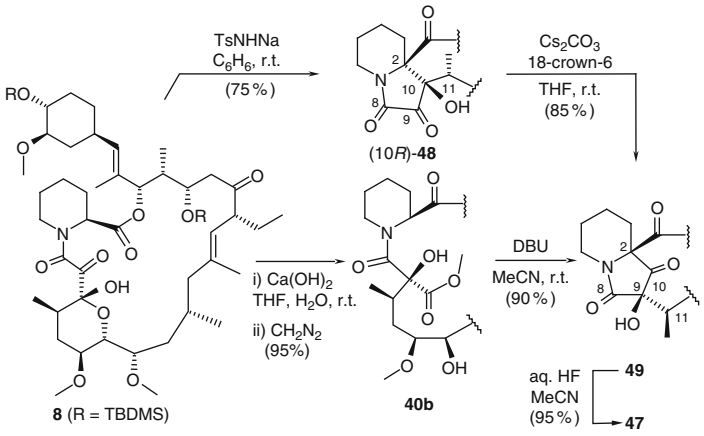


Scheme 15. Conversion of ascomycin (**2**) to the cyclo-ascomycin **44** (SDZ ASD732) and other isomers, and, transformation of **44** to 11-*epi*-ascomycin (11-*epi*-**2**) and *vice versa*

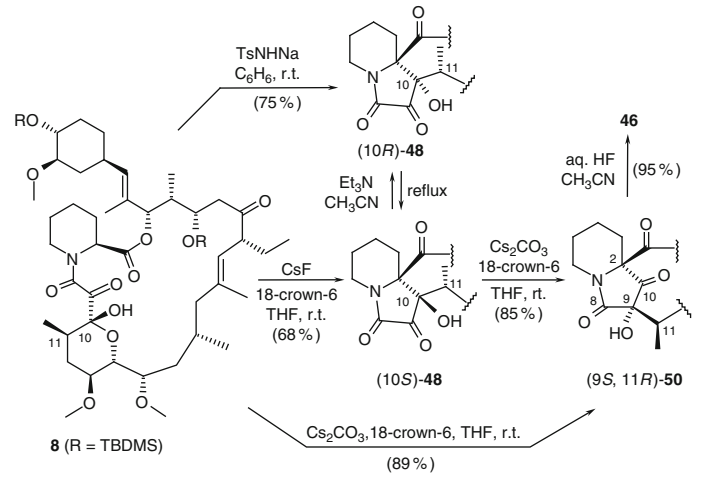
epimerization at C-11 as the first step, which is then followed by rearrangement and ring closure.

Starting from 24,33-bis-*O*-TBDMS-protected ascomycin (**8**), the isomeric cyclo-ascomycins **45**–**47** could be prepared as the sole products (*79*, *84*, *85*) (Scheme 16). Thus, treatment of **8** with the sodium salt of tosylamide provided with high stereoselectivity the cyclized compound **48** ((10*R*)-configuration), which, upon treatment with cesium carbonate, rearranged to **49**, and, after desilylation, afforded the (9*R*,11*R*),2,10-cyclo-ascomycin **47**. Alternatively, one could first carry out the rearrangement and then esterify to give **40b**, and then cyclize it to **49** (compare Scheme 14).

The isomer **46** can be prepared with a high stereoselectivity (Scheme 17). Thus, 24,33-bis-*O*-TBDMS-ascomycin (**8**) is transformed to (10*S*)-**48** with an (*S*)-configuration at C-10 using CsF in a 68% yield. Alternatively, **8** is converted



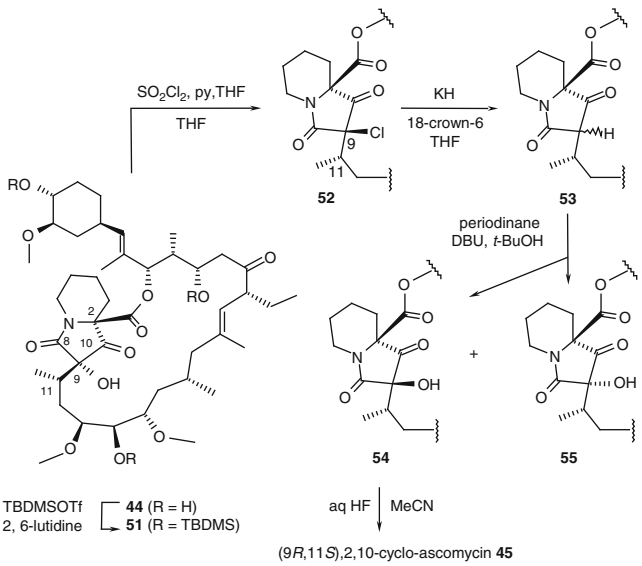
Scheme 16. Transformation of **8** to the cyclo-ascomycin **47**



Scheme 17. Transformation of **8** to the cyclo-ascomycin **46**

first to (10*R*)-**48** and then epimerized to (10*S*)-**48**. Treatment of isolated (10*S*)-**48** with Cs_2CO_3 in the presence of 18-crown-6 in tetrahydrofuran initiates a diastereoselective alkyl migration to give the 24,33-bis-*O*-TBDMS-derivative **50**, which after desilylation affords the (9*S*,11*R*),2,10-cyclo-ascomycin **46**. More conveniently, compound **50** is also obtained in a one-pot reaction starting from **8**.

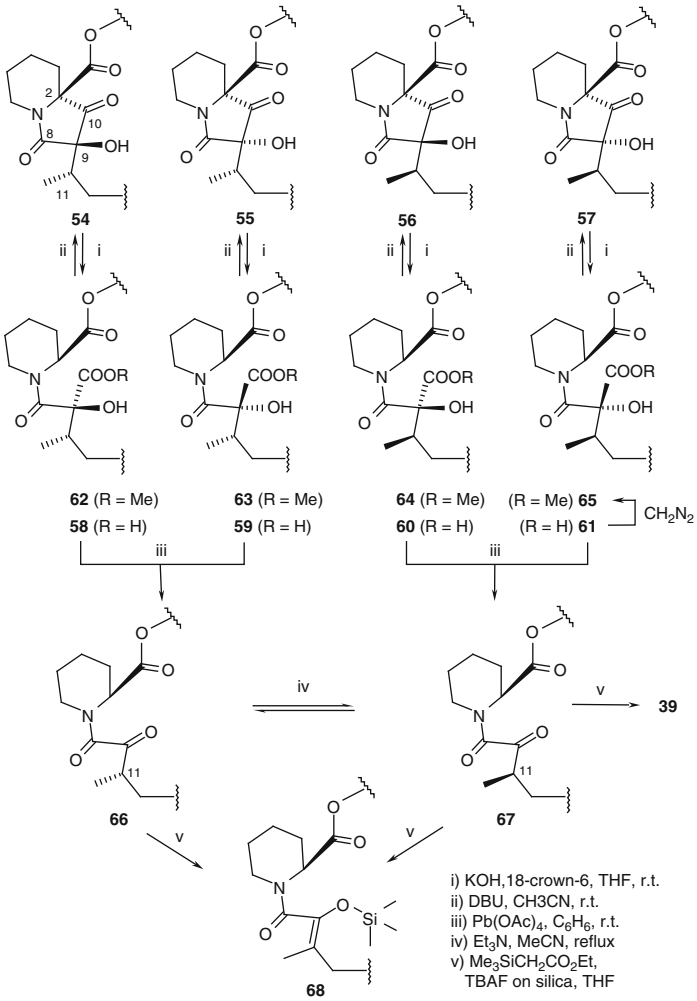
No methods are available so far for the preparation of the fourth stereoisomer (9*R*,11*S*),2,10-cyclo-ascomycin (**45**), in a good yield. The only available method involves silylation of **44** to give **51**, which is then transformed to its 9-chloro- derivative **52** by routine methods (Scheme 18). An unusual halogen/hydride exchange furnished the deoxo- derivative **53** in a low yield. Due to



Scheme 18. Transformation of the cyclo-ascomycin **44** to the isomer **45**

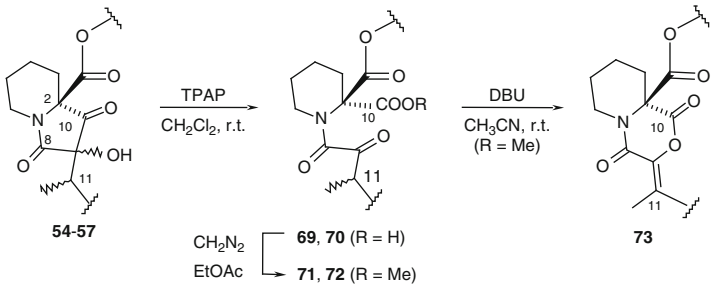
keto/enol tautomerism, **53** exists in $CDCl_3$-solution as a mixture of diastereomers. Oxidation of **53** resulted in the reintroduction of the hydroxy group to give the tris-*O*-TBDMS-protected cyclo-ascomycin-derivatives **54** and **55**. Finally, desilylation of **54** afforded the desired (9*R*,11*S*),2,10-cyclo-ascomycin **45**.

In summary, four diastereoisomeric cyclo-ascomycins (**44**–**47**) can be prepared stereoselectively by simple treatment of protected or unprotected ascomycin (**2**) in aprotic media with an appropriate base. Although C-2 is clearly involved during their formation, no C-2−epimeric cyclo-ascomycins have been found so far under any of the reaction conditions, thus indicating a powerful remote stereocontrol. Interestingly, it could be shown that upon treatment with powdered KOH in the presence of 18-crown-6, the 14,24,33-tris-*O*-TBDMS-cyclo-ascomycins **54**–**57** undergo ring cleavage to the corresponding α-hydroxy acids **58**–**61**, thereby leading to, in all the four isomers, C-2 in its natural (*S*)-configuration (Scheme 19). Furthermore, **58**–**61** can be transformed to esters **62**–**65** that may then be cyclized back diastereoselectively to give **54**–**57**. The α-hydroxy acids **58**–**61** have served as versatile tools for the determination of relative configurations. Thus, oxidative decarboxylation resulted in the formation of the epimeric 14,24,33-tris-*O*-TBDMS-α-ketoamides **66** and **67** differing in their configuration at C-11, as could be shown by equilibration experiments and by their conversion to the silyl enol ether **68**. Desilylation of **67** led to the already known derivative **39**, thus giving evidence for the outlined C-11−configuration and the confirmed (*S*)-configuration at C-2 for all the α-hydroxy acids. The stereochemistry at C-2 of the cyclo-ascomycins **54**–**57** (and thus also of their deprotected congeners **44**–**47**) has been established by another chemoselective oxidative degradation protocol (Scheme 20). Thus, reaction



Scheme 19. Ring-cleavage reactions of the 24,33-bis-TBDMS-cyclo-ascomycins **54**–**57** and their further transformations for establishing the stereochemistry

of **54**–**57** with excess tetrapropylammonium perruthenate (TPAP) provided the two C-11−epimeric α-ketoamides **69** and **70**, bearing a carboxylic acid functionality at C-2. After treatment with diazomethane they provided the corresponding esters **71** and **72**, which in an intramolecular base-mediated transesterification event provided the enol ester derivative **73**. The latter can only exhibit the intact original C-2−configuration of the starting cyclo-ascomycins **54**–**57**. Having ascertained the relative stereochemistry at C-2, C-9, and C-11 of all cyclo-ascomycins, the absolute configurations were established by X-ray crystal structure analysis of the



Scheme 20. Degradation reactions of the 24,33-bis-TBDMS-cyclo-ascomycins **54–57** towards establishing the C-2–configuration

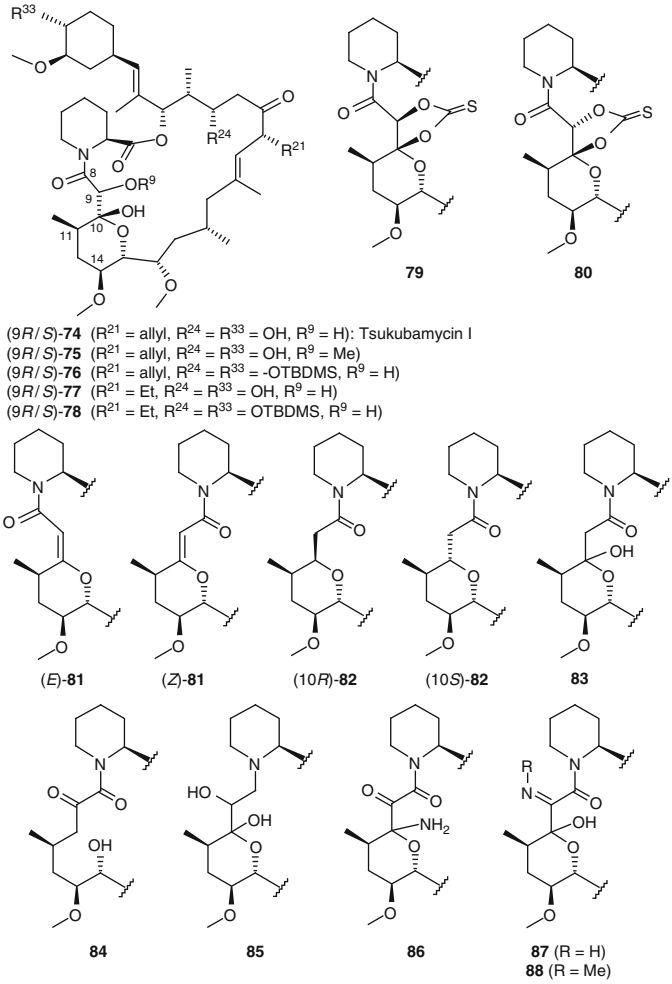
unprotected (9*S*,11*S*)-derivative **44** (*71*, *84*, *85*). The biological activities of these compounds have not been reported.

2.3.3. Reduction, Deoxygenation, Imine- and Aminal-Formation in the Binding Domain

9-Dihydro-FK506 (**74**, tsukubamycin I), together with its 9-dihydro-9-*O*-methyl-congener **75** (tsukubamycin A), was first isolated as a by-product from the fermentation broth of a tacrolimus producer strain (*8*) (Scheme 21). Synthetically, chemo- and stereoselective reductions of the 9-carbonyl group of ascomycin (**2**), tacrolimus (**1**), and 24,33-bis-O-protected congeners thereof, have been accomplished using either DIBAH or *Evans*' reagent (tetramethylammonium-triacetoxyborohydride) (*74*, *86*). The absolute stereochemistry of the 9-dihydro- derivatives could be deduced by NMR spectroscopy from their corresponding rigid thiocarbonates **79** and **80**, which were prepared readily by the action of thiocarbonyl diimidazole. The latter served as versatile starting materials for further modifications. Thus, radical deoxygenation/elimination of the thiocarbonates **79** and **80** gave the (*E*)- and (*Z*)-9,10-unsaturated compounds (*E*)-**81** and (*Z*)-**81**. Different (*E*)/(*Z*) ratios were obtained when Bu_3SnH or $(Me_3Si)_3SiH$ in combination with AIBN or triethylborane were used, allowing the preparation of either isomer selectively. Hydration of the enolether **81** provided the 9-deoxo-derivative **83** as a single diastereomer, whereas hydrogenation led to the two diastereomers, (10*S*/*R*)-9-deoxo-10-deoxy-**82**. Interestingly, the unusual reduction- and deoxygenation-product **85** was obtained when ascomycin (**2**) was reacted with diphenylsilane in the presence of cesium fluoride.

Action of ammonia in methanol converts ascomycin (**2**) to a mixture of the C-10−aminal **86** and the C-9−imine **87**, whereas reaction with methylamine led to the C-9−imine **88** exclusively (Scheme 21) (*87*).

9-Deoxoascomycin (**83**), together with minor amounts of the 10-deoxo- derivative **84**, could be prepared from ascomycin (**2**) directly through reaction with hydrogen sulfide in the presence of pyridine (Scheme 22) (*88*). Notably, the

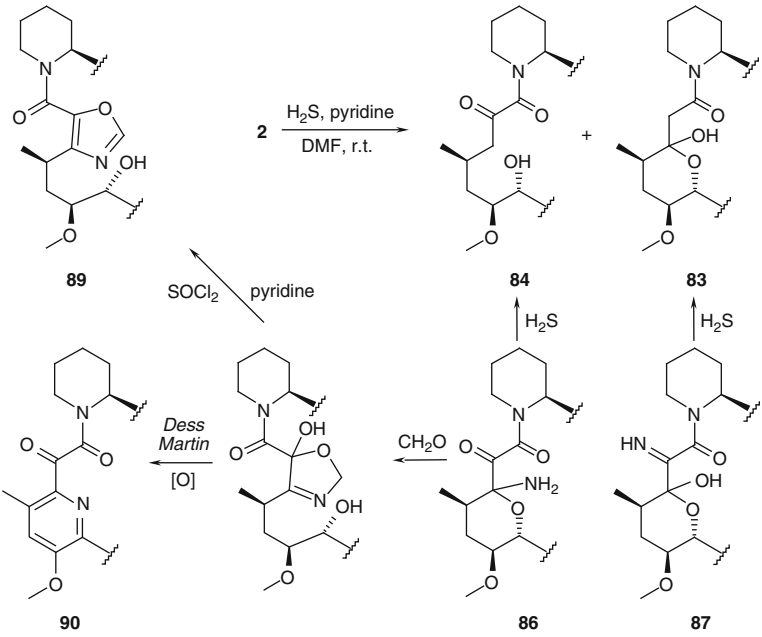


Scheme 21. Derivatives of ascomycin (**2**) and tacrolimus (**1**) with diverse modifications in the binding domain

exclusive formation of either the 9- or 10-deoxo- derivative was observed when the imine **87** or the aminal **86** was treated with hydrogen sulfide in the absence of base.

Semi-rigid derivatives of ascomycin (**2**), featuring heterocyclic structural elements in the binding region, were synthesized from the aminal **86** (*89*). The carbonyl C atoms, for example, were integrated into an oxazole ring giving **89**, and the tetrahydropyran ring of **86** was transformed into the pyridine unit in **90**.

Several of the analogues in this section have also been prepared with tacrolimus, but are omitted here for simplicity. Structure-activity relationships (SARs) have been reported (*74*) and are summarized as follows. The 9-dihydro- derivatives **74** and **77** showed good macrophilin binding but reduced inhibition of T-cell activation



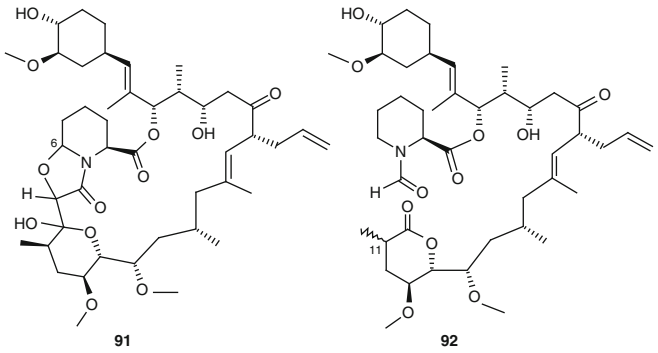
Scheme 22. Derivatives of ascomycin (**2**) featuring desoxygenations and heterocyclic rings in the binding domain

in cellular assays. The 9-desoxo derivative **83** showed lower binding affinity and reduced inhibition of T-cell activation. Interestingly, the 10-amino and 9-imino analogues **86** and **87** had higher macrophilin binding affinities than tacrolimus; the tacrolimus analogue of **86** was highly active in cellular assays. The pyridine analogue **90** was totally inactive on T-cells.

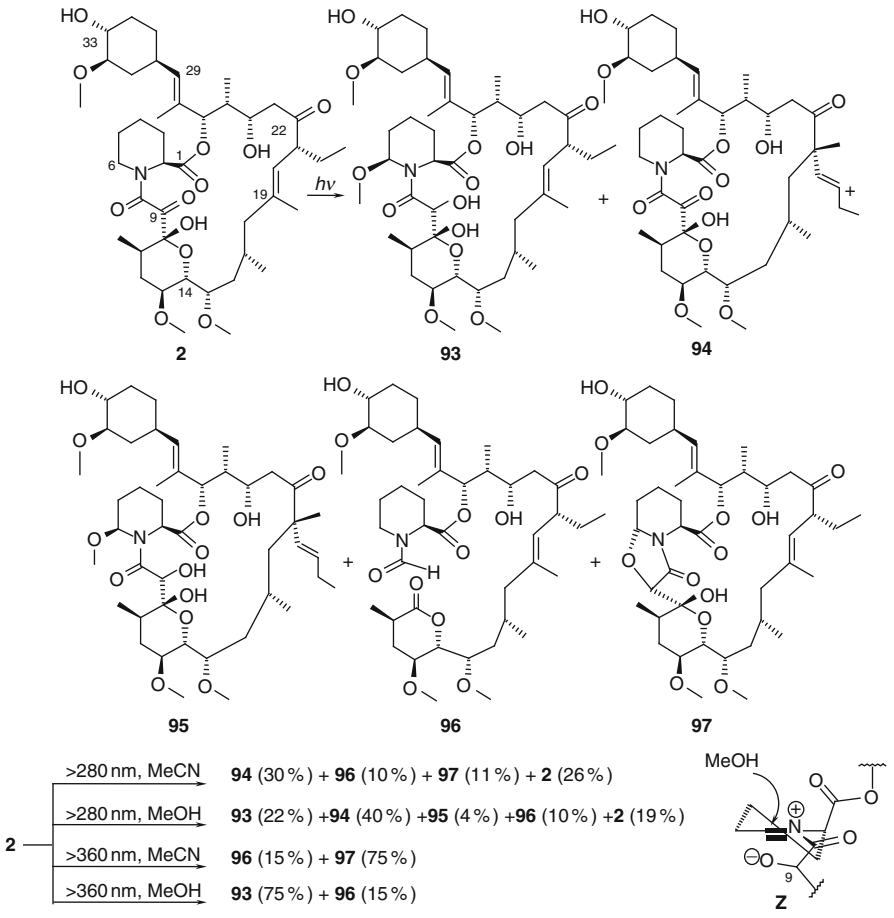
2.3.4. Modification in the Binding Domain Through Photochemistry

It has been reported that tacrolimus (**1**), on exposure to visible light (30,000 Lux) for 10 days, undergoes a rearrangement in the binding domain, leading to the oxazolidinone **91**, and degradation, affording the diastereomeric cleavage products (11*R*/*S*)-**92** (Scheme 23) (*90, 91*). The formation of **91** represents a modification on the amino acid unit, the type of which could not be achieved easily through routine chemical methods. This prompted Novartis researchers to investigate photochemical reactions in more detail.

Thus, irradiation of ascomycin (**2**) in acetonitrile using >280 nm light led to the [1,3]-sigmatropic shift product **94**, the cleavage product **96** and the oxazolidinone **97** (Scheme 24) (*92–94*). On the other hand, irradiation in methanol under the same conditions led to the methoxy derivative **93** in addition to the other products **94–96**; none of the intramolecular cyclization product **97** was observed under these



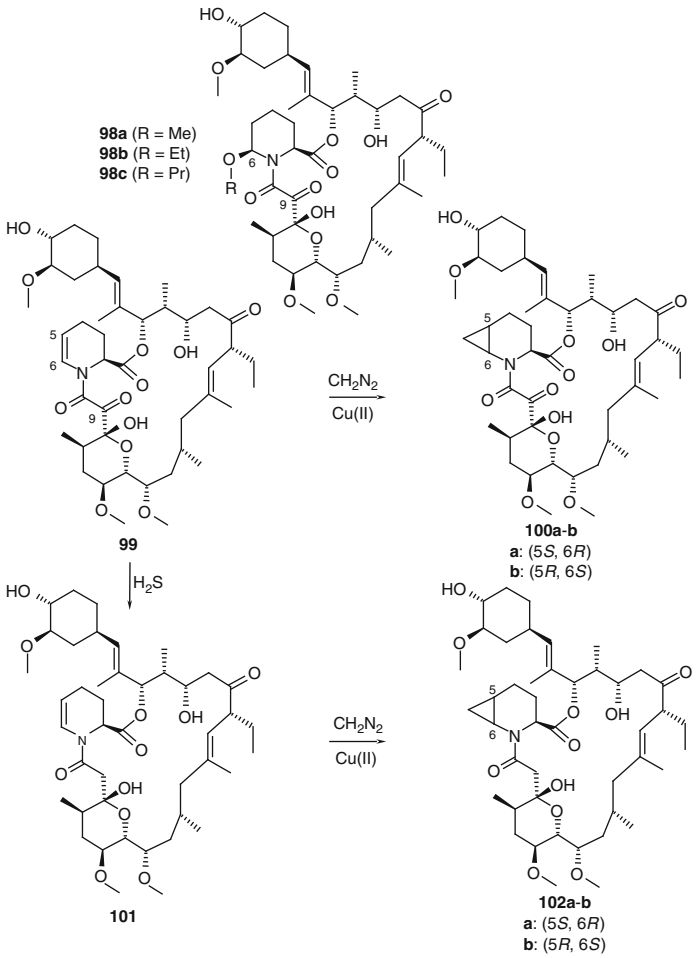
Scheme 23. Structures of the products formed after exposure of tacrolimus (**1**) to visible light



Scheme 24. Products formed upon irradiation of ascomycin (**2**) with ultraviolet light of different wavelengths in acetonitrile and methanol

reaction conditions. Interestingly, by employing light at >360 nm only the tricarbonyl chromophore is selectively excited and, hence, the formation of the [1,3]-shift product **95** could be suppressed leading to high selectivities. Thus, using these longer wavelengths the reaction in acetonitrile afforded the oxazolidinone **97**, whereas the reaction in methanol furnished the methoxy derivative **93** as the main product, hence providing preparatively useful protocols. The formation of these products could be explained through the intermediacy of the zwitterion **Z**.

The photoproduct **93** could be oxidized selectively using catalytic amounts of Cu $(OAc)_2$, pyridine, and oxygen in dichloromethane to afford 9-methoxyascomycin (**98a**) in excellent yield (Scheme 25). The ethoxy and propoxy analogues **98b** and **98c** could also be prepared analogously through irradiation of **2** in ethanol or propanol, followed by oxidation of the resulting 9-alkoxy photoproducts. On the

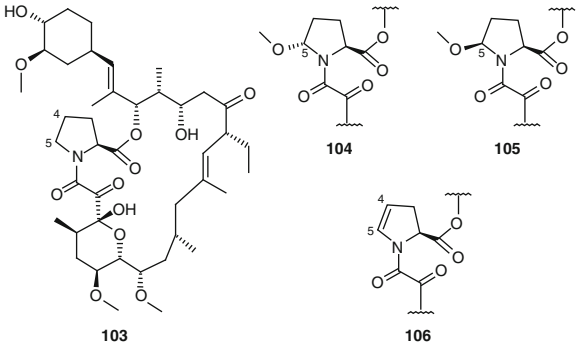


Scheme 25. 6-Alkoxy-ascomycins **98a**, **98b**, and **98c**, and 5,6-dehydroascomycin (**99**) and its further transformations

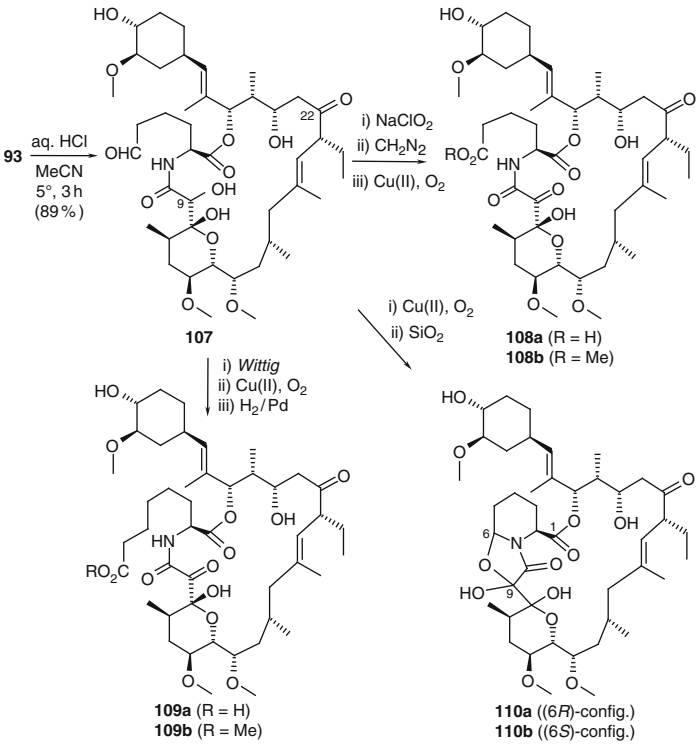
other hand, **93**, upon elimination of MeOH using ammonium chloride in DMF, gave 5,6-dehydro-9-dihydroacomycin, which after Cu(II)-catalyzed oxidation of the C-9−OH, afforded 5,6-dehydroascomycin (**99**), a close bioisostere of ascomycin in an excellent yield (*95*). Compound **99** served as a useful starting material for the preparation of 5,6-tritium labeled ascomycin in a single step through catalytic tritiation (*96*). Cu(II)-catalyzed cyclopropanation of **99** using diazomethane furnished both the α- and β- isopropano analogues **100a** and **100b** as further rigid derivatives of ascomycin (**2**). Further, **99** could be desoxygenated using hydrogen sulfide/pyridine giving the 9-desoxo analogue **101**, from which the two isomers of the cyclopropano analogues **102a** and **102b** could be obtained in low yields, in an analogous manner to the reactions described above. Starting from the proline analogue of ascomycin **103**, which is available as a side product in the fermentation of ascomycin (**2**), the 5-methoxy analogues **104** and **105**, and the 4,5-dehydro analogue **106** were prepared analogously (Scheme 26).

The hemiacetal photoproduct **93** can be hydrolyzed giving the aldehyde **107** in an excellent yield (Scheme 27). The reactivity of the aldehyde functionality can be used for preparing interesting derivatives. Thus, employing $NaClO_2$ and Cu(II)-catalyzed oxidations, *Wittig* and esterification reactions, **107** could be transformed to the linear amino acid analogues **108a**, **108b**, **109a**, and **109b** in a few steps and good yields. Furthermore, Cu(II)- catalyzed oxidation of **107** provided the corresponding C-9=O derivative, which underwent acid-catalyzed cyclization leading to the bicyclic analogues **110a** and **110b**.

For the purpose of broader derivatization in the amino acid region, the suitably protected key aldehyde **113** was synthesized starting from the easily available protected ascomycin derivative **111**, using the photoreaction described above (Scheme 28) (*97*). Thus, starting from **111** photolysis afforded the methoxy derivative **112**, which was hydrolyzed, and the C-33−*O*-silyl group that fell off was reintroduced giving **113**. Aldehyde **113** was transformed to the allyl carbonate **114** through a series of transformations. Interestingly, **114** exists exclusively as a seven-membered hemiketal in $CDCl_3$. Pd-Catalyzed cyclization of **114**, followed by desilylation and selective oxidation of the C-22−OH led to the 6-vinyl analogue **115** as an inseparable mixture of C-6−epimers. The two C-5−epimers of the 5-vinylproline analogue **116** were also synthesized starting from **113**, as individual isomers, using a similar strategy.

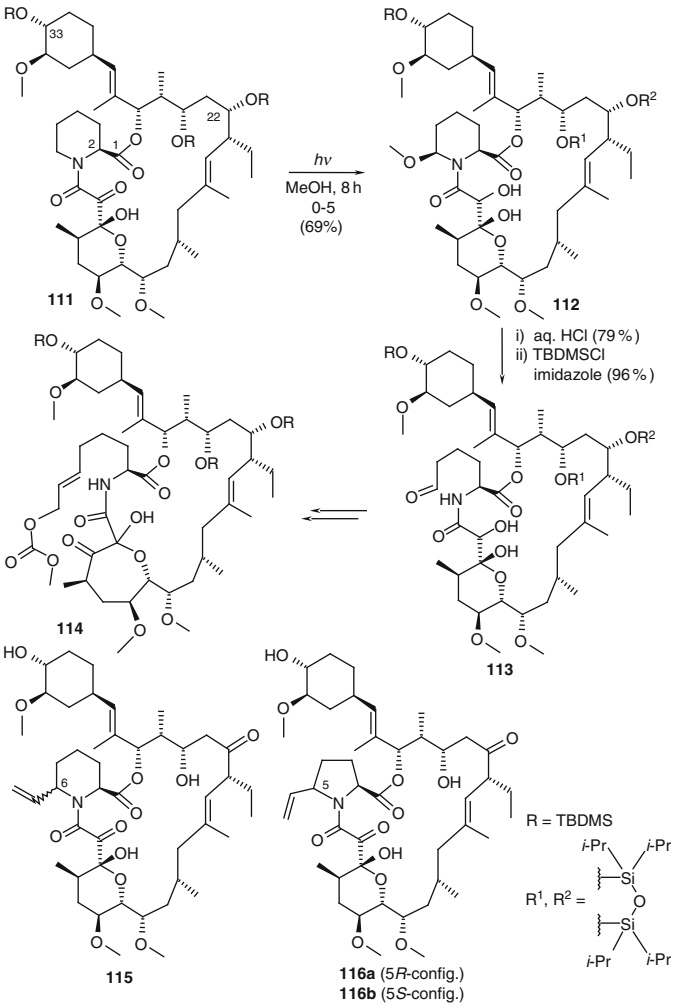


Scheme 26. 5-Alkoxy- and 4,5-dehydro- derivatives **104**, **105** and **106** of the proline analogue of ascomycin **103**



Scheme 27. Hydrolysis of the photoproduct **93** to the aldehyde **107** and its further transformations

The biological activities of these compounds resulting from photochemistry were discussed in detail in the light of the possible conformations (*92*). Briefly, the observed activities of the three 6-alkoxy derivatives **98a**, **98b**, and **98c** showed that larger alkoxy groups result in weaker binding to macrophilin. Among the cyclopropano analogues, those bearing the cyclopropano group in the α-face of the piperidine ring **100a** and **102a** showed higher activity than the β-counterparts **100b** and **102b**. The 9-oxo analogues **99**, **100a** and **100b** were more active than the 9-desoxo counterparts **101**, **102a**, and **102b**, indicating the importance of the C-9−carbonyl group to the binding with macrophilin. Other analogues, such as the vinyl analogues **115**, **116a**, **116b**, and the bridged analogues **110a** and **110b** were not active. The acyclic amino acid analogues **108a-110a** and **108b-110b** did not show any activity, reflecting on the importance of the necessity of the pipecolic acid ring structure, and hence the conformation of the macrolide. It is noteworthy that the ring-contracted butenyl analogue **94** and its saturated butyl derivative (not shown in the scheme), featuring unaltered binding domains, showed excellent binding to macrophilin, but were not active in T-cell assays. Thus, in these cases, modifications in the effector domain do not favor further interactions of the initially formed macrophilin-binding complex with calcineurin A and calcineurin B, and thus do not result in any T-cell modulatory activities. Of all these derivatives, the 5,6-dehydro-ascomycin **99** and the dehydroproline **106** showed activities close to



Scheme 28. Syntheses of the 6-vinyl-ascomycin **115** and the 5-vinylproline-analogues **116a** and **116b**

the parent ascomycin. Compound **99** (SDZ 283–871) had been under detailed investigations at Novartis, but its development was terminated later in preference to pimecrolimus.

2.3.5. Cleavage Reactions Within the Binding Domain: Amino Acid Exchange

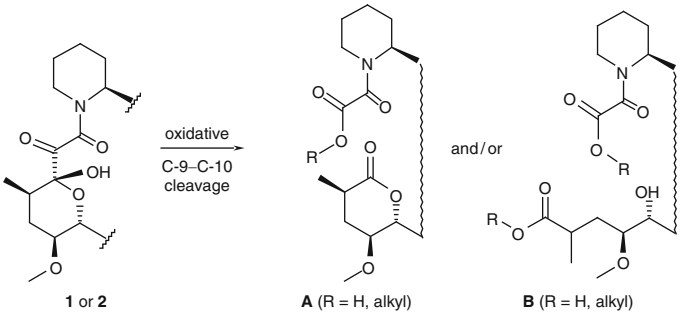
In addition to the already mentioned photochemical decarbonylative cleavage and the *Lewis* acid assisted cleavage of 9-oxirane derivatives (see Scheme 12), several other cleavage protocols have been elaborated. Thus, reaction of ascomycin (**2**) or

tacrolimus (**1**) derivatives with lead tetraacetate in methanol, or with *in situ* generated LiOOH or $Ca(OOH)_2$ in THF/aq. hydrogen peroxide solution, led to a facile oxidative cleavage of the C-9−C-10 bond, affording, depending on the work-up method, *seco*- structures of the general formula **A** or **B** (Scheme 29) (*64*, *73*, *98*–*100*).

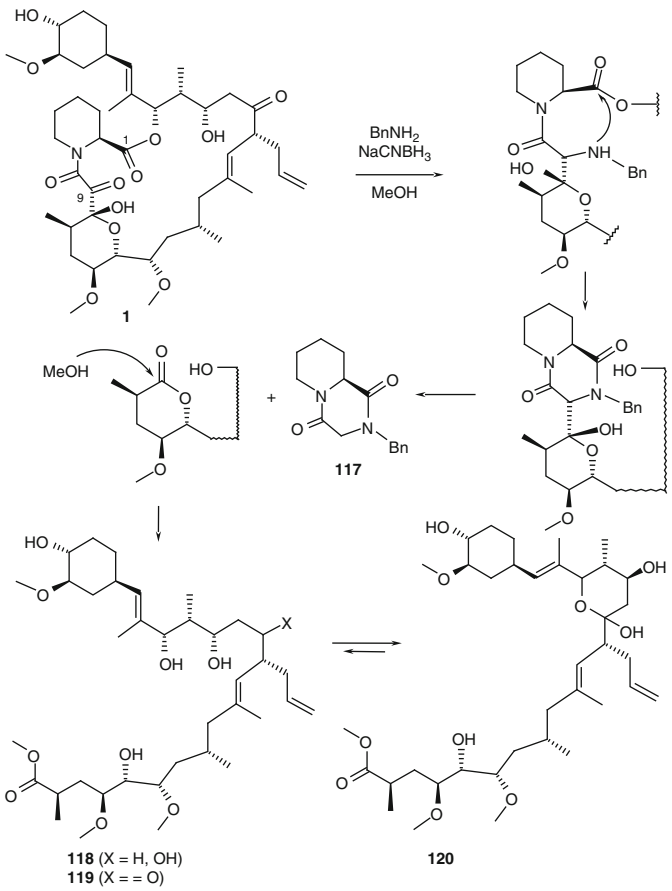
Two additional methods, allowing a reductive cleavage at the binding domain, have been described as well. Thus, reaction of tacrolimus with benzyl amine and sodium cyanoborohydride in methanol afforded the diketopiperazine **117** and a mixture of the C-10−C-34 fragments **118** and **119** (Scheme 30) (*73*). The hydroxyketone fragment **119** exists in equilibrium with its hemiketal tautomer **120**. Mechanistically, this interesting fragmentation might involve reductive amination of the C-9=O followed by an intramolecular aminolysis of the ester, and, retroaldol cleavage of the C-9−C-10 bond yielding the observed products.

Alternatively, reductive cleavage of 24,33-bis-*O*-TBDMS-ascomycin (**8**) has been achieved by the action of 9-BBN (9-bora-bicyclo[3.3.1]nonane) in THF giving the hydroxy-acetamide **121** in an excellent yield (Scheme 31) (*100*). Treatment of the latter with sodium hydride furnished the C-10−C-34 fragment **122** through an intramolecular transesterification reaction. Interestingly, **122** could also be obtained by a cyanide-induced cleavage. In the event, after addition of cyanide to the C-9−carbonyl, cleavage of the C-9−C-10 bond occurs to give the cyanohydrin **123** (*99*, *101*–*103*). Depending on the cyanide source used and the work-up conditions, either the cyanohydrin itself and/or the fragment **122** could be isolated.

The cleavage products having lost the pipecolic acid moiety could be elaborated to new analogues incorporating new amino acid units such as sarcosine, proline, D-pipecoline, *N*-methyl-alanine, 2-methyl-alanine, and β-alanine in place of the original amino acid (Schemes 32 and 33) (*100*, *104*). For example, coupling of a suitable N-protected amino acid to the most reactive, allylic C-26−OH of **122**, followed by a chemoselective reduction of the lactone unit, afforded the lactol **124**. Addition of a C_2 unit to the lactol through a *Wittig* reaction, protection of the C-14−OH and deprotection of the amine and the carboxylic acid groups followed by a macrolactamization reaction provided the enamide **125** as a key



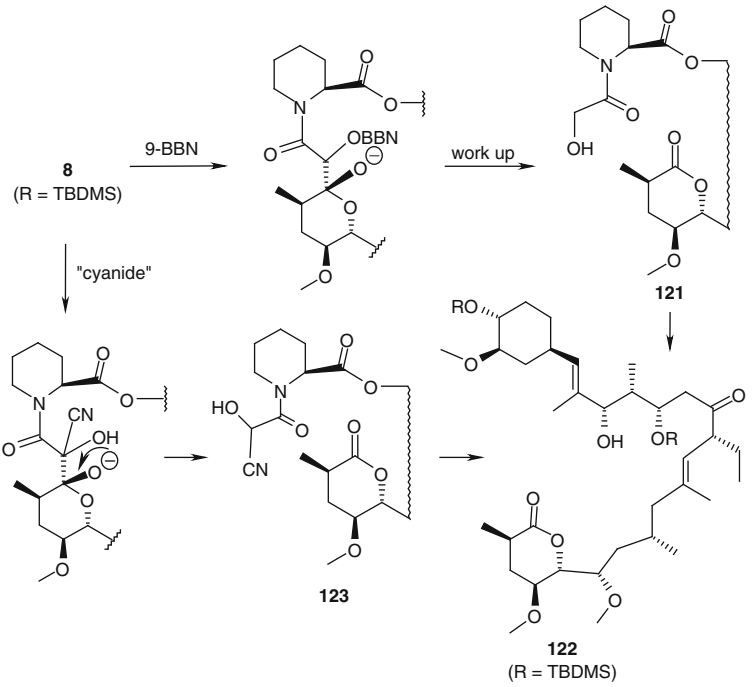
Scheme 29. Oxidative cleavage of derivatives of ascomycin (**2**) and tacrolimus (**1**). The *long wavy lines* indicate partial structures



Scheme 30. Reductive cleavage of the C-1–C-9 unit of tacrolimus (**1**). The *long wavy lines* indicate partial structures

intermediate. Bishydroxylation of the C-9−C-10 double bond followed by *Dess-Martin* periodinane oxidation led to the tricarbonyl product **126**. Finally, removal of all protecting groups led to the 23-membered macrocycles **127–130** incorporating the appropriate amino acid. Interestingly, the analogues featuring 2-methyl-alanine or β-alanine exist preferentially in the seven-membered hemiketal-form.

Finally, mention must be made here of a novel photochemical amide cleavage reaction (*92, 105*). Thus, the photoproduct **112** upon oxidation of the C-9−OH gave the protected 6-methoxyascomycin analogue **131** in an excellent yield (Scheme 34). Irradiation of **131** in MeOH afforded the amide cleavage product **132** in a good yield. The reaction most probably proceeds through the zwitterionic intermediate $\mathbf{Z_3}$, which is attacked by MeOH on C-8=O instead of the usually observed attack on C-6. It is interesting to note that similar cleavage reactions have also been reported on other protected and, surprisingly, also on the unprotected ascomycin

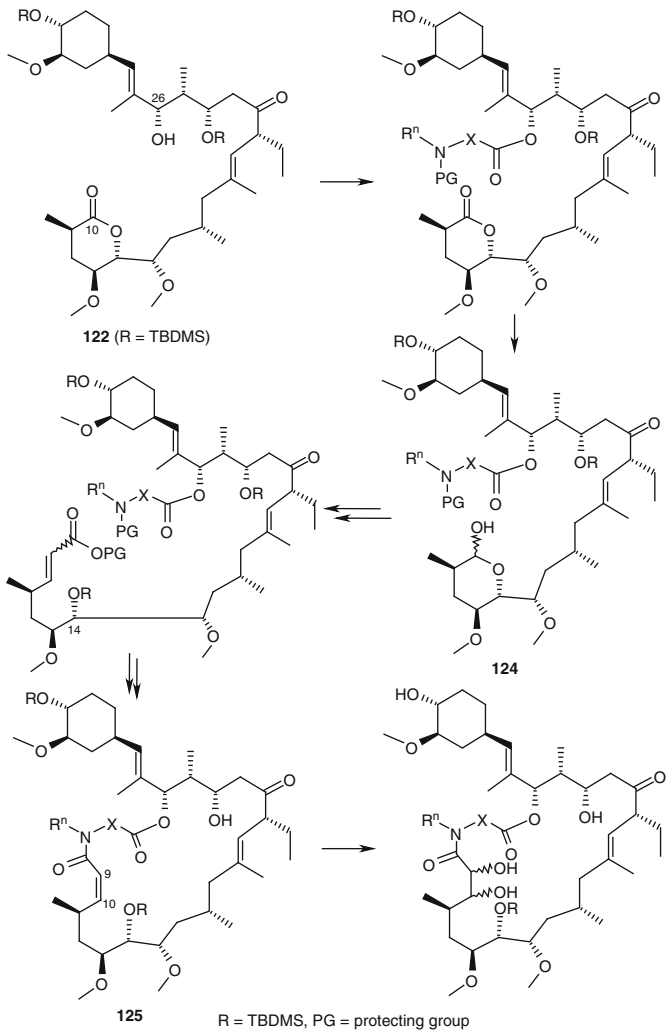


Scheme 31. Cleavage reactions in the binding domain of 24,33-bis-*O*-TBDMS-ascomycin (**8**). The *long wavy lines* indicate partial structures

analogues. The product **132** is a potentially useful intermediate for semisynthetic modifications, but this has not been explored further. Biological activities of the compounds in this section have not been reported.

2.4. Modifications in the Effector and Cyclohexyl Domains

Several modifications have been done on the effector parts of tacrolimus (**1**) and ascomycin (**2**). Whereas treatment of tacrolimus (**1**) with strong bases led to degradation of the molecule, treatment with 1,5-diazabicyclo[4,3,0]nonane (DBN) in CH_2Cl_2 at room temperature led to isomerization at C-21, affording a 1:2 equilibrium mixture of **1** and the C-21−epimer **133** (Scheme 35) (*106*). The isomer **133** was isolated and the structure established by X-ray crystal structure analysis. Compound **133** binds well to macrophilin but showed very weak immunosuppressive activities in cells (*74*). Acid-catalyzed dehydration of ascomycin (**2**) gave 23,24-dehydroascomycin (**134**), which was hydrogenated to give 24-desoxyascomycin (**135**) (*74, 107*). Whereas the dehydro derivative **134** showed only weak *in vitro* and *in vivo* activities, the desoxyascomycin **135** was as active as

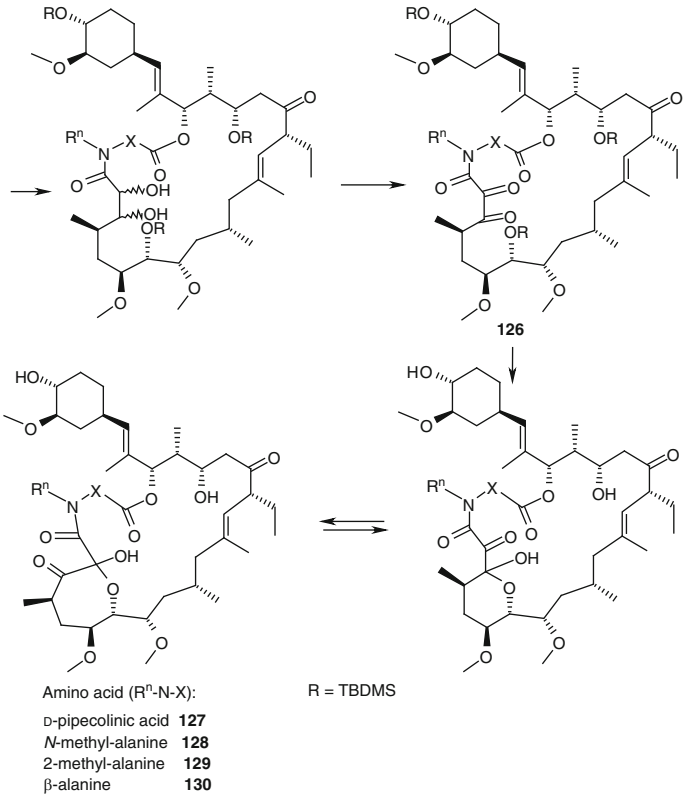


Scheme 32. Synthesis of amino acid analogues of ascomycin (**2**) starting from fragment **122**

ascomycin (**2**). NMR structures of the complexes formed between macophilin and **2** or **135** were found to be very similar, suggesting that hydrogen-bonding interactions with the C-24−OH are not important for complex formation.

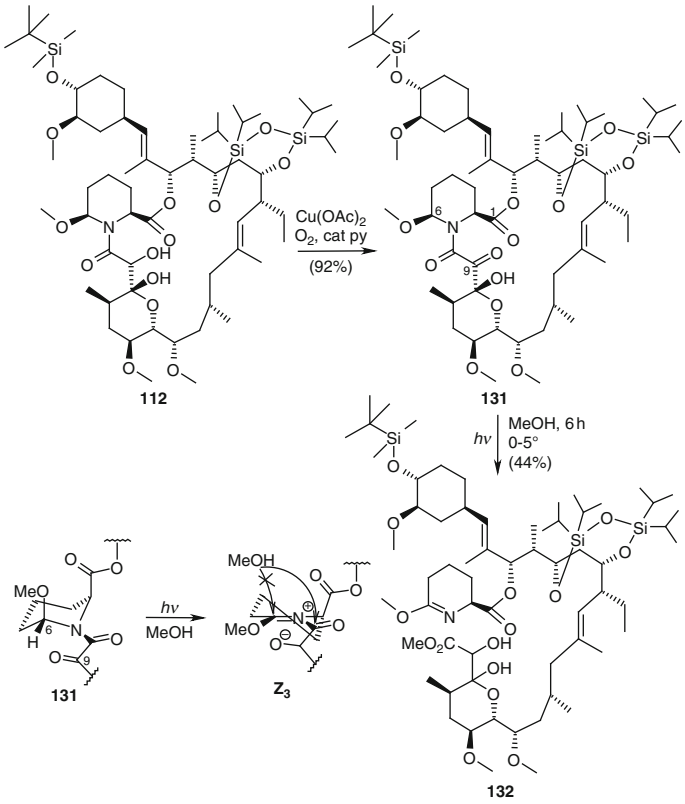
Numerous derivatives of the formula **136** with modifications on the terminal carbon of the allyl group of tacrolimus (**1**) have been prepared employing the olefin cross metathesis reaction (Scheme 35). These were employed as modules for the assembly of chemical inducers of dimerization (CIDs) (*108*, *109*).

Several derivatives of **2** with modifications at C-18 were prepared through allylic oxidation (Scheme 36) (*110*). Thus, oxidation of **2** by selenium oxide and *t*-butyl



Scheme 33. Synthesis of amino acid analogues of ascomycin (**2**) starting from fragment **122**, continued from Scheme 32

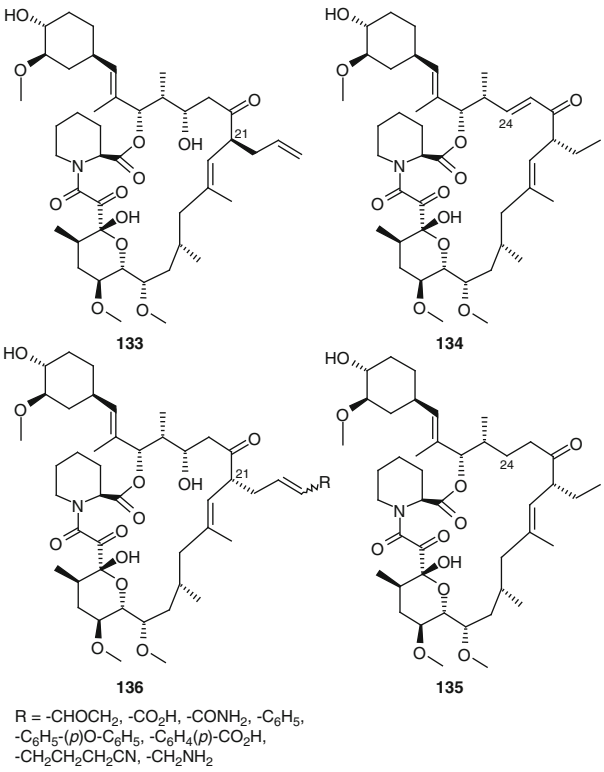
hydroperoxide in dichloromethane afforded the hydroxyascomycin **137** (R=(18*S*)-OH), also referred to in literature as L-685818, in addition to side products arising through dehydration at C-23−C-24. None of the (18*R*)-isomer was formed in this reaction. On the other hand, under the same reaction conditions, oxidation of the 24,33-bis-silyl derivative **8** gave the corresponding 18-hydroxy derivatives as a mixture of isomers from which the (18*R*)-isomer **137** (R=(18*R*)-OH) could be obtained after its separation and desilylation. The 24,33-bis-silyl protected (18*R*/*S*)-OH derivatives could be oxidized and desilylated to the oxo analogue **138**. The 24,33-bis-silyl-protected (18*S*)-OH derivative could be acylated and desilylated to give (18*S*)-acetoxy, -iodoacetoxy, and -benzoyloxy derivatives of the formula **137**. Whereas 24,33-bis-silyl-ascomycin could be deprotected in excellent yield, the corresponding 24,33-bis-silyl-18-hydroxy analogues were, because of the allylic nature of the additional hydroxy group, sensitive to the usual desilylation conditions and resulted in the cyclic derivatives **139** and **140** as side products. (18*R*/*S*)-Hydroxyascomycins bind tightly to macrophilin and are potent inhibitors of rotamase (PPIase) activity, but do not show any immunomodulatory activity



Scheme 34. A novel photochemical amide cleavage reaction of derivative **131**

in vitro or *in vivo* despite their good bioavailability. Further, they reversed the inhibition of calcineurin caused by the tacrolimus/macrophilin complex. This indicated clearly that the immunomodulatory activity has nothing to do with the inhibition of the PPIase activity of tacrolimus/macrophilin. Later, it was demonstrated that binding of the initially formed tacrolimus/macrophilin complex to calcineurins A and B is necessary for immunomodulatory activity.

Reaction of 33-*O*-TBDMS-ascomycin (**141b**) with *t*-butyldimethylsilyloxy-1-methoxyethene in the presence of $BF_3.Et_2O$ resulted in the addition of a methoxycarbonylmethyl unit to the C-22=O (Scheme 37) (*111*). The resulting aldol product upon treatment with 1 *N* HCl underwent 33-*O*-desilylation and concomitant intramolecular esterification leading to the lactone **142**. The product **142** then underwent facile acid-catalyzed dehydration to give the unsaturated lactone **143**. The enone **144**, which could be prepared through treatment of **141b** with methanesulfonyl chloride and excess of 4-dimethylaminopyridine, also underwent 1,2-addition upon treatment with *t*-butyldimethylsilyloxy-1-methoxyethane in the presence of $BF_3.Et_2O$, resulting in the corresponding silyl derivative, which could be desilylated to afford **145** as a single isomer with an unknown

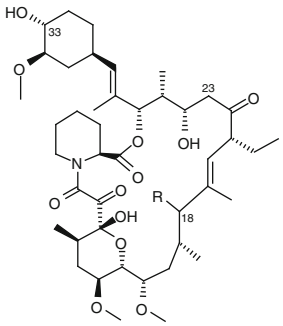


Scheme 35. 21-*epi*-FK506 (**133**), 24-desoxyascomycin (**135**) and olefin cross-metathesis derivatives of tacrolimus (**1**) **136**

C-22−configuration. On the other hand, the enone **144** underwent predominant 1,4-addition upon reaction with *t*-butyldimethylsilyloxy-1-methoxyethane in the presence of $LiClO_4$ in dichloromethane, providing the methyl ester **146** (as a single isomer of unknown stereochemistry) after desilylation (Scheme 38). Further, hydrosilylation of **144** gave the enol ether **147**, which could be employed in aldol reactions leading to **148a** and **148b**, as single isomers of unknown configuration at the newly formed stereocenters, featuring modifications at C-23.

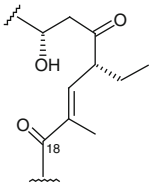
Novel cyclopropano dervatives could be prepared using carbene chemistry. Thus, both tacrolimus (**1**) and ascomycin (**2**) were transformed to their hydrazones **149a** (62%) and **149b** (56%) (Scheme 39) (*112*). Treatment of the hydrazones **149a** and **149b** with excess manganese dioxide in dichloromethane resulted in several products. However, reaction in methanol under the same conditions led to the cyclopropano derivatives **150a** and **150b** as the main products; no biological activities are reported.

Numerous pyrazole analogues of ascomycin (**2**) have been prepared (Scheme 40) (*113*). Thus, the 33-*O*-TBDMS derivative **141b**, upon oxidation with DMSO and oxalyl chloride followed by desilylation with HF/aceteonitrile, gave a 91% yield of

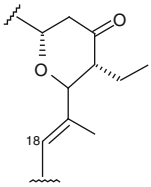


137

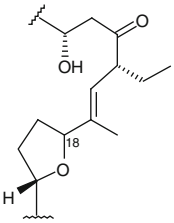
R = (18*R*)-OH, (18*S*)-OH, (18*S*)-OAc, (18*S*)-OBz, (18*S*)-$OCOCH_2I$



138



139



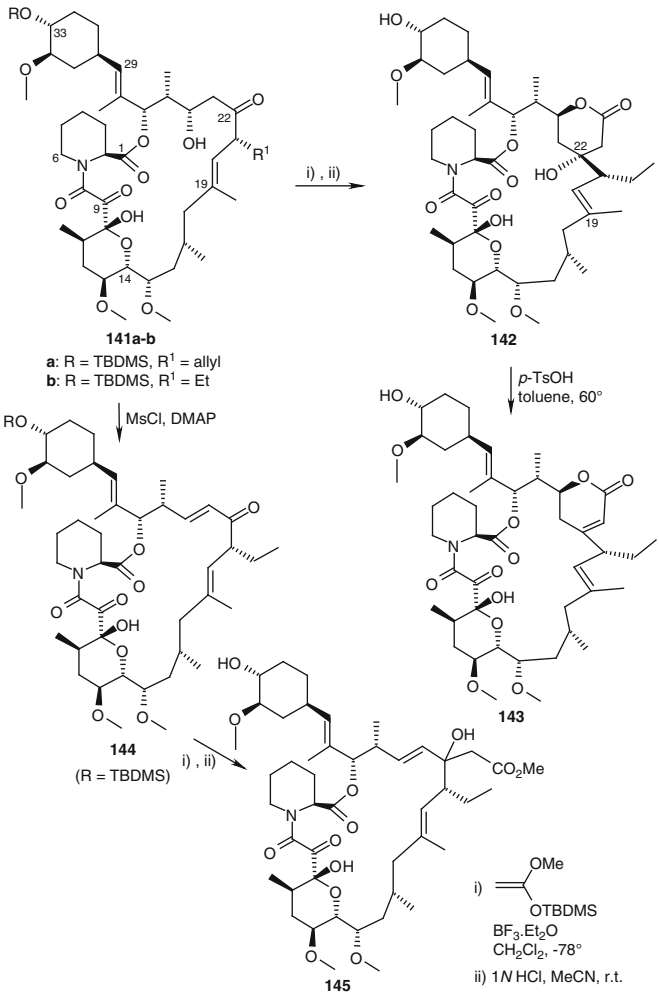
140

Scheme 36. 18-Hydroxyascomycin (**137**) and its derivatives **138–140**

151, which upon treatment with hydrazine, afforded a mixture of the isomeric pyrazoles **152** (R=H) and **153** (R=H). The substituted pyrazoles **152** and **153** were prepared either through alkylation of the 33-*O*-TBDMS-protected unsubstituted pyrazoles, or through reaction of **151** with substituted hydrazines followed by chromatographic separation of the isomers. No biological activities were reported.

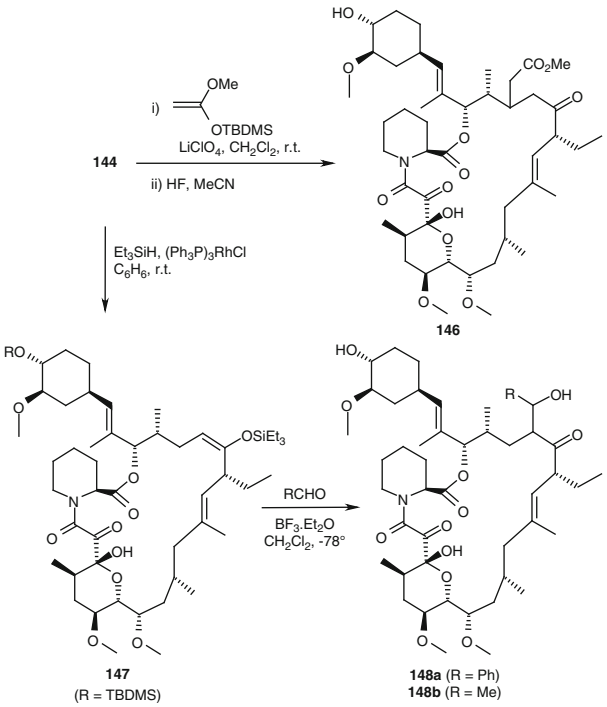
Several modifications in the cyclohexyl domain have been reported. Thus, activation of C-33−OH of the 24-*O*-TBDMS-ascomycin **154** as its triflate, followed by treatment with triethyl amine in dichloromethane at 45°C for 2 days and at room temperature for 2 days, resulted in an inseparable 3:1 mixture of the two isomers **155** and **156** in a 30% yield and the enol ether **157** (3% yield) (Scheme 41) (*114*). Osmium tetroxide-catalyzed c*is*-hydroxylation of the mixture **155** + **156** using *N*-methylmorpholine *N*-oxide in THF at room temperature for 2 days, followed by separation and desilylation of the individual isomers, afforded (34*R*)-hydroxyascomycin (**158**) and the 31,32-dihydroxy-33-methoxy analogue **159**. Interestingly, the introduction of an additional hydroxy group increased the solubility of compounds **158** and **159** by 300- and 150-fold compared to ascomycin (**2**) at pH 6.5. Compound **158** showed *in vitro* and *in vivo* activities comparable to those of ascomycin.

Several 33-*epi*-amino derivatives of ascomycin and its 24-desoxy analogue have been synthesized (Scheme 42) (*115*). Thus, the C-33−OH of 24-*O*-TBDMS-ascomycin (**154**) was activated as its *o*-nitrobenzenesulfonate (*o*-nitrobenzenesulfonyl

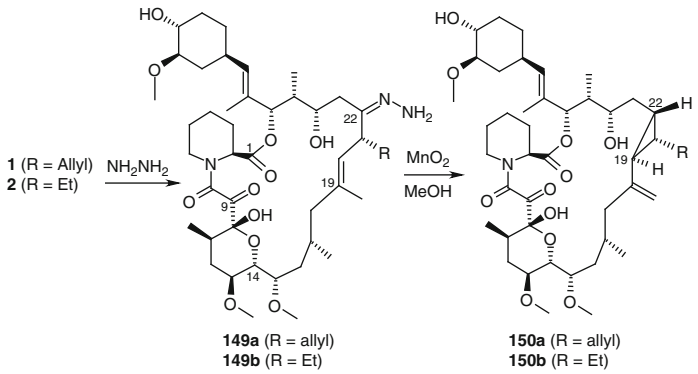


Scheme 37. Derivatives of ascomycin (**2**) with modifications in the C-22–C-24 region

chloride, triethyl amine, DMAP, CH_2Cl_2) and transformed to the 33-*epi*-azide through treatment with sodium azide in DMF at 60°C. Reduction of the azide with triphenyl phosphine in a mixture of THF and water at refluxing temperature afforded the 33-*epi*-amino-ascomycin **160** (R=H) in a 73% yield over three steps. Starting from **160** (R=H) several amides and carbamates have been synthesized. Similarly, from the 24-desoxyascomycin **135** the 33-*epi*-amino derivative **161** ($R=NH_2$) was synthesized, from which several amino analogues were prepared through condensation with aldehydes in THF, followed by reduction with sodium cyanoborohydride. The activities of these compounds in inhibiting T-cell proliferation were reported. Briefly, the 33-*epi*-amino analogue **160** (R=H) was tenfold less

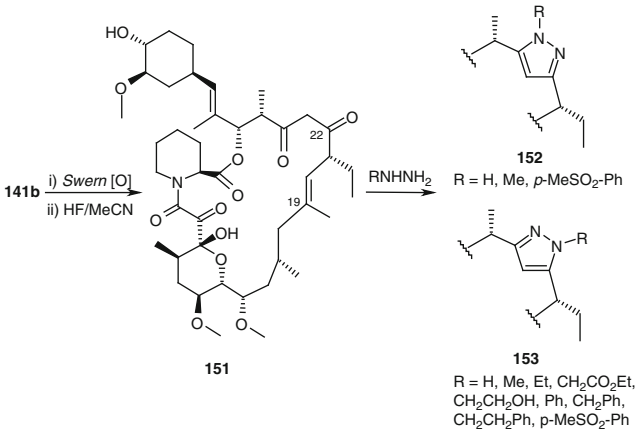


Scheme 38. Derivatives of ascomycin (**2**) with modifications in the C-22–C-24 region; continued from Scheme 37



Scheme 39. Transformation of tacrolimus (**1**) and ascomycin (**2**) to the cyclopropano- analogues **150a** and **150b**

active than ascomycin; this loss was partially recovered by removing the 24-OH group. Among the carbamate derivatives, the methyl carbamate **160** (R=MeOCO-) showed activity closest to that of ascomycin.

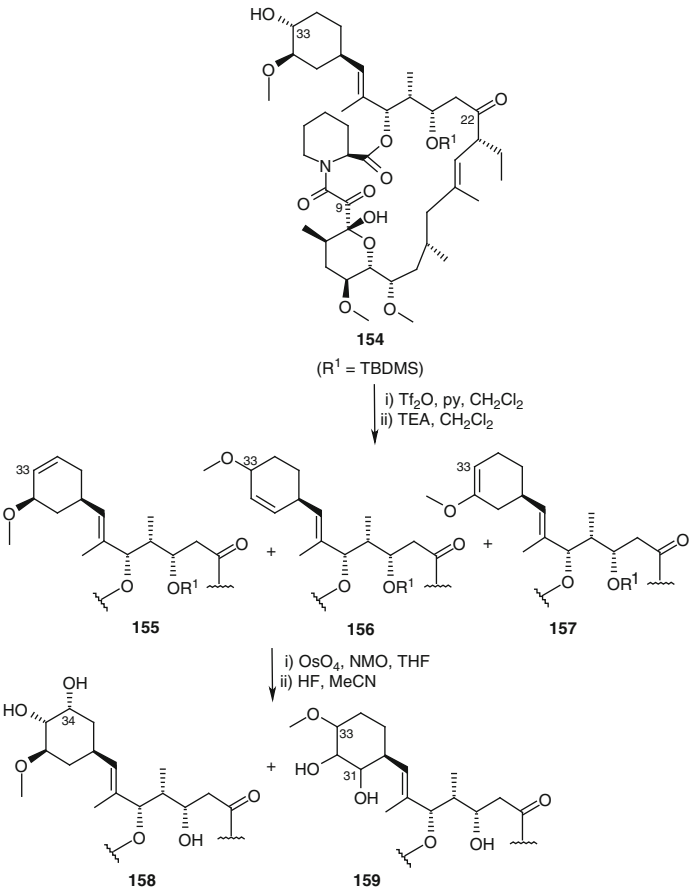


Scheme 40. Transformation of 33-*O*-TBDMS-ascomycin (**141b**) to the pyrazole analogues **152** and **153**

Pimecrolimus (**2a**) is prepared through selective chlorination of **2** using dichloro-triphenyl-phosphorane in a good yield (Scheme 43) (*116*). Other replacements of the C-33−OH of ascomycin by halogens or pseudohalogens were also reported (*116*, *117*, *118*). Further, the (33*R* or *S*)-*O*-cyano derivative **162** is easily accessible from **2** (*117*). Starting from **162** the derivatives **163**–**169** (SDZ 281–240 is a mixture of **169a** and **169b**) could be prepared easily (*117*, *118*). Of these derivatives, 33-*epi*-ascomycin (**163**) and 33-desoxy-32-oxo-ascomycin (**167**) showed good activities in a MLR (mixed lymphocyte reaction), but were, however, only weakly active in the animal models of ACD. Noteworthy is the observation that the *cis*-isomer **169b** is by a factor of 2 more active than the *trans*-isomer **169a**, in *in vitro* and *in vivo* models.

Several carbamate derivatives at C-33 have been prepared starting from 24-*O*-TBDMS-ascomycin (**154**) (Scheme 44) (*119*). Thus, reaction of **154** with the appropriate acyl isocyanate or acyl isothiocyanate followed by desilylation afforded the acylated carbamate **170a** and the acylated thiocarbamates **170b**-**h**. The hydrazide derivative **170i** was prepared through reaction of ascomycin (**2**) with triphosgene giving the corresponding 33-*O*-chloroformate selectively, followed by its reaction with phenyl hydrazine. Binding to macrophilin and the *in vitro* immunomodulatory activities were demonstrated; derivatives **170a** and **170i** showed activities similar to ascomycin (*119*).

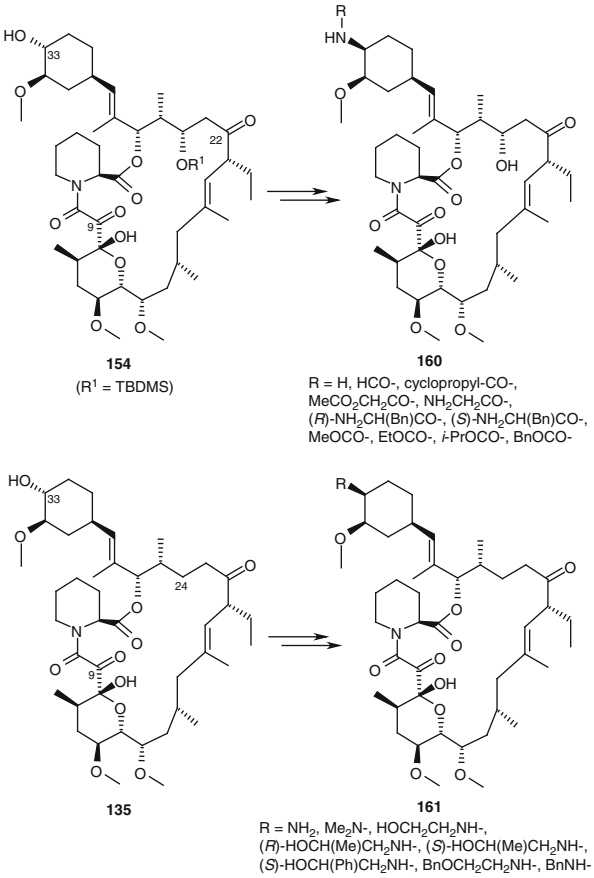
Numerous C-33−ether derivatives were prepared by O-alkylation of 24-*O*-TBDMS-ascomycin (Scheme 44). Thus, reaction of **154** with allyl 2,2,2-trichloroacetimidate in the presence of a catalytic amount of trifluoromethane sulfonic acid gave **171a**, which after desilylation using HF in acetonitrile, afforded the allyl ether **171b** in a 75% yield (*120*). Similarly, the unsubstituted cinnamyl ether **171c** and several analogues of **171c** with electron-withdrawing and -releasing substituents on the phenyl ring have been synthesized. Further, using the same method, several analogues of **171d** featuring alkyl, alkenyl, alkynyl, aralkyl, aralkenyl, and aralkynyl units, were prepared and their binding to macrophilin



Scheme 41. Synthesis of the hydroxylated derivatives **158** and **159**

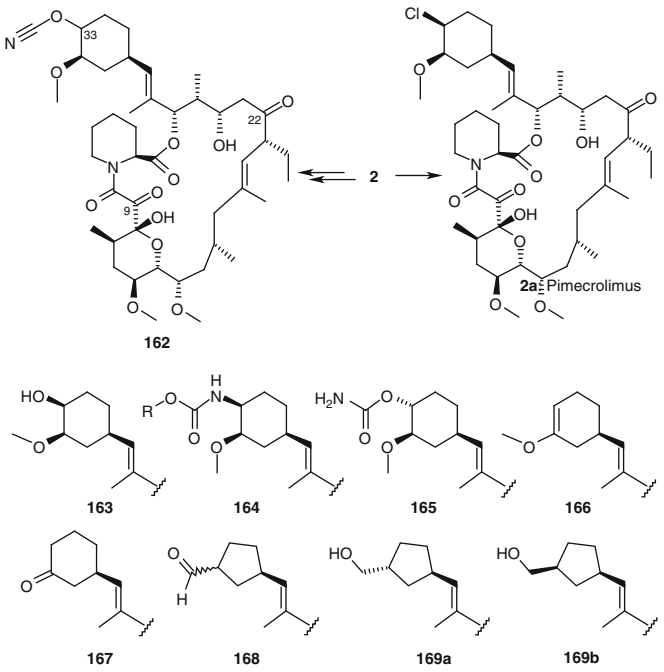
and inhibition of T-cells studied. It is interesting to note that **171c** and the corresponding *m*- and *p*-hydroxy cinnamyl ethers, in spite of their relatively weaker binding, showed inhibitory activities on T cells comparable to those of ascomycin. However, they were reported to be less efficacious *in vivo* especially upon oral administration (*121*). In further studies aimed at optimizing the linker between the C-33−O and the phenyl groups, the 24-silyl protected allyl ether **171a** was transformed to the aldehyde **171e** by osmium tetroxide-catalyzed hydroxylation followed by cleavage using sodium periodate in aqueous THF, in an overall yield of 64%. Starting from the valuable intermediate **171e**, using the sequences reductive amination followed by desilylation, or, reduction to alcohol, displacement of a trichloro acetimidate, followed by desilylation, ether derivatives of the type **172** (R=alkyl, aryl; tether=secondary amine or ether) with varying tethers were synthesized. Further, the aldehyde **171e** was transformed to several imidazol-2-yl-methyl ether derivatives of type **173** (R^1=H, Et; R^2=H, Me, Ph, substituted-Ph)

Scheme 42. Synthesis of the 33-*epi*-amino analogues **160** and **161** of ascomycin (**2**) and 24-desoxyascomycin (**135**)



with different substituents on the imidazole ring. Among the compounds investigated, derivative **173** (R^1=H, R^2=3,5-dimethoxyphenyl), referred to as L-733,725, was reported to have *in vivo* activities comparable to those of tacrolimus and a better therapeutic index. A convergent practical synthesis was developed and was used for synthesizing multi-kilogram quantities of L-733,725 of consistently high purity (*122*). Furthermore, labelled L-733,725 carrying a ^{14}C-label on the CH_2 of the side chain was also synthesized for utilization in animal and human drug metabolism studies (*123*).

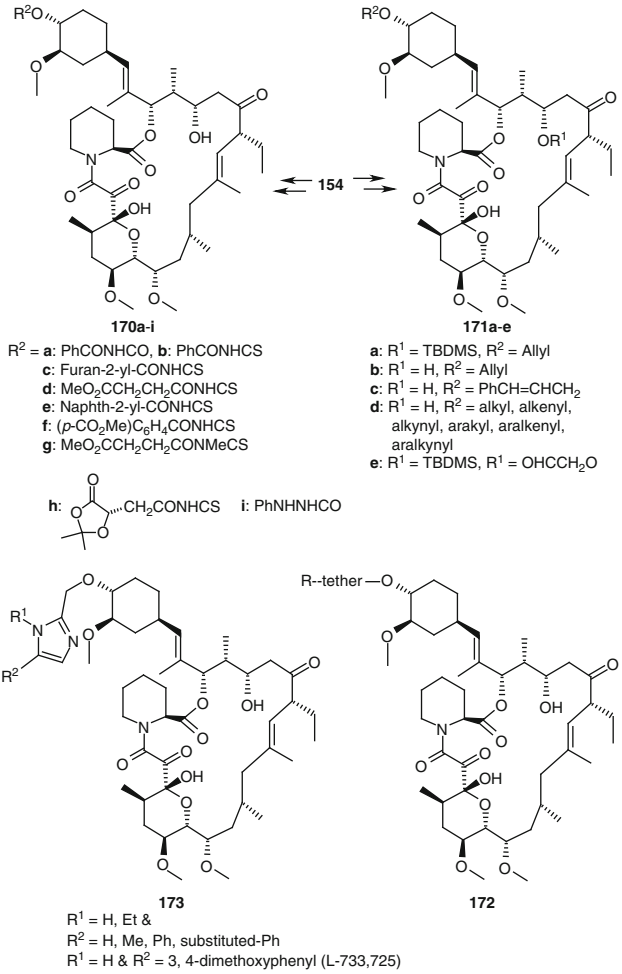
In a similar study, several 33-*O*-ether derivatives featuring a phenyl group connected through a carbon tether of varying length were synthesized (Scheme 45) (*124*). The synthesis started from the aldehyde **171e** and proceeded through addition of an organomagnesium bromide to the aldehyde, followed by transformation of the resulting alcohol to the corresponding trifluoro acetate, its elimination, selective hydrogenation of the resulting double bond and, finally, deprotection of the C-24−O−silyl group leading to the compounds **174a** (R=$PhCH_2CH_2$, $PhCH_2CH_2CH_2$); the biological data were compared with **174a** (R=Ph, $PhCH_2$)



Scheme 43. Transformation of ascomycin (**2**) to several analogues *via* the 33-*O*-cyano-derivative **162**, and, synthesis of pimecrolimus (**2a**) and the individual isomers **169a** and **169b** of SDZ 281–240

(*125*). The derivatives **174b** bearing hydroxy or keto groups on the carbon tether have also been synthesized using similar strategies. A two-carbon tether provided optimum *in vitro* activity. The acetophenone derivatives **174b** (R=$PhCOCH_2$) showed efficacy in models of immunosuppression. However, it was found that they were rapidly converted to the arylhydroxy ether products when incubated in rat blood. Hence, an extensive series of substituted arylhydroxy ethers **174c** were synthesized using analogous chemistry (*126*). Several of these compounds showed potent *in vitro* and *in vivo* immunosuppressive activities. The derivative **174c** (R=2-naphthyl-$CHOHCH_2$-) was claimed to have an improved therapeutic index compared to tacrolimus (**1**) (*126*).

Using pentavalent bismuth derivatives ($Ar_3Bi(OAc)_2$, CH_2Cl_2, THF) the C-33−OH of ascomycin (**2**) could be preferentially arylated leading to compounds of type **177a** in yields ranging from 14% to 72% (Scheme 45) (*125*, *127*). Similarly, starting from the C-33−O-desmethyl analogue **176**, derivatives **177b** and **177c** could be synthesized. Among the derivatives of the type **177a** (R^1=Me, R^2=Ph), **177b** and **177c**, those with electron-donating substituents such as HO and Me_2N in the *para*- position of the phenyl group, showed better *in vitro* immunosuppressive activities. The two indole ether derivatives of the type **177a** turned out to be the best in the series, being even superior to the parent macrolide **2** by a factor of 3 in *in vitro* immunosuppression. The two derivatives **175a** and **175b**, prepared from

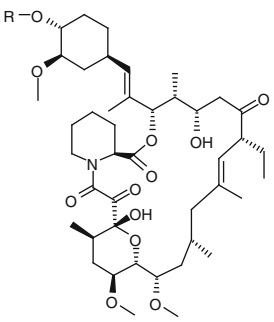


Scheme 44. Synthesis of 33-*O*-acyl, 33-*O*-thioacyl, and 33-*O*-ether derivatives of ascomycin (**2**)

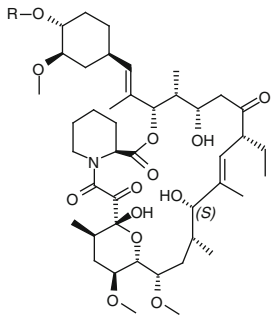
(18*S*)-hydroxyascomycin (**137** (R=(18*S*)-OH) using a similar method, showed 8- to 15-fold weaker immunosuppressive activities compared to **2**.

The C-33−OH group of **2** could be etherified selectively by rhodium (II) acetate-catalyzed insertion of benzyl diazoacetate in dichloromethane affording **178** (R=Bn), from which the corresponding acid **178** (R=H) could be obtained by hydrogenolysis of the benzyl ester (Scheme 46) (*128*). Starting from the acid, ester and amide derivatives of types **178** and **179** were prepared. While the carboxylic acid **178** (R=H) did not show any *in vitro* activity, the ester and amide derivatives showed potent T-cell inhibitory activities. Surprisingly, hydrophobic amides were reported to have weakened macrophilin binding by several orders of magnitude, while maintaining potent activity to inhibit T-cell activation (*128*).

Scheme 45. Synthesis of ether derivatives at the C-31 and C-33 positions of ascomycin (**2**)



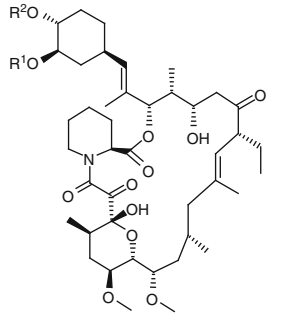
174a-c



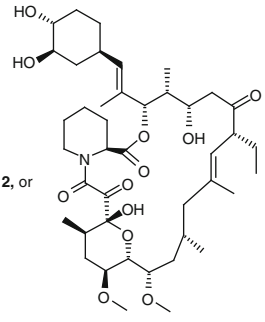
175a-b

a: R = 2-naphthyl
b: R = *N*-methyl-5-indolyl

a: R = Ph, $PhCH_2$, $PhCH_2CH_2$, $PhCH_2CH_2CH_2$
b: R = $OHCH_2CH_2$, $PhCHOHCH_2$, $PhCOCH_2$, $PhCH_2CO$, $PhC(=CH_2)CH_2$
c: R = $ArCHOHCH_2$

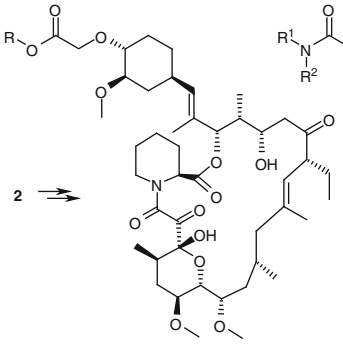


177a-c

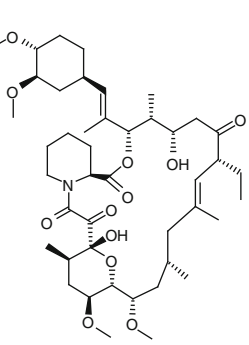


176

Prepared from **2:**
a: R^1 = Me, R^2 = substituted Ph, 2-naphthyl, 5-indolyl, *N*-methyl-5-indolyl
Prepared from **176:**
b: R^1 = H, R^2 = substituted Ph
c: R^1 = substituted Ph, R^2 = H



178
R = H, Et, Bn

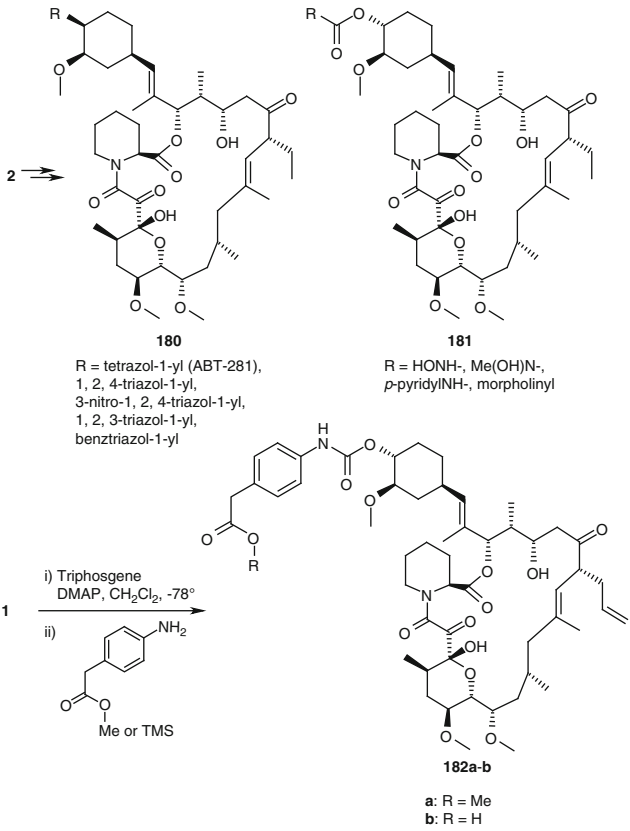


179a-b

a: R^1 = H, and R^2 = cyclohexyl, Ph, substituted aryl, Bn, CH_2CH_2Ph
b: R^1 = Me, and R^2 = Bn, CH_2CH_2Ph

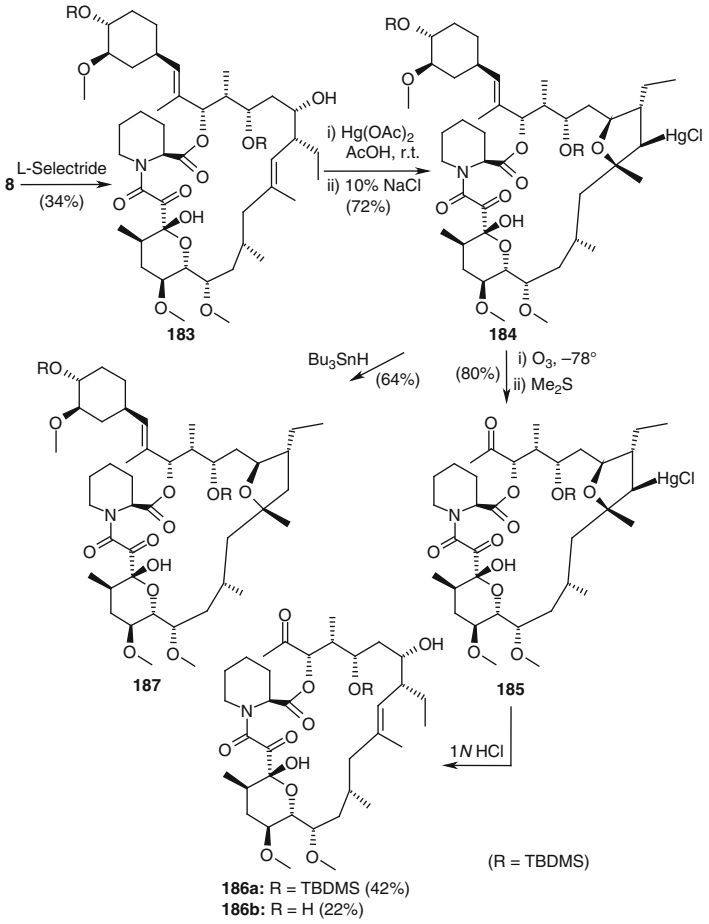
Scheme 46. Diverse esters and amides of the ascomycin-33-*O*-acetic acid skeleton

Derivatives like **180** featuring a heterocycle at C-33 have been prepared through selective activation of C-33−OH as its triflate, followed by its displacement by nitrogen of a small heterocycle (Scheme 47) (*129*). Further, selective activation of C-33−OH as a *p*-nitrophenylcarbonate, followed by reaction with nitrogen nucleophiles afforded the carbamates **181**. The tetrazole **180** (R=tetrazol-1-yl), also referred to as ABT-281, was reported to be equipotent to tacrolimus (**1**) in the swine contact hypersensitivity model after topical application, despite its several-fold lower potency for inhibiting swine T cells *in vitro*. The high activity was consistent with its superior skin penetration. Further, it was found to exhibit a three- to fivefold more rapid pharmacokinetic elimination in the rat, swine, and monkey, in addition to showing a substantial reduction in potency for immunologic responses in all three species after systemic administration. These features of ABT-281 were claimed to result in both efficacy and a high margin of safety for topical therapy of skin disease as compared to tacolimus (*129*).



Scheme 47. Derivatives of ascomycin (**2**) featuring a heterocyclic unit on C-33, and diverse 33-*O*-carbamoyl-type derivatives of **1** and **2**

Tacrolimus was transformed to the carbamates **182a** and **182b** through activation of the C-33−OH as its chloroformate followed by reaction with *p*-amino-phenylacetic acid methyl ester, leading to **182a** in a 70% yield, or, with *p*-amino-phenylacetic acid trimethylsilyl ester followed by hydrolysis providing the acid **182b** in a 26% yield (*130*). Compound **182a** could also be prepared through activation of C-33−OH as its *p*-ntrophenyl carbonate followed by quenching with the amine. The carbamate **182a**, also referred to as MLD987, inhibited the activation, proliferation and release of cytokines from T-cells with IC_{50} values in the low nanomolar range. Furthermore, in a brown Norway rat model of allergic asthma, **182a**, when given into the airways by intertracheal administration (ED_{50} = 1 mg/kg) or by inhalation (ED_{50} = 0.4 mg/kg), potently reduced the influx of leucocytes into the bronchoalveolar fluid. Interestingly, **182a** had an appreciably



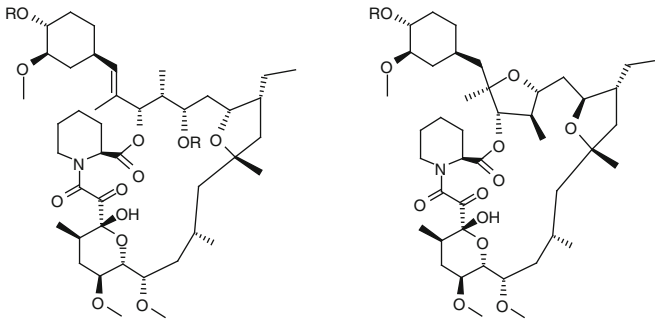
Scheme 48. Transformations using oxymercuration and ozonolysis resulting in the derivative **187** and the cleavage product **186b**

weaker activity when given orally and intravenously. Furthermore, pharmacokinetic evaluations showed that **182a** had a low oral and pulmonary bioavailability. In addition, **182a** was metabolized in the blood to the much less potent acid **182b**. These aspects indicated a potential of **182a** to serve as a soft drug after local application for therapy of asthma.

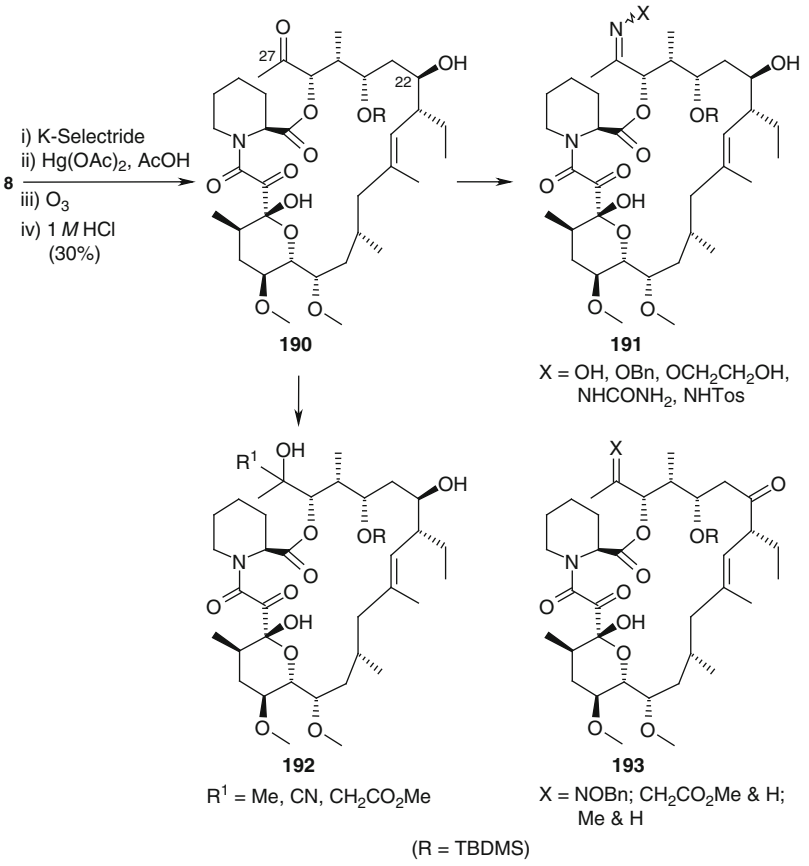
With a view to synthesizing derivatives with new substituents in place of the cyclohexyl moiety, a strategy was designed for chemoselective cleavage of the C-28 = C-29 double bond. Thus, the bis-silyl-protected ascomycin **8** was reduced stereo- and regioselectively at C-22=O giving the alcohol **183** in a 34% yield, which was then subjected to oxymercuration leading to the tetrahydrofuran **184**, thus protecting the C-19 = C-20 double bond against ozone (Scheme 48) (*131*). Ozonolysis of **184** afforded the ketone **185** in an excellent yield. Further, acidic hydrolysis of the organomercurial chloride afforded the required derivatives **186a** and **186b**. Finally, tributyl tin hydride reduction of **184** afforded the analogue **187**. The derivatives **188** and **189** could also be synthesized in an analogous manner (Scheme 49).

Starting from **8**, using K-selectride instead of L-selectride, the isomer **190** was prepared analogously to the synthesis of **186** (Scheme 50) (*132*). The C-27 = O keto group was then used for preparing derivatives of the types **191** and **192**. The required C-22 = O carbonyl could be re-established through oxidation leading to the 24-*O*-silyl derivatives **193** featuring novel replacements for the cyclohexylvinylidene unit. Several attempts to establish a carbon-carbon double bond at C-28 of **190** *via* a *Wittig* or *Wittig-Horner* olefination were unsuccessful and no reaction was observed. However, reaction of **190** with acetylmethylidenephosphorane ($CH_3COCH = PPh_3$) under forced conditions (65°C, 10 days) led to furano derivatives through reaction at the tricarbonyl region.

From the fermentation broth of *Steptomyces tsukubaensis* 9993, the novel ring-contracted metabolite *iso*-FK506 (**197a**) was isolated (Scheme 51) (*133*). A synthetic pathway for the transformation of tacrolimus to **197a** has been established (*134*). Thus, *Evans*' reduction of 33-*O*-TBMDS-FK506 (**141a**) afforded the (22*S*)-dihydro derivative **194a** in a good yield. Treatment of **194a** with DMAP in DMF



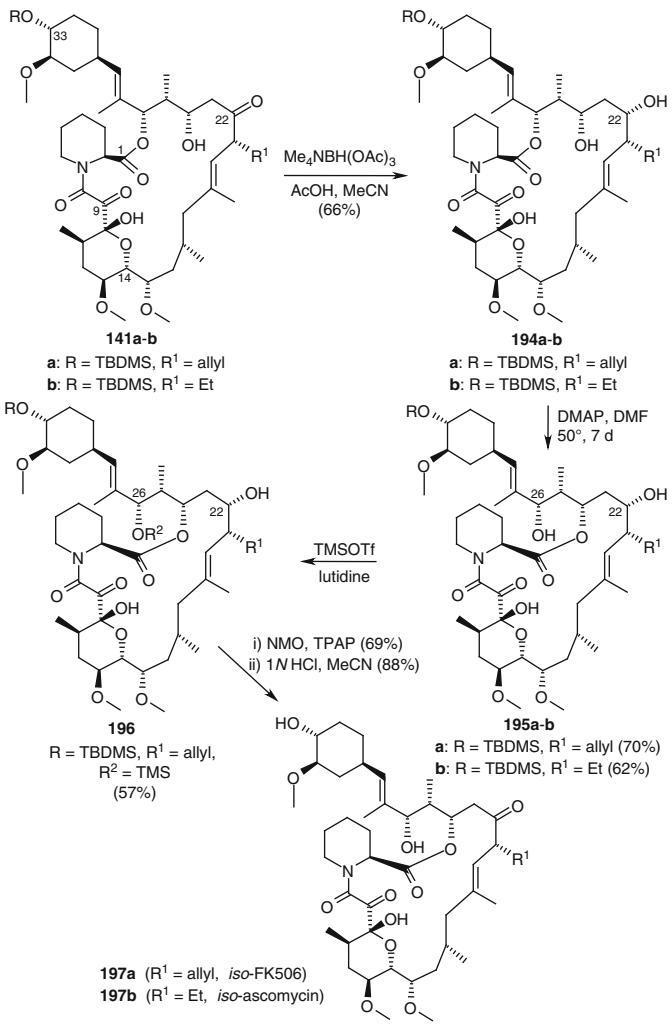
Scheme 49. Derivatives **188** and **189** prepared in an analogous manner to those in Scheme 48



Scheme 50. Derivatives of ascomycin (**2**) featuring novel replacements for the cyclohexylvinylidene subunit

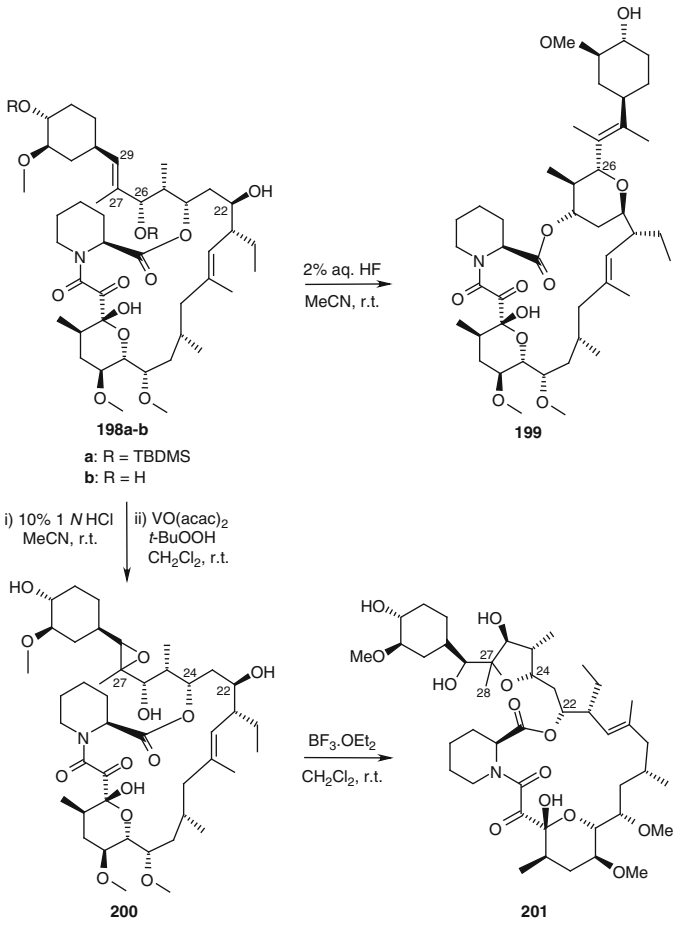
brought about acyl migration, giving **195a**. Attempts to oxidize the C-22−OH group selectively were not successful. However, after protection of the C-26−OH as its TMS derivative **196a**, oxidation using TPAP followed by desilylation afforded the *iso*-FK506 **197a**. The chemistry of the ethyl analogue **197b** followed in an analogous manner.

Under the usual desilylation conditions employing aqueous HF, the 22-dihydro *iso*-ascomycin derivative **198b** underwent intramolecular cyclization through attack of the C-22−OH on C-26 leading to the tetrahydropyran **199** in a 36% yield (Scheme 52) (*135*). On the other hand, **198a** could be desilylated using 1*N* HCl and transformed to the epoxide **200**. Interestingly, **200** upon treatment with $BF_3.Et_2O$, underwent acyl migration from 24-*O* to 22-*O*, followed by cyclization involving the released C-24−OH and the epoxide ring, affording the ring-contracted 19-membered macrolide **201** in a 38% yield.



Scheme 51. Synthesis of *iso*-FK506 (**197a**) and *iso*-ascomycin (**197b**)

Several derivatives of *iso*-ascomycin featuring broad modifications in the cyclohexyl region have been synthesized. Thus, selective *cis*-hydroxylation of the C-27=C-29 double bond in a 33-*O*-TBDMS-*iso*-ascomycin (**195b**) with osmium tetraoxide led to **202** as a mixture of diastereomers, which were cleaved with periodate to give the aldehyde **203** as a valuable intermediate (Scheme 53) (*136*). Compound **203** exists predominantly as a 1:1 mixture of the anomeric hemiacetals formed through intramolecular addition of the C-22−OH to the C-26−aldehyde. The aldehyde group of **203** could be transformed to **204a** through *Wittig* reaction and to the oxime and hydrazone derivatives **204b-d** through condensation reactions.

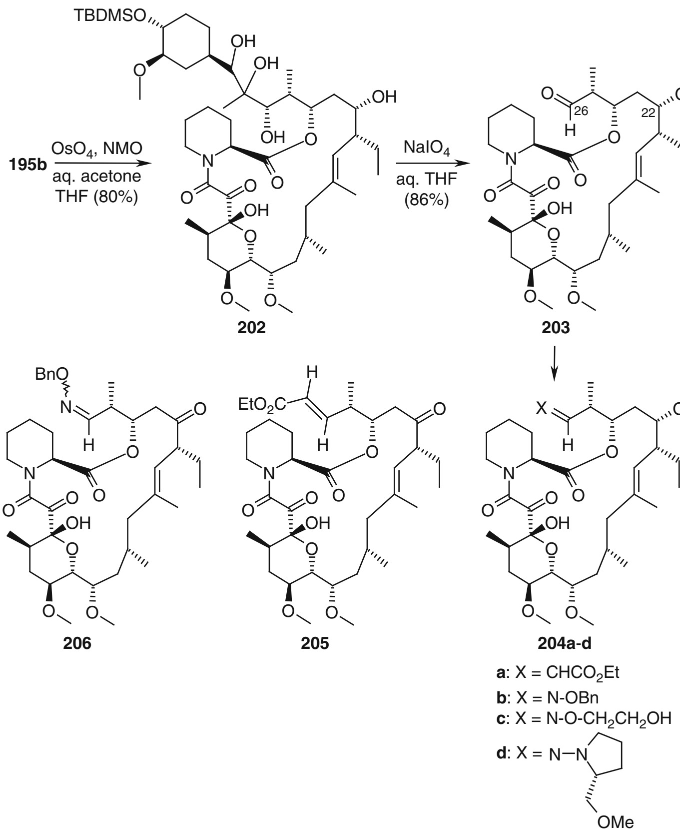


Scheme 52. Intramolecular cyclization, and ring-contraction reactions of 22-dihydro-*iso*-ascomycin (**198b**) leading to **199** and **201**

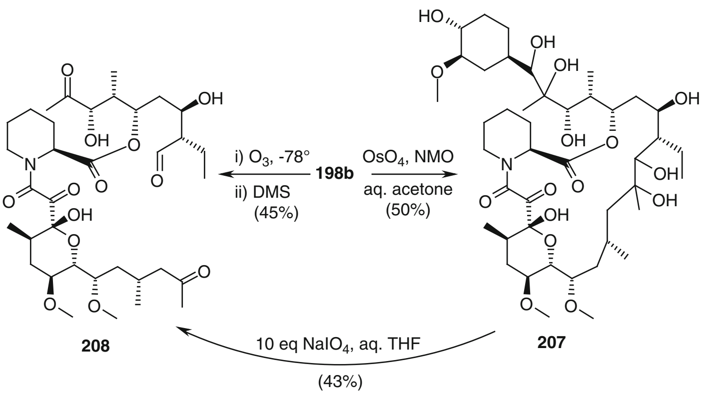
The derivatives **204a** and **204b** could be transformed to the analogues **205** (76%) and **206** (49%) through oxidation of the C-22−OH with *Dess-Martin's* periodinane.

In contrast to the chemistry observed with the (22*S*)-OH series (Scheme 52), osmium tetraoxide catalyzed dihydroxylation of the (22*R*)-dihydro-*iso*-ascomycin (**198b**) led to hydroxylation of both double bonds giving a 50% yield of the tetraol **207** as an isomeric mixture (Scheme 54) (*137*). Diol-cleavage using excess of sodium periodate led to the bis-*seco*-derivative **208**. Alternatively, **198b** could be transformed to **208** in a single step by ozonolysis followed by treatment with dimethyl sulfide.

Tacrolimus (**1**) is poorly soluble in water, and hence the conventional i.v. dosage forms contain surfactants such as cremophor EL or hydrogenated polyoxy 60 castor oil (HCO-60). Thus, towards increasing the water solubility of **1**, the methoxy-

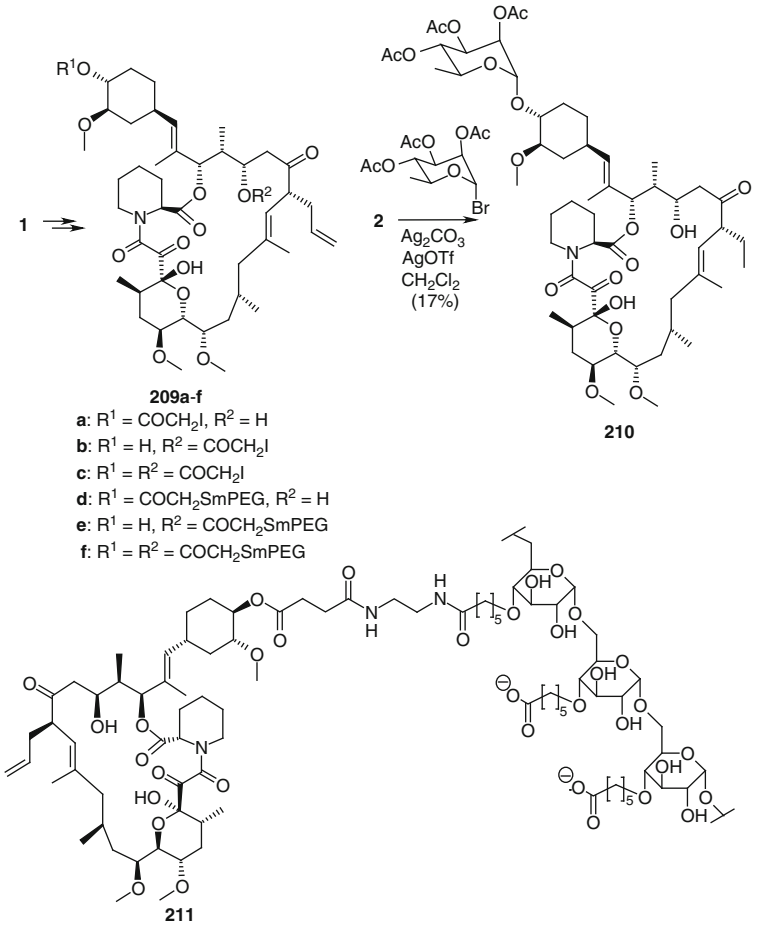


Scheme 53. Transformations of (22*S*)-diydro-33-*O*-TBDMS-*iso*-ascomycin (**195b**)



Scheme 54. Transformations of the (22*R*)-dihydro-*iso*-ascomycin **198b**

(polyethyleneglycol) (mPEG) conjugates **209d**, **209e**, and **209f** were synthesized by esterification of **1** with iodoacetic acid using dicyclohexylcarbodiimide followed by chromatographic separation giving the individual iodoacetates **209a** and **209b** and **209c**, and displacement of the iodide with mPEG-SH in a mixture of acetonitrile and 0.1 *M* aqueous sodium hydrogen carbonate (Scheme 55) (*138*). The half-life of these conjugates in phosphate buffer (pH 7.4, 37°C) was *ca*. 20 h, showing acceptable stability; in human liver homogenates they decomposed readily with a half-life of 10 min and released the drug tacrolimus (**1**). This indicates their potential as useful water-soluble prodrugs. The effects of the derivative **209d** on the proliferation of T-cells, B-cells and mast cells, and on IgG production in human B-cells, and histamine release, were investigated *in vitro* (*139*). Further, *in vivo*



Scheme 55. Water-soluble prodrugs of tacrolimus **209d**, **209e**, **209f**, featuring methoxy-poleyethyleneglycol units attached through an ester linkage, a rhamnose derivative of ascomycin (**210**), and tacrolimus-C-6-dextran conjugate (**211**)

studies were performed on skin graft rejection in mice and in *Freund*'s adjuvant arthritis in rats. The results demonstrated that the mPEG derivative **209d** and tacrolimus have similar effects both *in vitro* an *in vivo*, thus indicating the former to be a useful prodrug with its advantageous physicochemical properties for preparing formulations for different applications.

With a view to developing a macromolecular prodrug of tacrolimus (**1**) with modified pharmacokinetic properties for systemic administration, the dextran conjugate **211** has been prepared (Scheme 55) (*140*). Thus, dextran (T-70) was O-alkylated with 6-bromohexanoic acid to give carboxy-*n*-pentyl-dextran (C6D); the free carboxylic acid groups were then modified through coupling with ethylene diamine to give C6D-ED. The free amino groups were in turn coupled through an amide bond to FK506-33-*O*-hemisuccinate, affording the conjugate **211**. The conjugate was estimated to contain 0.45% of tacrolimus (**1**) and the coupling molar ratio was approximately 1:1 (dextran:tacrolimus) (*141*). After incubation of the conjugate **211** in phosphate buffer (pH 7.4), tacrolimus was released and the half-life of the conjugate was 150 h. The *in vitro* immunosuppressive activity was reported to be almost comparable to that of free tacrolimus. *In vivo* biodistribution studies demonstrated that conjugation dramatically changes the pharmacokinetic properties of tacrolimus. After i.v. injection in rats, the AUC of the conjugate was reported to be almost 2000 times higher than that of free tacrolimus and organ uptake and clearances of the conjugate were significantly smaller than those of the free drug. However, no further activities have been reported.

Ascomycin (**2**) can be glycosylated at C-33−OH selectively using triacetyl-L-rhamnosyl bromide giving a 17% yield of the derivative **210** (Scheme 55) (*142*). Compound **210** showed good efficacy in *in vitro*, and in animal models including rat heart allograft rejection and rat adjuvant arthritis, and indicated a lower potential for neurotoxicity compared to tacrolimus.

Tacrolimus has been linked at C-33−OH through an ester or carbamate bond to several polymers for the purpose of affinity chromatography (*143*, *144*). Finally, several total syntheses (*145*–*155*) of tacrolimus and ascomycin, and also segments thereof, have been reported, and biosynthetic pathways discussed (*156*).

3. Summary

The discovery of the potent anti-inflammatory activity of the natural compounds ascomycin and tacrolimus in a new animal model of skin inflammation provided the first pharmacological evidence that calcineurin inhibitors of this type may represent a novel class for topical treatment of inflammatory skin diseases. A subsequent extensive medicinal chemistry effort finally resulted in the selection of the ascomycin derivative pimecrolimus for development, due to its favorable pharmacology and safety profile. Since 2001/2 the new class of topical calcineurin inhibitors represented by pimecrolimus cream and tacrolimus ointment has become

the first and only alternative to topical corticosteroids for treatment of inflammatory skin diseases such as atopic dermatitis.

Biological properties and clinical data of pimecrolimus are summarized. This compound differs from corticosteroids by selective action on T- and mast cells only, and by the lack of induction of skin atrophy and by less permeation through the skin. Pimecrolinus differs from other calcineurin inhibitors by specifically targeting skin inflammation, by a lower potential to affect systemic immune responses, and by its favorable skin penetration properties. Pimecrolimus cream 1% (Elidel®) has proven to be well tolerated, safe, and highly effective in extensive clinical studies in patients with atopic dermatitis and other inflammatory skin diseases.

Medicinal chemistry efforts aiming at understanding the structure-activity relationships required a detailed study of the chemical properties of the highly complex macrolactam structure of ascomycin and its derivatives, and elaboration and establishment of methodologies for selective transformations in various regions of the molecule. This chapter summarizes for the first time the chemical investigations in our laboratories and discusses the work published by others in this field as well. Furthermore, certain structure-activity relationships are described qualitatively.

Ascomycin (**2**) and tacrolimus (**1**) both exist as a mixture of several isomers in the macrophilin-binding region. Potential equilibrium products including the "furano-ascomycins" **13a**, **13b**, **13c**, and **13d** have been synthesized. The tricarbonyl moiety is highly sensitive and undergoes a benzilic acid-type of rearrangement, giving two diastereomers through two different pathways depending on the reaction conditions. Based on this, protocols were developed for the synthesis of labeled ascomycins. A new class of derivatives termed "cyclo-ascomycins" **44–47**, arising through cyclization in the binding domain, has been synthesized. In addition to other selective transformations in the binding region, photochemical investigations and further elaboration of the intermediates led to the biologically active 5,6-dehydro-ascomycin **99** and several other analogues. Using cyanide or 9-BBN, selective cleavage of C-1−C-9 was accomplished and semisynthetic strategies starting from the resulting fragment **122** led to the new analogues **127–130** featuring new amino acids. In the effector side of the molecule, several selective transformations such as allylic oxidation on C-18, epimerization of C-21, enumeration of the C-21−allyl side chain through *Grubbs*' cross metathesis reactions, and dehydration of C-24−OH leading to the enone 23,24-dehydroascomycin (**134**) were achieved. The protected enone **144** could be further transformed through selective addition reactions. Whereas the 21-*epi* derivative **133** did not show useful activities, 24-desoxyascomycin (**135**) was as active as ascomycin (**2**). On the cyclohexyl part of the molecule, demethylations, or introduction of additional hydroxy groups led to the more water-soluble derivatives **158** and **159**. Extensive derivatization of the C-33−OH has been achieved. Noteworthy is the ring contraction leading to SDZ 281–240, which was the first topical calcineurin inhibitor to demonstrate clinical proof of concept in patients with inflammatory skin disease. The cyclohexyl-methylidene group was cleaved off and new moieties could be

incorporated instead. Furthermore, *iso*-tacrolimus (**197a**), a ring-contracted derivative, also isolated from the fermentation broths, has been synthesized and its further chemistry summarized.

In conclusion, this chapter summarizes the extensive chemistry and biology studies on a natural product, which have resulted in a novel therapy approved worldwide. This helps underscore the importance of natural products as a versatile source of novel structures with unique biological activities.

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