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Published on Web 03/06/2003

Sanglifehrin−Cyclophilin Interaction: Degradation Work, Synthetic Macrocyclic Analogues, X-ray Crystal Structure, and Binding Data

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Abstract: Sanglifehrin A (SFA) is a novel immunosuppressive natural product isolated from *Streptomyces sp.* A92-308110. SFA has a very strong affinity for cyclophilin A ($IC_{50} = 6.9 \pm 0.9$ nM) but is structurally different from cyclosporin A (CsA) and exerts its immunosuppressive activity via a novel mechanism. SFA has a complex molecular structure consisting of a 22-membered macrocycle, bearing in position 23 a nine-carbon tether terminated by a highly substituted spirobicyclic moiety. Selective oxidative cleavage of the C_{26}═C_{27} exocyclic double bond affords the spirolactam containing fragment **1** and macrolide **2**. The affinity of **2** for cyclophilin ($IC_{50} = 29 \pm 2.1$ nM) is essentially identical to SFA, which indicates that the interaction between SFA and cyclophilin A is mediated exclusively by the macrocyclic portion of the molecule. This observation was confirmed by the X-ray crystal structure resolved at 2.1 Å of cyclophilin A complexed to macrolide **16**, a close analogue of **2**. The X-ray crystal structure showed that macrolide **16** binds to the same deep hydrophobic pocket of cyclophilin A as CsA. Additional valuable details of the structure−activity relationship were obtained by two different chemical approaches: (1) degradation work on macrolide **2** or (2) synthesis of a library of macrolide analogues using the ring-closing metathesis reaction as the key step. Altogether, it appears that the complex macrocyclic fragment of SFA is a highly optimized combination of multiple functionalities including an (*E*,*E*)-diene, a short polypropionate fragment, and an unusual tripeptide unit, which together provide an extremely strong affinity for cyclophilin A.

Introduction

More than two decades ago, the discovery of cyclosporin A (CsA, Figure 1) allowed a spectacular progress in the field of organ transplantation.[1] Since then, the number of transplanted organs has grown continuously and the search for novel immunosuppressants has intensified.[2] These efforts resulted in the isolation of several immunosuppressants from natural sources which interfere with T cell activation or proliferation. The most prominent examples are FK506 and rapamycin (Figure 1). Besides their important therapeutic use, these drugs have also proven to be powerful tools for dissecting signal transduction pathways at the molecular level.[3] It has been shown that the biological activity of CsA, FK506, and rapamycin is mediated by intracellular binding proteins called immunophilins. CsA binds to cyclophilins (CyP), whereas FK506 and rapamycin bind to FK506-binding proteins (FKBP) (Figure 2). However, although immunophilin binding is required, it is not sufficient for the immunosuppressive activity of these drugs. Biological effects are observed only upon interaction of the drug/immunophilin complexes with a third partner, the effector protein. Thus, the CyP−CsA and FKBP−FK506 complexes inhibit the serine/threonine phosphatase activity of calcineurin, thereby blocking the production of cytokines including interleukin-2.[4] On the other hand, the FKBP−rapamycin complex inhibits a kinase called FRAP (also known as RAFT or mTOR)[5] that is involved in interleukin-2 receptor-mediated T cell proliferation.[6]

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(1) For a review on cyclosporin, see: Kallen, J.; Mikol, V.; Quesniaux, V. F. J.; Walkinshaw, M. D.; Schneider-Scherzer, E.; Schörgendorfer, K.; Weber, G.; Fliri, H. G. In *Biotechnology, Vol. 7*; Kleinkauf, H., von Döhren, H., Eds.; VCH: Weinheim, Germany, 1997; Chapter 12, pp 535−591.
(2) (a) Dumont, F. J. *Exp. Opin. Ther. Patents* **2001**, *11*, 377−404. (b) *Perspectives in Drug Discovery and Design, Vol. 2*; Anderson, P. S., Kenyon, G. L., Marshall, G. R., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1994; pp 3−248.
(3) (a) Schreiber, S. L.; Albers, M. W.; Brown, E. J. *Acc. Chem. Res.* **1993**, *26*, 412−420. (b) Schreiber, S. L.; Crabtree, G. R. *Immunol. Today* **1992**, *13*, 136−142.
(4) (a) Liu, J.; Farmer, J. D., Jr.; Lane, W. S.; Friedman, J.; Weissman, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807−815. (b) Friedman, J.; Weissman, I. *Cell* **1991**, *66*, 799−806.

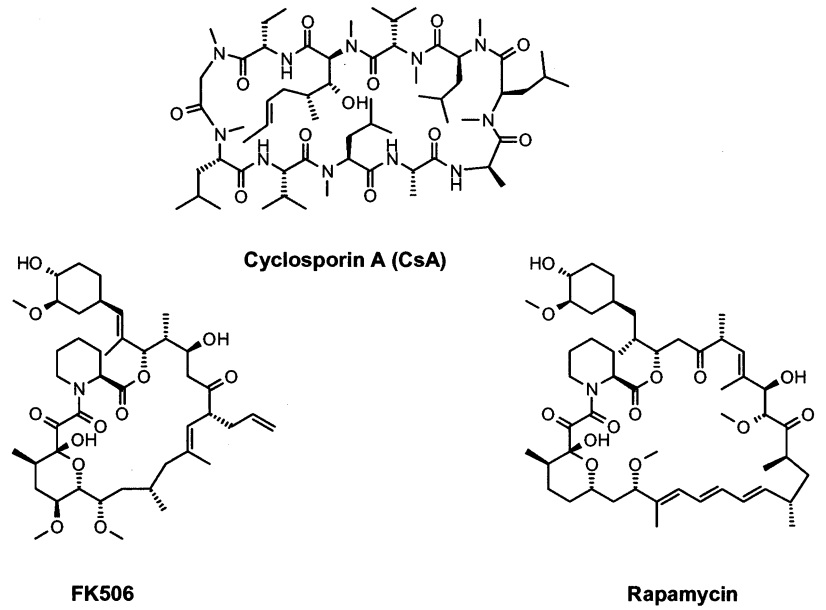


Figure 1. Molecular structures of the immunosuppressive natural products interfering with T cell signaling pathways: cyclosporin A, FK506, and rapamycin.

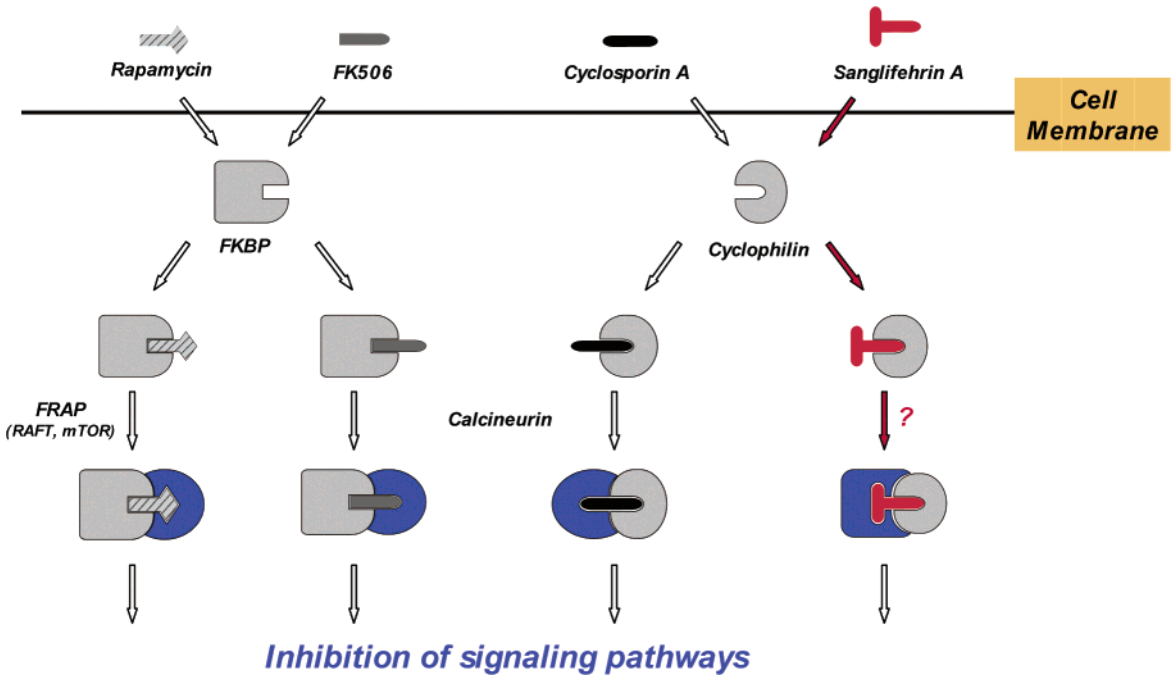


Figure 2. Mode of action of the immunosuppressants and the discovery of the sanglifehrins.

Considering that different ligands for FKBP exert their biological effects by different mechanisms, we wondered whether besides CsA other ligands of cyclophilin might exist and whether these ligands would exhibit different modes of action than CsA. Screening of microbial broth extracts for cyclophilin-binding substances led to the isolation from *Streptomyces sp.* A92-308110 of a new class of compounds named sanglifehrins.[7] Among the 20 different sanglifehrins isolated so far, sanglifehrin A (SFA) is the most abundant component (Scheme 1). The affinity of SFA for cyclophilin in a cell-free competitive binding assay is remarkably high (IC_{50} = 6.9 ± 0.9 nM),[8] approximately 60-fold higher than that of CsA (IC_{50} = 420 ± 56 nM). SFA displays potent immunosuppressive activity in the murine mixed lymphocyte reaction (IC_{50} = 170 ± 30 nM), an in vitro immune response assay. The details of the mechanism by which this compound exerts its immunosup-

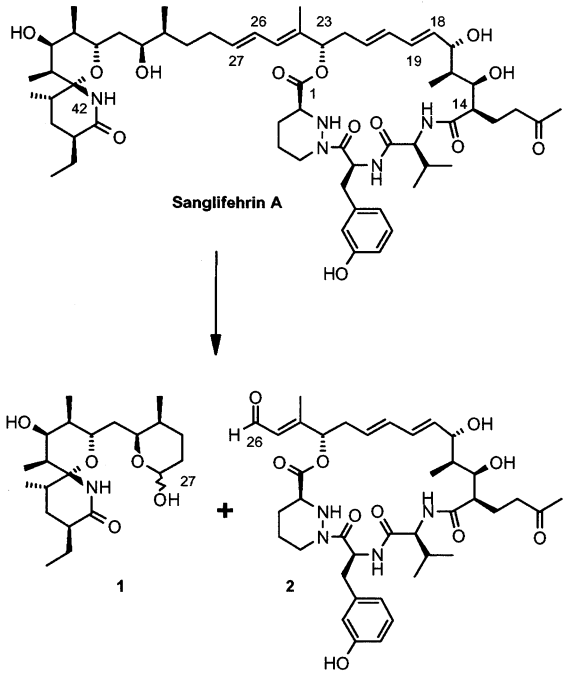
(5) (a) Brown, E. J.; Albers, M. W.; Shin, T. B.; Ichikawa, K.; Keith, C. T.; Lane, W. S.; Schreiber, S. L. *Nature* **1994**, *369*, 756−758. (b) Sabers, C. J.; Martin, M. M.; Brunn, G. J.; Williams, J. M.; Dumont, F. J.; Wiederrecht, G.; Abraham, R. T. *J. Biol. Chem.* **1995**, *270*, 815−822. (c) Sabatini, D. M.; Erdjument Bromage, H.; Lui, M.; Tempst, P.; Snyder, S. H. *Cell* **1994**, *78*, 35−43.

(6) (a) Bierer, B. E.; Mattila, P. S.; Standaert, R. F.; Herzenberg, L. A.; Burakoff, S. J.; Crabtree, G.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9231−9235. (b) Dumont, F. J.; Staruch, M. J.; Koprak, S. L.; Melino, M. R.; Sigal, N. H. *J. Immunol.* **1990**, *144*, 251−258.

(7) (a) Sanglier, J.-J.; Quesniaux, V.; Fehr, T.; Hofmann, H.; Mahnke, M.; Memmert, K.; Schuler, W.; Zenke, G.; Gschwind, L.; Maurer, C.; Schilling, W. *J. Antibiot.* **1999**, *52*, 466−473. (b) Fehr, T.; Kallen, J.; Oberer, L.; Sanglier, J.-J.; Schilling, W. *J. Antibiot.* **1999**, *52*, 474−479.

(8) Zenke, G.; Strittmatter, U.; Fuchs, S.; Quesniaux, V. F. J.; Brinkmann, V.; Schuler, W.; Zurini, M.; Enz, A.; Billich, A.; Sanglier, J.-J.; Fehr, T. *J. Immunol.* **2001**, *166*, 7165−7171.

Scheme 1. Structure of Sanglifehrin A and Oxidative Cleavage of the C_{26}═C_{27} Bond[13]



pressive activity at the molecular level is currently under investigation, and the first results were published recently.[9] These data clearly indicate that SFA represents a novel type of immunosuppressant whose mode of action is different from that of all other known immunophilin-binding compounds, namely CsA, FK506, and rapamycin.

In addition to its fascinating biological activity, sanglifehrin A has a complex and unique molecular structure. The compound consists of a 22-membered macrocycle, bearing in position 23 a nine-carbon tether terminated by a highly substituted spirobicyclic moiety. The macrolide contains an (*E*,*E*)-diene, a short polypropionate fragment, and a tripeptide unit composed of valine and two rather unusual amino acids, piperazic acid and *meta*-tyrosine. Interestingly, it is the β-nitrogen of piperazic acid that is involved in the amide bond formation. This stands in contrast to all other piperazic acid containing natural products isolated so far. The structural attractiveness of sanglifehrin, combined with its immunosuppressive activity, generated broad interest in industrial and academic laboratories. Their efforts resulted in the preparation of several fragments[10] and finally culminated in the total syntheses by Nicolaou et al.[11] and Paquette et al.[12]

Despite the structural complexity of SFA, selective cleavage of the C_{26}═C_{27} exocyclic double bond could be achieved by a two-step procedure (Sharpless asymmetric dihydroxylation, periodate oxidation) (Scheme 1).[13] This approach allowed an efficient preparation of the spirolactam containing fragment **1** and macrolide **2**. Fragment **1** by itself does not bind to cyclophilin ($IC_{50} \geq 5000$ nM). On the other hand, the removal of the $C_{27}-N_{42}$ subunit has only a minor effect on the affinity of macrolide **2** for cyclophilin ($IC_{50} = 29 \pm 2.1$ nM). But surprisingly, neither of the two fragments exhibits immunosuppressive activity. Based on these initial observations, we initiated a two-phase synthetic effort. First, we aimed at further reducing the complexity of the macrocycle while maintaining its affinity for cyclophilin. During this phase, particular emphasis was put on reducing the overall polarity of the molecule. Indeed, it is believed that the high number of polar functional groups of the sanglifehrin class of substances is responsible for their poor cell permeation.[14] In the second phase, we planned to recombine suitable macrolides with various side chains in order to restore the immunosuppressive activity.[15]

Herein, we will discuss in detail our findings related to the interaction between the macrolide fragment of sanglifehrin A and cyclophilin. An overview of the structure−activity relationship (SAR) was obtained either by degradation work on **2** or by synthesis of a library of macrolide analogues based on the ring-closing metathesis reaction. In addition, we disclose the first X-ray crystal structure of a sanglifehrin derivative, macrolide **16**, bound to cyclophilin A.

Chemistry

Degradation Work on Macrolide 2. The syntheses of the new derivatives are summarized in Schemes 2 and 3. To assess the role of the ketone in the side chain attached at C_{14}, sanglifehrin A was reduced with $NaBH_4$. The resulting mixture of epimeric alcohols could be selectively converted into the epimeric iodo derivatives **3**, which were not separated and were obtained in 48% yield over two steps. The other hydroxy groups in the molecule did not react under these conditions, most probably due to steric hindrance. Compound **3** could be selectively dihydroxylated at the C_{26}═C_{27} double bond, and the resulting diol could be oxidatively cleaved under the same conditions used for sanglifehrin A to afford macrolide **4**.

We then concentrated our efforts on modifying the conjugated $C_{18}-C_{21}$ (*E*,*E*)-diene system of the macrocyclic fragment. Hydrogenation of **2** in the presence of Pd/C resulted in reduction of both the $C_{18}-C_{21}$ (*E*,*E*)-diene system and the C_{24}═C_{25} exocyclic double bond, affording the fully saturated macrocycle **5** as an epimeric mixture at C_{24}. Selective hydrogenation of the (*E*,*E*)-diene system only could not be achieved under a variety of conditions. The lack of selectivity during the hydrogenation of macrolide **2** might be associated with the electron-withdrawing effect of the aldehyde at C_{26}. In the case of sanglifehrin A, it is indeed possible to obtain selectively the $C_{18}-C_{21}$ tetrahydro derivative using Wilkinson's rhodium catalyst.

The reduction of both carbonyl groups of **2** could be achieved in quantitative yield using $NaBH_4$ to afford the tetraol **6**. Under acidic conditions (aqueous HCl, dioxane), **6** was converted into

(9) (a) Zhang, L.-H.; Liu, J. O. *J. Immunol.* **2001**, *166*, 5611−5618. (b) Zhang, L.-H.; Youn, H.-D.; Liu, J. O. *J. Biol. Chem.* **2001**, *276*, 43534−43540.

(10) (a) Bänteli, R.; Brun, I.; Hall, P.; Metternich, R. *Tetrahedron Lett.* **1999**, *40*, 2109−2112. (b) Hall, P.; Brun, I.; Denni, D.; Metternich, R. *Synlett* **2000**, *3*, 315−318. (c) Nicolaou, K. C.; Ohshima, T.; Murphy, F.; Barluenga, S.; Xu, J.; Winssinger, N. *Chem. Commun.* **1999**, *9*, 809−810. (d) Paquette, L. A.; Konetzki, I.; Duan, M. *Tetrahedron Lett.* **1999**, *40*, 7441−7444. (e) Duan, M.; Paquette, L. A. *Tetrahedron Lett.* **2000**, *41*, 3789−3792. (f) Gurjar, M. K.; Chaudhuri, S. R. *Tetrahedron Lett.* **2002**, *43*, 2435−2438.

(11) (a) Nicolaou, K. C.; Xu, J.; Murphy, F.; Barluenga, S.; Baudoin, O.; Wei, H.-X.; Gray, D. L. F.; Ohshima, T. *Angew. Chem., Int. Ed.* **1999**, *38*, 2447−2451. (b) Nicolaou, K. C.; Murphy, F.; Barluenga, S.; Ohshima, T.; Wei, H.; Xu, J.; Gray, D. L. F.; Baudoin, O. *J. Am. Chem. Soc.* **2000**, *122*, 3830−3838.

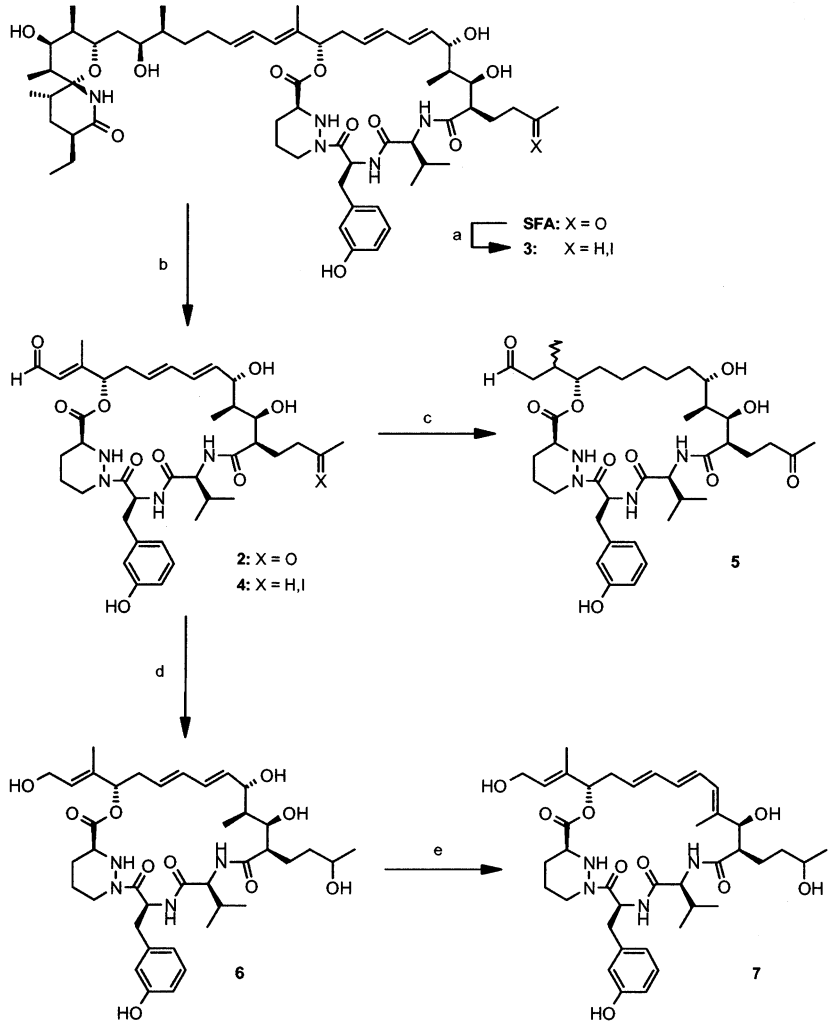
(12) Duan, M.; Paquette, L. A. *Angew. Chem., Int. Ed.* **2001**, *40*, 3632−3636. Paquette, L. A.; Duan, M.; Konetzki, I.; Kempmann, C. *J. Am. Chem. Soc.* **2002**, *124*, 4257−4270.

(13) Metternich, R.; Denni, D.; Thai, B.; Sedrani, R. *J. Org. Chem.* **1999**, *64*, 9632−9639.

(14) Bänteli, R.; Wagner, J.; Zenke, G. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1609−1612.

(15) The results of the second part of the program will be disclosed in a separate publication.

Scheme 2. Degradation Studies on Sanglifehrin A[a]



[a] Reagents and conditions: (a) (i) $NaBH_4$, MeOH, 0 °C, (ii) PPh_3, imidazole, I_2, CH_2Cl_2, 0 °C to rt, 48% (two steps); (b) (i) $(DHQ)_2PHAL$, OsO_4, $CH_3SO_2NH_2$, $K_3Fe(CN)_6$, K_2CO_3, *t*-BuOH/H_2O 1:1, (ii) $NaIO_4$, THF/H_2O 2:1, 32% for **4** (two steps), see ref 13 for the preparation of **2**; (c) Pd/C 10%, H_2, EtOH, 59%; (d) $NaBH_4$, MeOH, 0 °C, quantitative; (e) 2.0 M aqueous HCl, dioxane, 30%.

the conjugated (*E*,*E*,*E*)-triene **7** by elimination of the allylic alcohol at C_{17}. The fully conjugated tetraene, which could potentially be generated by the additional elimination of the alcohol at C_{15} of **7** was not detected even after prolonged acidic treatment. Triene **7** proved to be unstable even when stored at −4 °C.

We next focused our attention on modifying the potentially reactive α,β-unsaturated aldehyde (Scheme 3). The reaction of macrolide **2** with phosphonoacetates under modified Wittig−Horner conditions provided the esters **8** and **9** in 50% and 34% yield, respectively. In this case, no protection of the other functionalities in the macrolide was required. But for further derivation of the aldehyde under strongly basic conditions, the keto-diol was transformed quantitatively into the ketal **10** under mild acidic conditions. For the olefination of the aldehyde with aliphatic groups, the procedure developed by Sylvestre Julia et al.[16] and later modified by Kocienski et al.[17] was applied to **10** as described previously for the resynthesis of sanglifehrin A from degradation products.[13] The desired 5-(alkane-1-sulfonyl)-1-phenyl-1*H*-tetrazoles were prepared from the commercially available primary alcohols in two steps according to the procedure outlined by Kocienski et al.[18] In the presence of 2 equiv. of NaHMDS, the olefination procedure afforded the ketal-protected forms of dienes **11** and **12** in 74% and 49% yield, respectively. In both cases, the (*E*,*E*)-diene products were formed with very high selectivity (≥10:1). Acidic hydrolysis of the intramolecular ketal afforded the desired macrolides **11** and **12**. Typically, the latter reaction reached an equilibrium at 50% conversion.[13] Finally, the reaction between the macrolide and an unstabilized Wittig reagent required an additional protection of the phenol with a *tert*-butyldimethylsilyl group. The fully protected aldehyde **13** was conveniently obtained using *N*-(*tert*-

(16) (a) Baudin, J. B.; Hareau, G.; Julia, S. A.; Ruel, O. *Tetrahedron Lett*. **1991**, *32*, 1175−1178. (b) Baudin, J. B.; Hareau, G.; Julia, S. A.; Ruel, O. *Bull. Soc. Chim. Fr*. **1993**, *130*, 336−357. (c) Baudin, J. B.; Hareau, G.; Julia, S. A.; Lorne, R.; Ruel, O. *Bull. Soc. Chim. Fr*. **1993**, *130*, 856−878.

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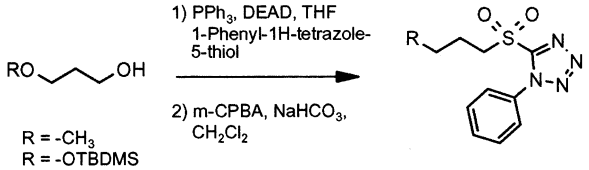
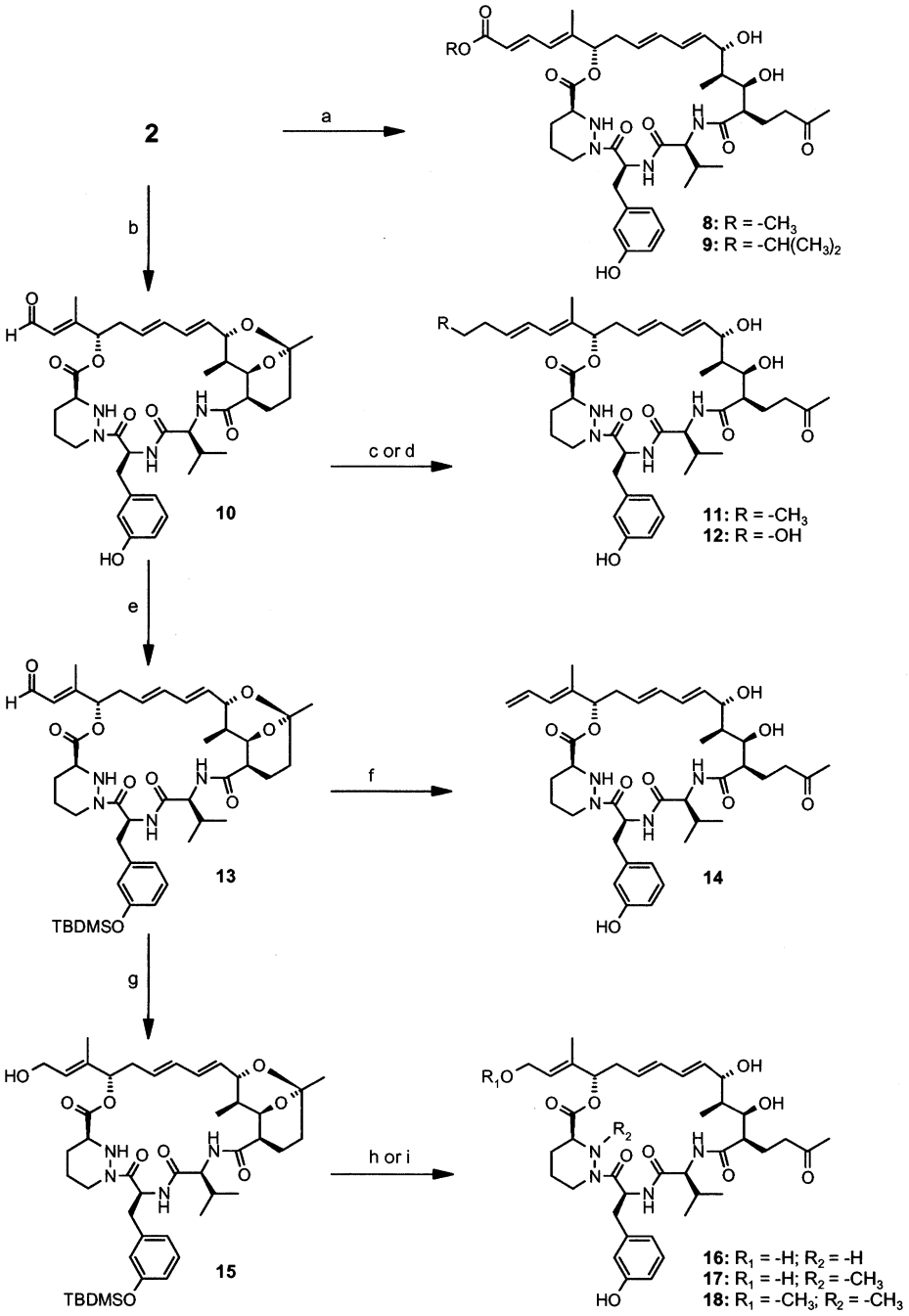
(18) The sulfones were prepared according to following scheme:



Scheme 3. Derivation of the Aldehyde in Macrolide **2**[a]



[a] Reagents and conditions: (a) $RO_2CCH_2PO(OCH_3)_2$, K_2CO_3, CH_3CN, 50% for **8**, 34% for **9**; (b) HF·pyridine, CH_3CN, 0 °C, 92%; (c) (i) 5-(butane-1-sulfonyl)-1-phenyl-1*H*-tetrazole, NaHMDS, DME, −78 °C, (ii) TsOH, $B(OH)_3$, THF/H_2O 1:1, 15% for **11** (two steps); d) (i) 5-[3-(*tert*-butyldimethylsilyloxy)propane-1-sulfonyl)]-1-phenyl-1*H*-tetrazole, NaHMDS, DME, −78 °C, (ii) TBAF, THF, (iii) TsOH, $B(OH)_3$, THF/H_2O 1:1, 7% for **12** (three steps); (e) MTBSTFA, CH_3CN, 50 °C, 88%; (f) (i) Methyltriphenylphosphonium bromide, LiHMDS, THF, −78 °C, (ii) TBAF, THF, (iii) TsOH, THF/H_2O 1:1, 6% (three steps); (g) $NaBH_4$, MeOH, 0 °C, 90%; (h) (i) TBAF, THF, (ii) TsOH, THF/H_2O 1:1, 15% for **16** (two steps); (*i*) (i) $CF_3SO_3CH_3$, 2,6-di-*tert*-butylpyridine, toluene, (ii) TBAF, THF, (iii) TsOH, THF/H_2O 1:1, 8% for **17**, 7.5% for **18** (three steps).

butyldimethylsilyl)-*N*-methyltrifluoroacetamide as the silylating agent. Aldehyde **13** was then allowed to react with methyltriphenylphosphonium bromide in the presence of LiHMDS at −78 °C to afford the fully protected form of **14** in 39% yield. The latter was sequentially deprotected using tetrabutylammonium fluoride followed by standard acidic conditions.

The primary alcohol **15** was obtained in excellent yield by reduction of aldehyde **13** with $NaBH_4$. Complete deprotection of **15** led to alcohol **16**, which was used for cocrystallization with cyclophilin A (see X-ray crystal structure section). Macrolide **15** was also used to modify the free α-nitrogen of piperazic acid and probe its role in mediating the binding to cyclophilin. Methylation of macrolide **15** using trifluoromethanesulfonic acid methyl ester in the presence of a hindered base, 2,6-di-*tert*-butylpyridine, afforded a mixture of di- and monomethylated products, which, upon deprotection, gave **17** and **18**, respectively. Interestingly, while all sanglifehrin derivatives in DMSO solution contain approximatively 20% of the hemiket-

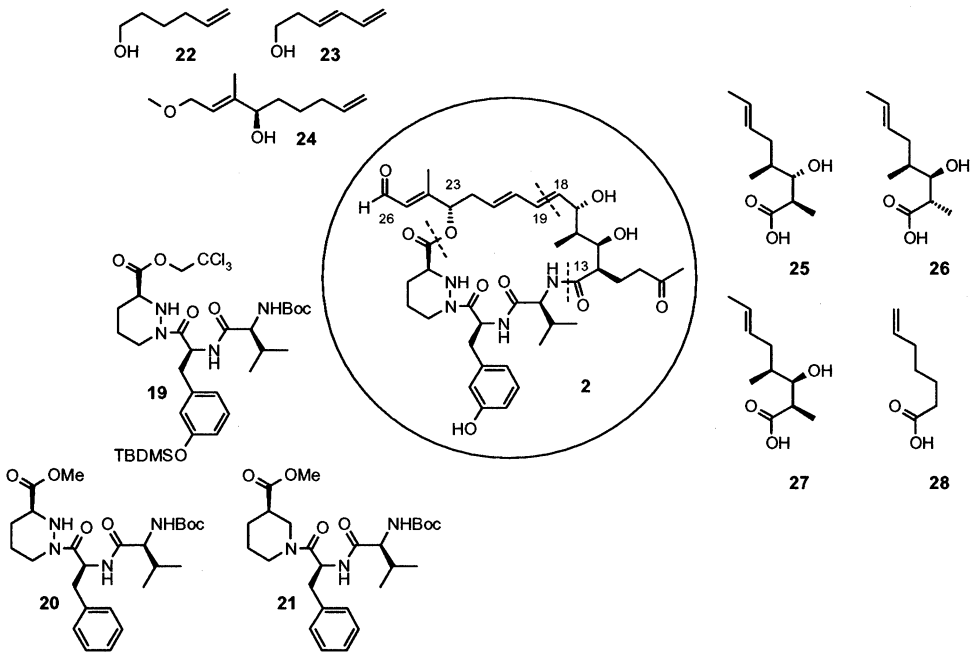


Figure 3. Building blocks used for the preparation of a library of analogues of the sanglifehrin macrolide.

al form resulting from addition of the C_{15} hydroxyl to the carbonyl in the side chain,[13] the content of the hemiketal form of **17** and **18** was increased to ~50%.

Design and Construction of a Library of Macrolide Analogues. Further manipulation of the functional groups present in macrolide **2** was difficult. Therefore, we switched to a fully synthetic approach to prepare a library of simplified macrolides.[19] For our first generation of analogues, we planned to keep the tripeptide fragment, which forms all the hydrogen bonds with cyclophilin, mostly intact. In addition, at least one double bond was maintained in the $C_{13}-C_{23}$ subunit to reduce the conformational flexibility of the 22-membered macrocycle. With this basic scaffold in mind, a retrosynthetic analysis showed that several routes can be considered for the construction of the macrocycles: macrolactonization, macrolactamization, Stille coupling, and ring-closing metathesis (RCM). The latter approach was particularly attractive because the macrolides can be rapidly assembled from relatively simple building blocks in a highly convergent manner.

For the selection of the building blocks, the macrolide was divided into three fragments: the tripeptide, the polypropionate fragment $C_{13}-C_{18}$, and the carbon skeleton extending from C_{19} to C_{26}. An overview of the 10 selected building blocks is shown in Figure 3.

Three different protected tripeptide fragments were prepared. Tripeptide **19** corresponds to the original motive found in the sanglifehrins. In the two other tripeptides, **20** and **21**, the unusual amino acids *meta*-tyrosine and piperazic acid were replaced by phenylalanine and/or nipecotic acid, respectively. These modifications were introduced to investigate the influence of the phenolic hydroxyl and of the α-nitrogen of piperazic acid on cyclophilin binding. Three building blocks were considered for the $C_{19}-C_{26}$ fragment. In two of them, **22** and **23**, the side chain attached at C_{23} was neglected, thus eliminating one asymmetric center. The comparison between the final products resulting from alkene **22** and diene **23** would allow us to assess the relative importance of the $C_{18}-C_{21}$ diene. The possible role of the side chain attached at C_{23} in stabilizing the 3-dimensional conformation of the macrolide could be tested using fragment **24**. Finally, four fragments were prepared as analogues of the polypropionate portion extending from C_{13} to C_{18}. Based on the results obtained by degradation of macrolide **2**, the hydroxy group at C_{17} was not considered during the planning phase. For similar reasons, the side chain attached at C_{14} was simplified to a methyl group. The stereochemistry of the methyl group at C_{16} was maintained in fragments **25**−**27**, because this substituent, directed toward the internal face of the macrolide (see discussion of the X-ray crystal structure), was thought to play an important role in stabilizing the three-dimensional structure of the macrolide. Fragments **25**−**27** were synthesized in order to analyze the influence of the stereochemistry at C_{14} and C_{15} on the affinity of the macrolides for cyclophilin. Fragment **27** corresponds to the original stereochemistry found in these positions for SFA. Commercially available 6-heptenoic acid **28** was added to the list in order to study the overall impact of the substituents of the polypropionate fragment on cyclophilin binding.

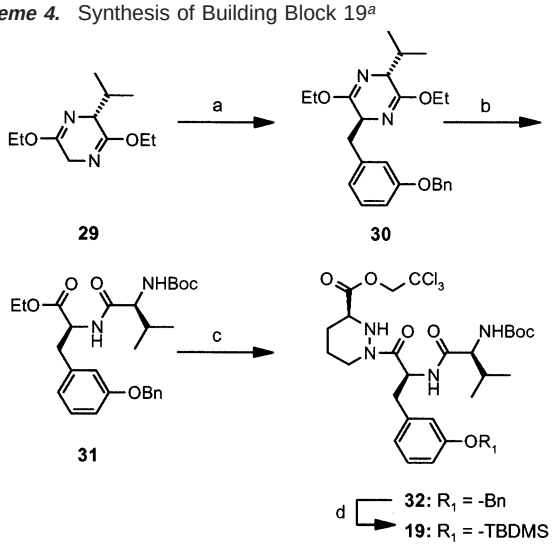
The details of the synthesis of the tripeptide unit **19** are described in Scheme 4.[19,20] The selection of the three orthogonal protecting groups (Tce, TBDMS, and Boc) was crucial to allow the desired synthetic flexibility during the final stage of macrolide assemblage. The synthesis of the tripeptide fragment **19** began with the preparation of benzyl-protected *meta*-tyrosine in a chiral form. The alkylation of the bis(lactim) ether (Schöllkopf auxiliary) **29** with 3-benzyloxybenzyl bromide provided intermediate **30** with 89% diastereoselectivity.[21] The undesired isomer was readily separated by flash chromatogra-

(19) For a preliminary communication on this appraoch, see: Martin Cabrejas, L. M.; Rohrbach, S.; Wagner, D.; Kallen, J.; Zenke, G.; Wagner, J. *Angew. Chem., Int. Ed.* **1999**, *38*, 2443−2446.

(20) The syntheses of the tripeptides **20** and **21** were described previously: Wagner, J.; Martin Cabrejas, L. M.; Grossmith, C. E.; Papageorgiou, C.; Senia, F.; Wagner, D.; France, J.; Nolan, S. P. *J. Org. Chem.* **2000**, *65*, 9255−9260.

(21) Schöllkopf, U.; Groth, U.; Deng, C. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 798−799.

Scheme 4. Synthesis of Building Block 19[a]

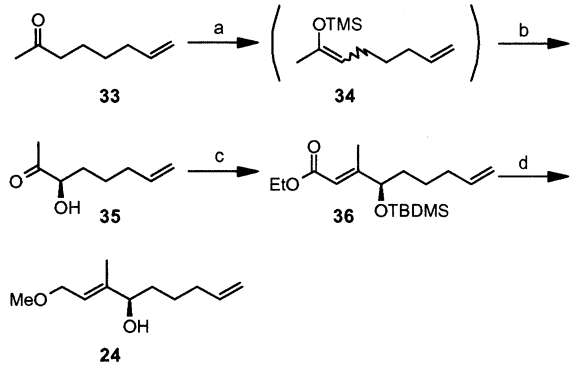


[a] Reagents and conditions: (a) *n*-BuLi, 3-benzyloxybenzylbromide, THF, −78 °C, 62%; (b) (i) 0.5 M HCl, CH_3CN, (ii) L-Boc-Val-OH, NMM, isobutylchloroformate, THF, −15 °C to rt, 50% (two steps); (c) (i) LiOH, THF/H_2O 3:1, (ii) $H_2N-Pip-OCH_2CCl_3$, NMM, isobutylchloroformate, THF, −15 °C to rt, 62% (two steps); (d) (i) Pd/C 10%, H_2, *i*-PrOH, (ii) TBDMSCl, imidazole, DMF, 40 °C, 95% (two steps).

phy. The bis(lactim) ether **30** was cleaved under strongly acidic conditions, and the resulting amine was coupled to Boc-protected valine to afford the dipeptide **31** in 50% yield over two steps. The latter was saponified and coupled to 2,2,2-trichloroethyl-protected piperazic acid using the mixed anhydride method to provide tripeptide **32**. Optically pure piperazic acid was obtained in multigram quantities by the procedure described by Hale et al.[22] The benzyl group of **32** was removed by hydrogenolysis in 2-propanol and replaced by the TBDMS group to afford the desired fragment **19**. Use of a less hindered alcohol during the debenzylation resulted in partial transesterification of the trichloroethyl ester. Tripeptide **19** was thus prepared in seven steps and 18% overall yield.

5-Hexen-1-ol **22** is commercially available, and 3,5-hexadien-1-ol **23** was prepared according to published procedures.[23] The synthesis of optically pure **24** is described in Scheme 5.[24] 7-Octen-2-one **33** was prepared according to known procedures on a multigram scale.[25] Sharpless dihydroxylation of the intermediate silylenolether **34** afforded the highly volatile α-hydroxy ketone **35** in 27% yield from **33**. The reaction proceeded in 89% ee as determined by ^{19}F NMR of the Mosher ester derivative of **35**. Protection of **35** with a *tert*-butyldimethylsilyl group followed by a Wittig−Horner reaction with triethyl phosphonoacetate in the presence of sodium hydride afforded the ester **36** in good yield. The ester was reduced with DIBAL-H to the primary alcohol, which was methylated. Final deprotection of the secondary alcohol afforded the desired

Scheme 5. Synthesis of Building Block **24**[a]



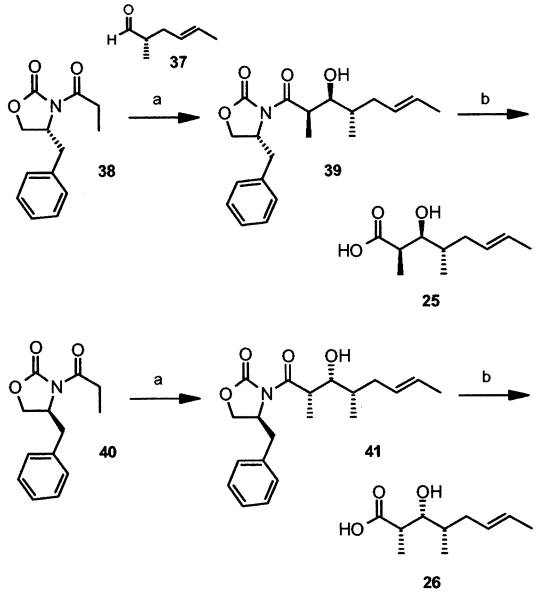
[a] Reagents and conditions: (a) TMSI, HMDS, pentane, −20 °C to rt, 48%; (b) AD-mix-*β*, *t*-BuOH/H_2O 1:1, 0 °C to rt, 89% ee, 56%; (c) (i) TBDMSCl, imidazole, DMF, 0 °C to rt, (ii) NaH, triethylphosphonoacetate, toluene, rt to 60 °C, 59% (two steps); (d) (i) DIBAL-H, CH_2Cl_2, 0 °C to rt, (ii) NaH, MeI, THF, 0 °C to rt, (iii) TBAF, THF, 0 °C to rt, 69% (three steps).

building block **24** in 69% yield over three steps. This synthetic route allowed straightforward access to **24** on a multigram scale.

Finally, we focused our attention on the synthesis of building blocks **25**−**27** for the polypropionate fragment $C_{13}-C_{18}$. The preparation of all three fragments was based on the same chiral aldehyde, (*S*,*E*)-2-methyl-hex-4-enal **37**, which was available in multigram quantities.[26] The corresponding (*R*)-enantiomer of **37** is a crucial intermediate for the synthesis of a unique amino acid, MeBmt, which is part of the structure of cyclosporin A.[27] The additional methyl group attached to the double bond would be lost during the final ring-closing metathesis step. Building blocks **25** and **26** with the *syn* stereochemistry between C_{15} and C_{16} were prepared using Evans' aldol methodology (Scheme 6).[28] The reaction between the (*R*)-imide **38** and aldehyde **37** in the presence of freshly prepared dibutylboron triflate[29] at −78 °C gave the desired aldol adduct **39** as a single diastereoisomer in excellent yield (92%). On the other hand, the reaction of the (*S*)-imide **40** with **37** resulted in a sluggish reaction, which afforded **41** in 43% yield. HPLC analysis showed that **41** contained approximately 10−20% of the C_3 epimer.[30] The slow reaction is probably caused by the mismatch situation between the α-methyl group of the aldehyde and the benzyl of the imide. The epimers could be separated by reverse phase HPLC, but the bulk material was carried through and separated only at the macrolide stage. The chiral auxiliary was removed under standard conditions to afford the acids **25** and **26** in 76% and 64% yield, respectively. While the formation of *syn* aldol adducts is well documented, only few efficient methodologies allowing the formation of *anti* aldol products in high diastereoselectivity and yield have been published. In the case of aldehydes possessing a chiral center in the α position, examples are very scarce. The synthesis of fragment **27**, possessing the

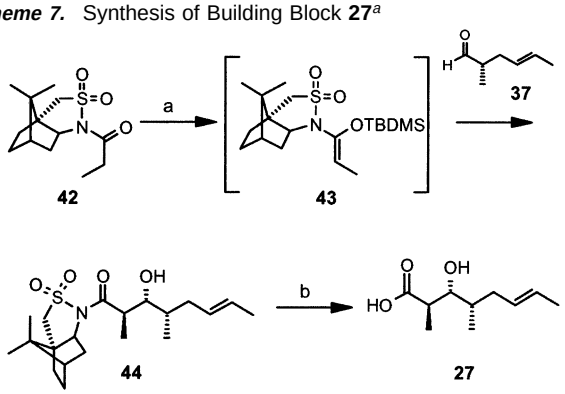
(22) Hale, K. J.; Cai, J.; Delisser, V.; Manaviazar, S.; Peak, S. A.; Bhatia, G. S.; Collins, T. C.; Jogiya, N. *Tetrahedron* **1996**, *52*, 1047−1068.
(23) (a) Stevens, R. V.; Cherpeck, R. E.; Harrison, B. L.; Lai, J.; Lapalme, R. *J. Am. Chem. Soc.* **1976**, *98*, 6317−6321. (b) Hoye, T. R.; Magee, A. S.; Trumper, W. S. *Synth. Commun.* **1982**, *12*, 183−187.
(24) A very short racemic route based on the alkylation of commercially available (*E*)-3-methyl-4-oxo-but-2-enoic acid ethyl ester with the required Grignard reagent was evaluated first. But attempts to resolve the secondary alcohol with chiral auxiliaries, for example, camphanic acid, or separation of the diastereoisomers at the macrolide stage were unsuccessful.
(25) 7-Octen-2-one **33** was prepared by saponification and acidic decarboxylation of the corresponding alkenylacetoacetate. See: (a) Huckin, S. N.; Weller, L. *J. Am. Chem. Soc*. **1974**, *96*, 1082−1087. (b) Crombie, L.; Harper, S. H. *J. Chem. Soc*. **1952**, 869−875.
(26) Deyo, D. T.; Aebi, J. D.; Rich, D. H. *Synthesis* **1988**, *8*, 608−610.
(27) (a) Blaser, D.; Ko, S. Y.; Seebach, D. *J. Org. Chem*. **1991**, *56*, 6230−6233. (b) Togni, A.; Pastor, S. D.; Rihs, G. *Helv. Chim. Acta* **1989**, *72*, 1471−1478. (c) Evans, D. A.; Weber, A. E. *J. Am. Chem. Soc*. **1986**, *108*, 6757−6761.
(28) (a) Evans, D. A.; Dart, M. J.; Duffy, J. L.; Rieger, D. L. *J. Am. Chem. Soc*. **1995**, *117*, 9073−9074 and references therein. (b) For a review on the use of oxazolidinones as chiral auxiliaries, see: Ager, D. J.; Prakash, I.; Schaad, D. R. *Aldrichimica Acta* **1997**, *30*, 3−11.
(29) For the preparation of dibutylboron triflate, see: Evans, D. A.; Nelson, J. V.; Vogel, E.; Taber, T. R. *J. Am. Chem. Soc.* **1981**, *103*, 3099−3111.
(30) Similar fragments in a deuterated form were prepared previously. See: Hailes, H. C.; Handa, S.; Leadlay, P. F.; Lennon, I. C.; Ley, S. V.; Staunton, J. *Tetrahedron Lett*. **1994**, *35*, 315−318.

Scheme 6. Synthesis of Building Blocks **25** and **26**[a]



[a] Reagents and conditions: (a) Et_3N, Bu_2BOTf, (*S*,*E*)-2-methyl-hex-4-enal **37**, CH_2Cl_2, −78 °C, 92% for **39**, 43% for **41**; (b) LiOH, H_2O_2, THF/H_2O 3:1, 0 °C to rt, 76% for **25**, 64% for **26**.

Scheme 7. Synthesis of Building Block **27**[a]



[a] Reagents and conditions: (a) (i) Et_3N, TBDMSOTf, CH_2Cl_2, (ii) (*S*,*E*)-2-methyl-hex-4-enal **37**, $TiCl_4$, CH_2Cl_2, −78 °C, 91%; (b) LiOH, THF/H_2O 3:1, 60 °C, 53%.

same stereochemistry as sanglifehrin, required an *anti* aldol reaction. First, we tested the Heathcock modification of Evans' aldol reaction.[31] Unfortunately, complexation of the additional Lewis acid, Et_2AlCl or $TiCl_4$, to aldehyde **37** caused racemization. We then turned our attention to Oppolzer's sultam as the chiral auxiliary.[32] The condensation of aldehyde **37** with the intermediate silyl enolate **43** obtained from sultam **42** formed the desired *anti* aldol product with excellent diastereoselective control (Scheme 7). Indeed, the *anti* aldol adduct **44** was isolated as a single diastereoisomer and in excellent yield (91%) after flash chromatography. The relative stereochemistry was confirmed by X-ray crystallography (Figure 4). To the best of our knowledge, this is the first example of the formation an *anti* aldol adduct obtained by reacting sultam **42** with an α-methylated *chiral* aldehyde. Cleavage of the chiral auxiliary under standard basic conditions afforded the acid **27** in 53% yield.

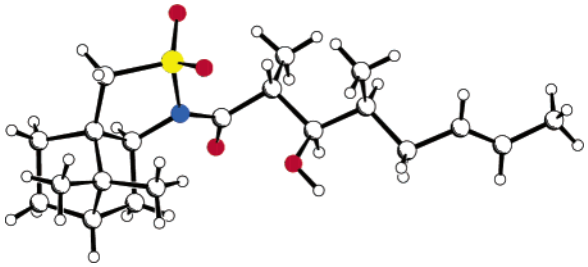
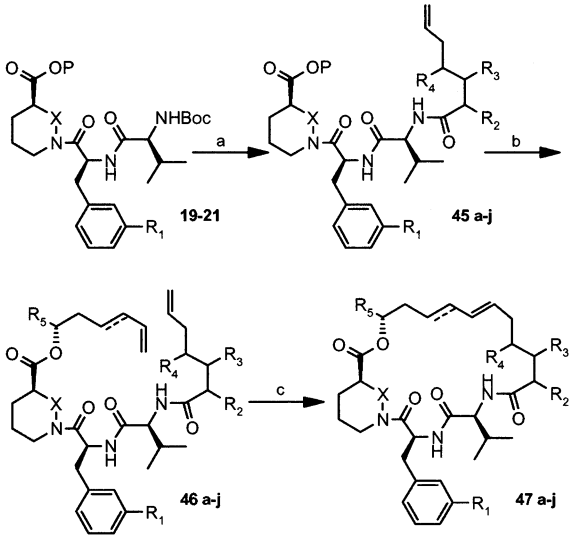


Figure 4. X-ray crystal structure of the aldol product **44** possessing the $C_{14}-C_{15}$ *anti* stereochemistry.

Scheme 8. Synthesis of a Library of Sanglifehrin Macrolide Analogues via the Ring-Closing Metathesis Reaction[a]



[a] Reagents and conditions: (a) (i) TMSOTf, CH_2Cl_2, 0 °C or TFA, CH_2Cl_2, 0 °C, (ii) NMM, isobutylchloroformate, building blocks **22**−**24**, THF, −10 °C to rt or NMM, EDC, HOBT; (b) (i) Zn, 1.0 M aqueous NH_4OAc, THF or LiOH, THF/H_2O 5:1, (ii) PPh_3, DEAD, building blocks **25**−**28**, THF, (iii) TBDMSOTf, pyridine, CH_2Cl_2, 0 °C; (c) (i) $(PCy_3)_2Cl_2Ru$=CHPh, CH_2Cl_2, (ii) TBAF, THF. Symbols: X = C or N; P = $-CH_3$ or $-CH_2CCl_3$; For R_1 to R_5, see Table 1.

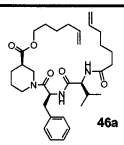
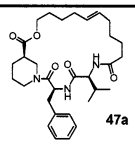
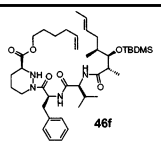
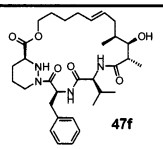
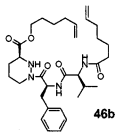
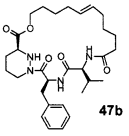
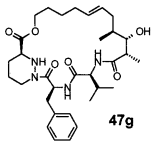
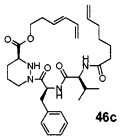
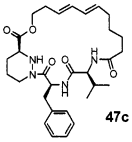
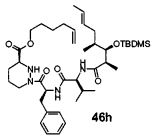
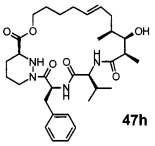
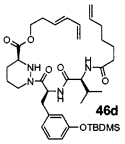
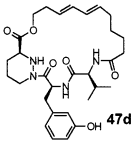
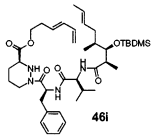
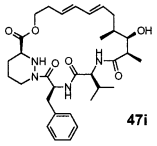
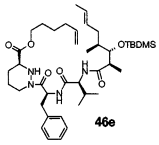
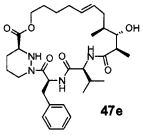
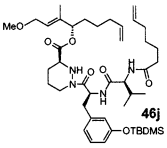
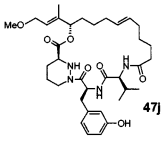
With all the building blocks in our hands, we started to assemble the macrolide library according to Scheme 8. Removal of the Boc group from tripeptides **19**−**21** in the presence of TFA or TMSOTf in CH_2Cl_2, followed by coupling of the resulting amines to building blocks **25**−**28**, afforded compounds **45a**−**j**. Methyl ester hydrolysis or selective deprotection of the trichloroethyl ester group with zinc provided the corresponding acids, which were esterified with the alcohols **22**−**24** under Mitsunobu conditions. The precursors **46a**−**j** for the ring-closing metathesis reaction were thus obtained in good overall yield. At this stage, the precursors with a free hydroxy group at C_{15}, **46e**−**i**, were protected with a *tert*-butyldimethylsilyl group. In the presence of this protecting group, the subsequent metathesis reaction was faster and proceeded in higher yields. The ruthenium-based catalyst first described by Grubbs et al. was used for the critical macrocyclization step.[33] The best yields were obtained in CH_2Cl_2 under reflux and high dilution (<5 mM). Under these conditions, the alkene or diene macrolides **47a**−**j** were obtained in good to excellent yields (50−80%) after

(31) Walker, M. A.; Heathcock, C. H. *J. Org. Chem.* **1991**, *56*, 5747−5750. The Heathcock procedure was used recently to synthesize a very similar fragment contained in callipeltin A. The *anti* aldol product was obtained in 27% yield. See: Guerlavais, V.; Carroll, P. J.; Joullié, M. M. *Tetrahedron: Asymmetry* **2002**, *13*, 675−680.

(32) (a) Oppolzer, W.; Starkemann, Ch.; Rodriguez, I.; Bernardinelli, G. *Tetrahedron Lett.* **1991**, *32*, 61−64. (b) Oppolzer, W.; Rodriguez, I. *Helv. Chim. Acta* **1993**, *76*, 1275−1281.

(33) Schwab, P.; France, M. B.; Ziller, J. W.; Grubbs, R. H. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2039−2041.

Table 1. Macrocyclic Analogues and Yields of the Ruthenium-Catalyzed Ring-Closing Metathesis Reaction

Entry	Precursor	Macrolide	Yield RCM	Entry	Precursor	Macrolide	Yield RCM
1	 46a	 47a	88%	6	 46f	 47f	81%
2	 46b	 47b	60%	7	-----	 47g	-----
3	 46c	 47c	57%	8	 46h	 47h	52%
4	 46d	 47d	47%	9	 46i	 47i	57%
5	 46e	 47e	51%	10	 46j	 47j	68%

HPLC purification (Table 1). The additional methyl group attached to the alkene (entries 5−9) did not affect the yield of the RCM reaction. Macrolide **47g**, the C_{15} epimer of **47f**, could be separated as a side product (<5%) by reverse-phase HPLC from **47f** after macrocyclization and desilylation. This product arises from the presence of a minor isomer present in the macrocyclization substrate **46f**. This isomer has its origin in the Evans aldol reaction leading to acid **26**, as indicated previously.

X-ray Crystal Structure of Macrolide 16 Bound to Cyclophilin. The X-ray crystal structure of macrolide **16** bound to cyclophilin was determined and allowed further insight into the ligand−protein interactions. The resolution of the complex was 2.1 Å, and the final *R*-factor, 16.4%. The crystal structure revealed the details of the interaction between the sanglifehrin macrolide and cyclophilin (Figure 5a and b). Similarly to cyclosporin A, the macrocyclic subunit of sanglifehrin A binds to the active site of cyclophilin A, which is an enzyme that catalyzes the *cis*−*trans* isomerization of prolyl peptide bonds.[1] Accordingly, both CsA and SFA inhibit the catalytic activity of the enzyme.[8] The tripeptide Pip-*meta*-Tyr-Val is located deep in the binding pocket and forms all hydrogen bonds with the protein. This portion of the ligand will be referred to as the *recognition domain*. The C_{13}−C_{26} fragment, that we would call the *spacer domain*, extends on the surface of the complex and does not undergo any direct interactions with cyclophilin. The spacer domain might play a critical role in maintaining the molecule in the optimal three-dimensional conformation.

Six direct hydrogen bonds (cutoff 3.4 Å) could be identified between the recognition domain and four amino acids of the protein. Three amino acids of the protein (Arg^{55}, Gln^{63}, and His^{126}) make intermolecular hydrogen bonds through their side chains, while one amino acid (Asn^{102}) forms an antiparallel β-sheet interaction with the ligand. In detail, these hydrogen bonds are located between NE2-His^{126} and the *meta*-tyrosine hydroxy group (2.69 Å/2.65 Å), between the main chain nitrogen of Asn^{102} and the main chain carbonyl oxygen of *meta*-tyrosine (2.97 Å/2.92 Å), between the main chain carbonyl oxygen of Asn^{102} and the main chain nitrogen of *meta*-tyrosine (2.75 Å/2.83 Å), between NE2-Gln^{63} and the main chain carbonyl oxygen of valine (3.04 Å/3.01 Å), between OE1-Gln^{63} and the α-nitrogen of the piperazic acid (3.03 Å/3.00 Å), and between NH2-Arg^{55} and the carbonyl oxygen of the ester moiety (3.31 Å/3.20 Å). In the latter list, distances are given between heteroatoms in the two independently refined complexes of the asymmetric unit (the averaged values are displayed in Figure 5b). The piperazic acid moiety of macrolide **16** occupies the same deep hydrophobic groove of cyclophilin A that is used by the side chain of Me-Val^{11} of cyclosporin A[1] or by the proline ring of the model substrate Ala-Ala-Pro-Ala.[34] The hydrophobic pocket is formed by Phe^{60}, Met^{61}, Phe^{113}, and Leu^{122}. The 3-oxo-butyl side chain attached at C_{14} extends on the surface of the complex and does not form any obvious interaction with the protein. The C_{15} hydroxy group points toward a pocket in the cyclophilin structure occupied by water molecules. As mentioned earlier, the methyl group at C_{16} is directed toward the interior surface of the macrolide and the C_{17} hydroxyl is exposed to the solvent. Altogether, it appears that the spacer domain is essential to induce the optimal 3-dimensional conformation of the macrolide, which would allow a perfect fit of the recognition domain in the catalytic site of cyclophilin A.

(34) Kallen, J.; Walkinshaw, M. D. *FEBS Lett.* **1992**, *300*, 286−290.

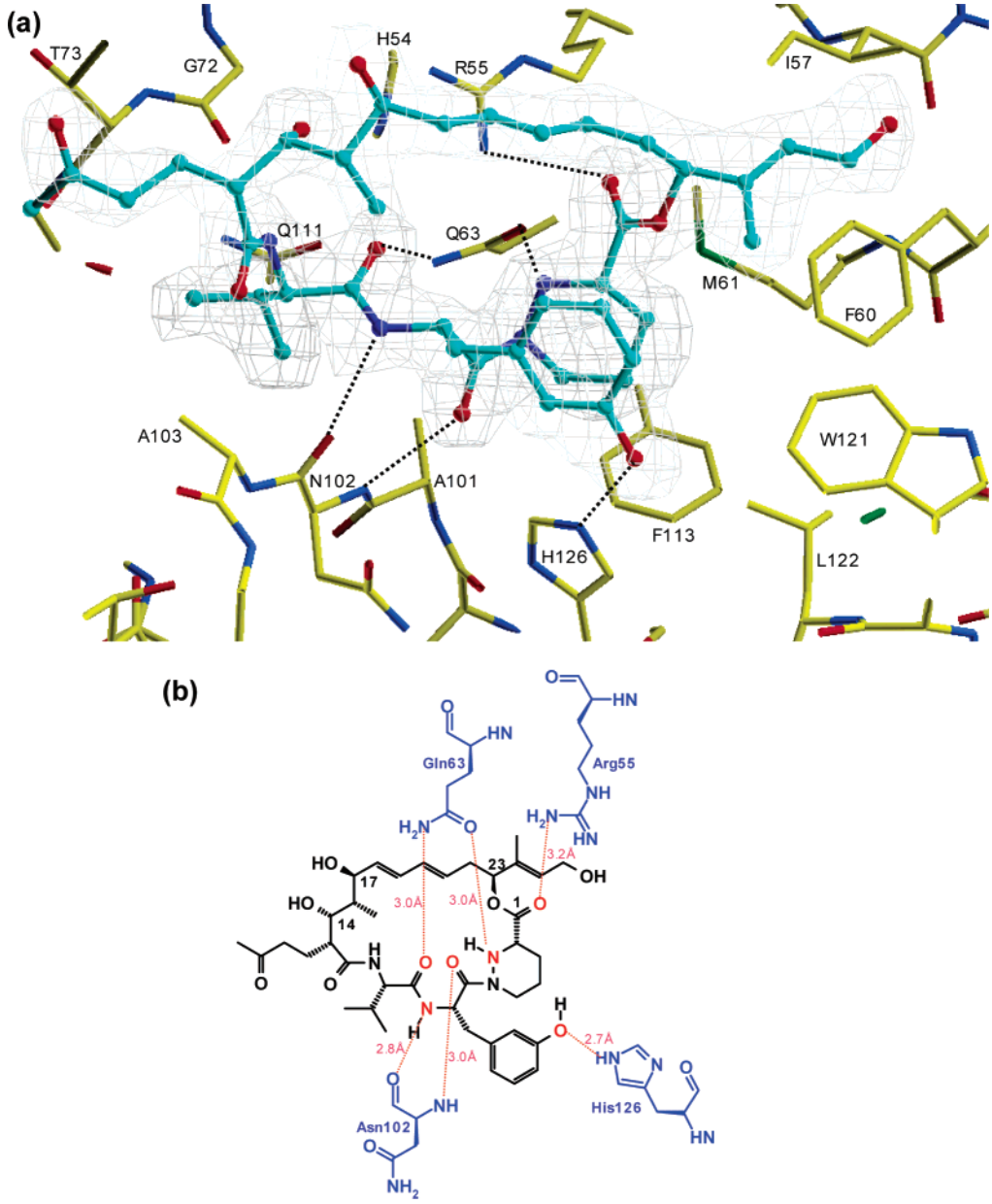


Figure 5. (a) Final $2F_{obs} - F_{calc}$ electron density map covering macrolide **16** and the refined model. Direct hydrogen bonds (cutoff 3.4 Å) between **16** and cyclophilin A are indicated as dashed lines. (b) Schematic view of the direct intermolecular hydrogen bonds between macrolide **16** and cyclophilin A in the binding site. The distances are for the separation between heteroatoms, averaged over the two independently refined complexes in the asymmetric unit. The representation of **16** was rotated in order to match the view in part a.

Biological Evaluation of Macrolides and Discussion. The macrolides obtained by derivatization of **2** as well as the synthetic analogues were screened for their affinity for cyclophilin A in a competitive binding assay. In this assay, the interaction between solid-phase coated SFA−BSA conjugates and biotinylated cyclophilin is inhibited by free SFA in solution.[35] The IC_{50} value for sanglifehrin A in this assay is 6.9 ± 0.9 nM.[8] The results obtained with the new derivatives, summarized in Table 2, reveal a number of important structure−activity relationships. Macrolide **16** exhibits the same affinity to cyclophilin as the parent compound, clearly indicating that the binding of sanglifehrin A is fully mediated by the macrocyclic portion. Various modifications of the side chain attached at C_{23} (derivatives **8**, **9**, **11**, **12**, and **14**) have little effect on the binding affinity. Sufficient space is available to accommodate side chains of different lengths and containing ester or hydroxy functionalities. This is in agreement with the fact that removal of the spirolactam moiety of SFA results only in the loss of immunosuppressive activity but not in affinity for cyclophilin A. In contrast, full hydrogenation of all double bonds to form the hexahydro derivative **5** results in a 7- or 35-fold loss in affinity in comparison to **2** and **16**, respectively. Thus, the diene system is important for fine-tuning the three-dimensional conformation of the macrocycle and confirms our initial working hypothesis. The C_{54} carbonyl group, which is in equilibrium with its intramolecular hemiketal form in solution, is not important for the binding activity. Indeed, reduction to the alcohol, as in macrolide **6**, does not affect the affinity. Nevertheless, the reduced affinity of **4** over **2** suggests that

(35) For details of the ELISA binding assay, see ref 8.

Table 2. Cyclophilin Binding Data for Selected Analogues of the Sanglifehrin Macrocycle[a]

compd	cyclophilic binding[a]	compd	cyclophilin binding[a]
2	29 ± 2.1	**17**	>5000
4	86 ± 28	**18**	>5000
5	200 ± 40	**47a**	>5000
6	12 ± 0.8	**47b**	10 270 ± 405
7	49 ± 15	**47c**	7130 ± 640
8	16 ± 1.8	**47d**	5870 ± 320
9	11 ± 2.0	**47e**	9370 ± 950
11	21 ± 1.7	**47f**	12 930 ± 970
12	8.9 ± 2.9	**47h**	6430 ± 710
14	6.5 ± 1.0	**47i**	3830 ± 740
16	5.7 ± 1.0	**47j**	5100 ± 530

[a] The cyclophilin binding data were measured as described elsewhere.[8] IC_{50} values [nM] represent mean ± SD of 3−4 independent experiments. The IC_{50} value for SFA is 6.9 ± 0.9 nM.

oxygen is preferred over iodine in this position. Finally, the good affinity retained by triene **7** shows that the C_{17} hydroxyl is not critical for binding. This conclusion is supported by analysis of the X-ray crystal structure, which shows that this hydroxyl is exposed to the solvent. Furthermore, it appears that the overall conformation of the macrocycle is not greatly affected by the additional double bond. Derivatives **17** and **18**, in which the free α-nitrogen of piperazic acid is methylated, are inactive. This observation confirms that the hydrogen bond formed between the free α-nitrogen of piperazic acid and OE1-Gln63 is crucial for binding to cyclophilin. It is interesting to note that this interaction is mediated by a structural feature, *which is unique to the sanglifehrins*. Therefore, it is not surprising that the replacement of piperazic acid by nipecotic acid in compound **47a** also leads to a substance that does not bind to cyclophilin.

All the synthetic macrolide analogues, **47b**−**47j**, retain only modest affinity in the micromolar range. Among those, **47d** combines the complete recognition domain with the (*E*,*E*)-diene unit. The binding data of **47d** prove that these two fragments of the macrolide are certainly necessary but not sufficient to achieve high affinity for cyclophilin as hypothesized initially. The role of the three stereocenters at C_{14}, C_{15}, and C_{16} of the polypropionate fragment was assessed with compounds **47e**−**47i**. To our surprise, the stereochemistry at C_{14} and C_{15} had only a minor impact on the binding to cyclophilin. Compound **47i**, which combines the absolute configurations of the C_{14}−C_{16} stereocenters found in sanglifehrin A with the (*E*,*E*)-diene moiety, remained the most potent synthetic derivative prepared so far. Finally, the addition of a C_{23} side chain and the *meta*-tyrosine hydroxyl to **47b**, leading to **47j**, did not significantly improve the affinity.

Conclusions

The interaction between the macrocyclic moiety of sanglifehrin A and cyclophilin was studied in detail. The X-ray crystal structure of the cyclophilin A-macrolide **16** complex was resolved at 2.1 Å and provided detailed insight into the ligand-protein interactions. In addition, a series of macrolide analogues was prepared either by derivatization of macrolide **2**, resulting from selective degradation of sanglifehrin A, or via a synthetic approach using the ring-closing metathesis reaction as the key step. The latter work confirmed the potential of the RCM reaction for rapidly assembling a library of macrolides of variable complexity. Altogether, the X-ray crystal structure and the synthetic work proved that the high affinity of sanglifehrin A for cyclophilin A is mediated exclusively by the macrocyclic portion of the molecule. More surprising was the finding that the complex macrocyclic structure is a highly optimized combination of multiple functionalities including a rather unusual tripeptide, an (*E*,*E*)-diene unit, and a polypropionate portion, which together provide an extremely strong affinity for cyclophilin. The "original" sanglifehrin A macrocycle **2** allows for some, mostly single or double, modifications which do not greatly affect binding. On the other hand, simultaneous modifications of several positions, including removal of substituents, configurational changes, and functional group modifications, as in the synthetic macrolides **47a**−**j**, result in a significant loss of affinity for cyclophilin.

Acknowledgment. We thank Beatrix Wagner for her help with the X-ray crystal structure, Serge Fuchs of his technical assistance, Francis Roll for the HRMS measurements, and Frédéric Zecri for his contribution to the paper.

Supporting Information Available: Details of crystallization conditions and structure resolution. Complete experimental procedures and spectral data for all compounds generated in the course of the investigation reported here, including copies of the ^{1}H NMR spectra of **3**−**9**, **11**−**12**, **14**−**19**, **24**−**27**, **30**−**32**, **34**−**36**, **39**, **41**, **44**, $\mathbf{45_{d-f,\ h}}$, $\mathbf{46_{d-f,\ h-j}}$, and $\mathbf{47_{d-j}}$. This material is available free of charge via the Internet at http://pubs.acs.org.

JA021327Y