0031-6997/89/4103-0243$02.00/0
PHARMACOLOGICAL REVIEWS

Vol. 41, No. 3
Printed in U.S.A.

Pharmacology of Cyclosporin (Sandimmune)
II. Chemistry

ROLAND M. WENGER
Preclinical Research, Sandoz Ltd., Basel, Switzerland

A. The Molecule

1. Structure

The isolation, culture conditions, and taxonomy of microorganisms isolated from soil samples collected at Hardanger Vidda (Norway) and in Wisconsin (USA) have been described by Dreyfuss et al. (4). The discovery of the immunosuppressive effects of the secondary metabolites produced by these microorganisms provoked an extensive chemical and pharmacological investigation which in 1973 led to the isolation of the main component in pure crystalline form, which first named cyclosporin A (CS).* [For its first description see Rüegger et al. (25a) and Petcher et al. (15a).]In addition to this compound, the microorganisms produce a plethora of related minor metabolites. These compounds have been recently reviewed by Traber et al. (29) and by von Wartburg and Traber (30).

Because the main compound was named cyclosporin A (CS), the minor congeners were designated cyclosporins B, C, D. . .Z. Subsequently, the structure of CS was elucidated by a combination of spectroscopic methods and chemical degradation experiments and shown to be that of a cyclic undecapeptide (fig. 1). Total hydrolysis of this cyclic peptide yielded N-methyl-L-leucine (4 mol), sarcosine, N-methyl-L-valine, L-2-aminobutyric acid, L-valine, racemic alanine (2 mol), and artifacts of a previously unknown amino acid consisting of nine carbon atoms. At that time it was not possible to obtain this amino acid from CS hydrolysis. The artifacts which were obtained, however, gave some clues about the structure of this amino acid, and after completion of the X-ray crystal structure analysis it was possible to assign its absolute configuration as (4*R*)-4[(E)-2-butenyl]-4,N-dimethyl-L-threonine (MeBmt). The total synthesis of this amino acid (32) first allowed its isolation in pure form, and recently chemical procedures have been developed by Wenger (manuscript in preparation) to obtain this compound by degradation of CS. Treatment of CS under acidic conditions causes a molecular rearrangement to give isocyclosporin with a free methylamino group accessible to chemical derivation, including a modified Edman degradation. This established MeBmt as the first amino acid of the sequence of CS.

The structure of CS as determined by X-ray crystal structure analysis is shown in fig. 2. Amino acids 11-7 form a beta fragment containing a type-II′ beta-turn consisting of amino acids 2-5. Residues 7-11 form the second characteristic feature, the loop. The side chain of MeBmt in the crystal structure folds back into the ply of the beta-pleated sheet, whereas in solution in nonpolar solvents it is extended. The structural and conformational aspects of CS have been discussed in detail by Wenger (35; see also ref. 28).

2. Conformational Studies

Of particular interest is the conformation of CS in biological fluids. In recent years important progress has been made in the development of methods and techniques to elucidate the three-dimensional structure of peptides and proteins in aqueous solution (10). These methods, however, are not applicable to CS because of the very low solubility of this molecule in water and because of its tendency to form micelles. The solution conformation of CS in aprotic* solvents has been determined by nuclear magnetic resonance spectroscopy (NMR) (11).

CS has also been the subject of molecular dynamics calculations using the programs GROMOS (developed at

* The abbreviations used are: CS, cyclosporin A; MeBmt, (4R)-4[(E)-2-butenyl]-4,N-dimethyl-L-threonine; NMR, nuclear magnetic resonance spectroscopy.

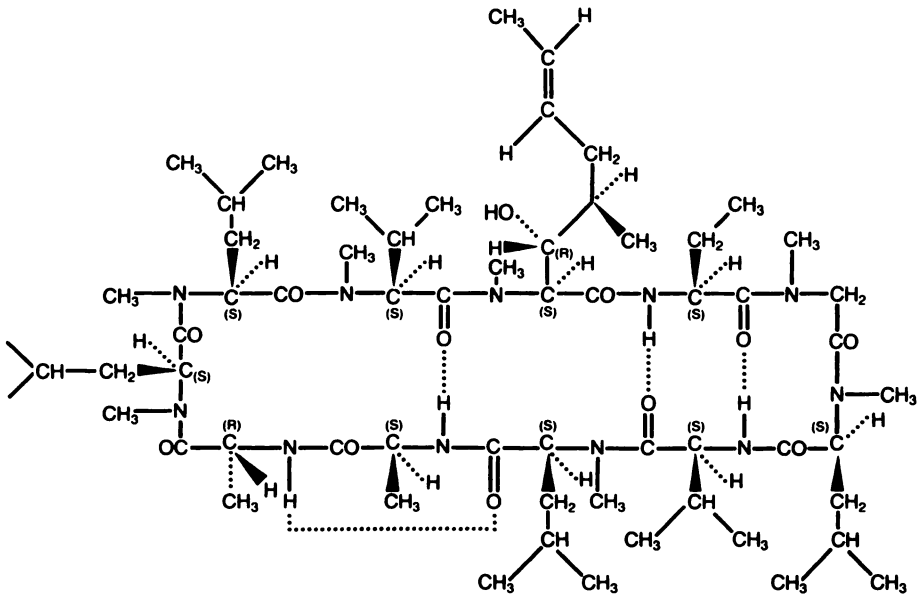

FIG. 1. Cyclosporin A (cyclic undecapeptide).

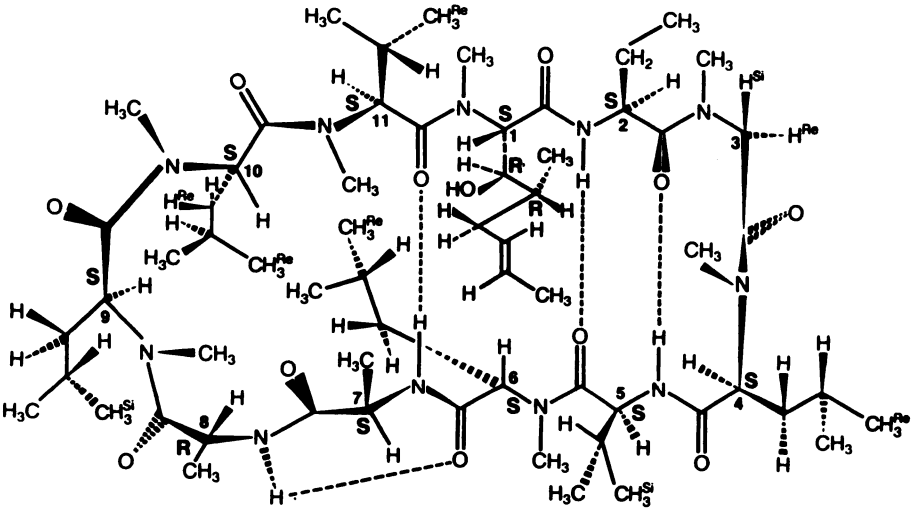

FIG. 2. Structure of cyclosporin A (schematic) corresponding to the conformation observed in the crystal.

the University of Groningen, The Netherlands) and DISCOVER (Biosym Technologies, Inc., San Diego, CA) (14, 31, 37). Molecular dynamics simulations of the CS crystal structure and dynamic modeling of the structure determined by NMR suggest that in water the conformation observed in the crystal structure is the most stable one. In a hypothetical experiment which simulates placement of the CS molecule in its conformation as determined in aprotic solvents (NMR) in water results in immediate conformational changes until the conformation as observed in the crystal is obtained. This result indicates that the conformation as determined experimentally in an aprotic solvent is destabilized in an aqueous environment, whereas the conformation found experimentally in the crystal is favoured.

Because of the aforementioned difficulties in obtaining structural information about the conformation of CS in aqueous media, a series of immunochemical studies was performed (16–21). After a suitable CS derivative was coupled to a protein carrier, polyclonal and monoclonal antibodies against CS were obtained. Then, the fine specificity of the monoclonal antibodies for CS was determined by studying the cross-reactivities of the antibodies for different CS analogues showing preserved skeletal conformation. It was possible to identify contact residues of the CS molecule for the various antibodies by using enzyme-linked immunosorbent assay inhibition tests. Also, it became feasible to attach the molecule at different positions to the carrier proteins and to prepare a large number of monoclonal antibodies recognizing different epitopes on the surface of the molecule by means of some especially designed derivatives of CS. It was found that monoclonal antibodies recognizing modifications at the end of the MeBmt carbon chain (but not at the carbon atoms 3 or 4) also recognized amino acids 6, 8, and 9. By contrast, those monoclonal antibod-

ies that distinguished chemical changes at carbon atoms 3 and 4 of the MeBmt carbon chain at the same time recognized amino acids 1, 2, 3, 10, and 11. These findings suggest that when CS is in an aqueous environment the terminal carbon atoms of MeBmt are part of a contiguous surface including amino acids 6, 8, and 9. These structural characteristics are also found in the CS crystal structure, whereas the MeBmt carbon chain extends away from the skeleton of the ring in chloroform.

3. Biosynthesis

As has been found for many other cyclic, partially N-methylated peptides containing unusual amino acids (e.g., the gramicidins or the antibiotics of the vancomycin/ristocetin group), the biosynthesis of the cyclosporins does not occur on ribosomes but is effected by a multifunctional enzyme complex. The chemical mechanism of the biosynthetic assembly of these compounds resembles in some way the biosynthesis of fatty acids (15, 38). Feeding experiments by Kobel et al. (12) indicated that the seven N-methyl groups of CS and the methyl group at carbon atom 4 of MeBmt originate from methionine, and the linear carbon chain of MeBmt is assembled from acetate units. Recently, the group of Billich and Zocher (2) was able to isolate an enzyme preparation from Tolypocladium capable of synthesizing CS from the constituent amino acids with concomitant consumption of adenosine triphosphate and S-adenosyl methionine.

Previously, it had been found by Kobel and Traber (13) that adding certain amino acids to the fermentation media resulted in a shift in the ratio of CS components. Thus, adding L-threonine or L-valine significantly raises the proportion of cyclosporins C and D, whereas adding DL-2-amino butyric acid suppresses the formation of other cyclosporins to a high degree, allowing almost exclusive formation of cyclosporin A. Without adding external norvaline, cyclosporin G is produced only in minimal amounts; adding norvaline causes production of cyclosporin G as the major metabolite.

4. Production

Tolypocladium inflatum grows well at ambient temperatures on yeast-malt agar. For submerged organisms, culture media containing glucose and casein peptone, supplemented with mineral salts, can be used. The optimal concentrations of CS are commonly reached after a fermentation time of 10 to 12 days. The biomass is harvested by centrifugation of the broth; cyclosporins are extracted with organic solvents and defatted, and the crude extract is subjected to several chromatographic procedures. The final step in the production of CS is crystallization (30).

B. Chemical Synthesis

When the therapeutic potential of CS was fully recognized, the need for derivatives that were not provided by nature or could not be obtained by chemical modification of naturally occurring compounds was obvious. Not only would studies of structure-activity relationships require a series of different derivatives, but because the drug exerts its biological activity by a mechanism that at that time was unknown (and is not yet completely understood) various tools for biochemical investigations such as affinity labels or derivatives, which could be bound to affinity columns or protein carriers, would also be required. The first total synthesis of CS was reported by Wenger (33), who also achieved the first total synthesis of the unusual amino acid, MeBmt (32). After the real value of CS to the medical community had become common knowledge, many other groups embarked on efforts to synthetize CS derivatives. Stereo-specific syntheses of MeBmt have been reported by several groups (1, 6, 22, 24, 26, 27). Rich and coworkers also reported the synthesis of analogues of MeBmt and their incorporation into synthetic CS derivatives (3, 25). Further efforts to synthesize CS and CS derivatives have been reported by two other groups (5, 7–9).

Although the approaches toward MeBmt synthesis reflect the fundamentally individual nature of endeavors and each has its unique features, the efforts to synthesize the cyclic peptide framework were mostly based on the first synthesis of CS A reported by Wenger (33). The strategic considerations were centered around the following aspects: (*a*) the linear peptide precursor to the macrocycle should be cyclized at a peptide bond where the least opposition by steric factors was to be expected (the bond between the alanines was chosen); (*b*) introduction of the rare synthetic amino acid MeBmt should occur as late as possible; (*c*) construction of peptide fragments to allow the combination of building units; and (*d*) the synthesis of N-methyl peptides is considerably more difficult than the synthesis of nonmethylated peptides. The problems encountered are primarily due to the greater steric bulk presented by the additional methyl groups (which means longer reaction times) but also due to an increase in acidity at the alpha-carbon of N-methyl amino acids, which makes them prone to racemisation during peptide coupling steps.

A detailed account of the considerations of synthesis, the problems encountered, and their solution has been given by Wenger (33).

C. Chemical Modifications and Structure-Activity Relationships

CS and the natural congeners have been subjected to a variety of chemical modifications. A major part of these efforts focused on the unusual amino acid. The hydroxyl group as well as the carbon-carbon double bond of MeBmt can be subjected to a variety of more or less obvious chemical transformations. It is worth mentioning in this context that most of these variations resulted in diminished immunosuppressive activities. The double bond can be reduced to give dihydro compounds which

generally show slightly diminished immunosuppressive activity. The OH group can be acylated or even removed completely to give nearly inactive compounds. Omission of the methyl group at carbon 4 of MeBmt or addition of an extra methyl group reduces the activity significantly (3, 34).

Interestingly and unexpectedly, CS can be converted into a polyanion with an excess of a strong base, and this polyanion reacts with a variety of electrophiles to give derivatives of an N-methyl-D-amino acid in position 3, i.e., in place of the sarcosine (28). Even more surprisingly, many of these derivatives are potent immunosuppressants.

As mentioned in the discussion of the three-dimensional structure of CS, amino acids 2-5 form a type-II′ beta-turn. The cyclic pseudodipeptides A (23) and B (fig. 3) (Fliri, H., unpublished work) have been incorporated in place of amino acids 3 and 4 into the CS molecule to make this particular fragment less flexible and thus potentially achieve stronger receptor binding.

Both derivatives showed only weak immunosuppressive activity. Similar considerations led to the synthesis of L-pro-3-cyclosporin and D-pro-3-cyclosporin, both of which are nearly devoid of immunosuppressive activity (36). These modifications of amino acid 3 suggest that the CS receptor imposes severe steric restrictions at that position and that only unbranched D-substituents are tolerated.

As exemplified by the potent immunosuppressive activities of the natural cyclosporins C and G, amino acid 2 presents some potential for variations. Several modifications at this position, notably by introducing amino acids containing fluorine or sulphur atoms, were reported by Durette et al. (5). The immunosuppressive activities of the reported compounds were lower than that of CS. Similar results were obtained by the same group for derivatives in position 6 (5). Little room for modification seems to exist in position 11 without considerable loss in immunosuppressive potency. Addition of an additional carbon atom to the methyl groups of MeVal-11 furnishes derivatives that are practically inactive. Insertion of a methylene group between the isopropyl group of MeVal-11 and the alpha carbon (MeLeu-11-cyclosporin) also gives a compound that is almost inactive. Similar results are obtained when the two methyl groups of MeVal-11 or in position 10 are removed: MeAla-11- and MeAla-10-cyclosporin are inactive compounds.

In summary, the immunosuppressive activity of CS seems to be associated with a large part of the molecule and variations are permitted only at a few positions without considerable loss of potency. Clearly, the unusual amino acid MeBmt is intimately involved but, by itself, is not sufficient for immunosuppressive activity. Based on the results presented, it may safely be concluded that the biological activity of the CS molecule resides in a part of the structure that includes amino acids 1, 2, 3, 10, and 11, which are clustered together on the surface of the molecule.

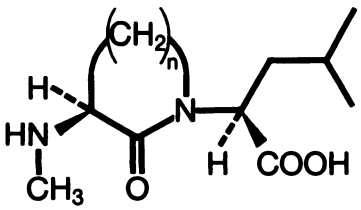

FIG. 3. Pseudodipeptide A (n = 3) and pseudodipeptide B (n = 2).

REFERENCES

1. AEBI, J. D., DHAON, M. K., AND RICH, D. H.: A short synthesis of enantiomerically pure (25,3R,4R,6E)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid, the unusual C-9 amino acid found in the immunosuppressive peptide cyclosporine. J. Org. Chem. **52:** 2881–2886, 1987.
2. BILLICH, A., AND ZOCHER, R.: Enzymatic synthesis of the immune suppressor cyclosporin A. J. Biol. Chem. **262:** 17258–17259, 1987.
3. DEYO, D., TUNG, R. D., AND RICH, D. H.: Enantioselective syntheses of beta-hydroxy,alpha-amino acids for cyclosporine analogs. Presented at the American Chemical Society Meeting, New Orleans, LA, Aug. 30, 1987, abstract 104.
4. DREYFUSS, M., HAERRI, E., HOFMANN, H., KOBEL, H., AND TSCHERTER, H.: Cyclosporine A and C: new metabolites from Trichoderma polysporum (Link. ex Pers.) Rifai. Eur. J. Appl. Microbiol. **3:** 125–133, 1976.
5. DURETTE, P. L., DUMONT, F., FIRESTONE, R., FRANKSHUN, R. A., KOPRAK, S. L., LIN, C. S., MELINO, M. R., PISSOLANO, A. A., PISANO, J., SCHMIDT, J. A., SIGAL, N. H., STARUCH, M. J., AND WITZEL, B. E.: A study of the correlation between cyclophilin binding and in vitro immunosuppressive activity of cyclosporine A and analogues. Transplant. Proc. **20 (suppl. 2):** 51–57, 1988.
6. EVANS, D. E., AND WEBER, A. E.: Asymmetric glycine enolate aldol reactions: synthesis of cyclosporine's unusual amino acid, MeBmt. J. Am. Chem. Soc. **108:** 6757–6761, 1986.
7. GALPIN, I. J., KARIM, A., MOHAMMED, A., AND PATEL, A.: Synthesis of cyclosporine analogues. Tetrahedron Lett. **28:** 6517–6520, 1987.
8. GALPIN, I. J., MOHAMMED, A. K. A., AND PATEL, A.: Synthetic studies of cyclosporin analogues. Tetrahedron *44:* 1783–1794, 1988.
9. GALPIN, I. J., MOHAMMED, A. K. A., AND PATEL, A.: Synthesis of linear undecapeptide precursors of cyclosporin analogues. Tetrahedron **44:** 1773–1782, 1988.
10. KESSLER, H., LOOSLI, H. R., AND OSCHKINAT, H.: *In* Peptides 1984, Proceedings of the 18th European Peptide Symposium, ed. by U. Ragnarsson, pp. 65–83, Almqvist and Wiksell Int., Stockholm, 1984.
11. KESSLER, H., LOOSLI, H. R., AND OSCHKINAT, H.: Assignment of the 1-H-, 13-C-, and 15-N-NMR spectra of cyclosporin A in CDCl3 and C6D6 by a combination of homo- and heteronuclear two-dimensional techniques. Helv. Chim. Acta **68:** 661–681, 1985.
12. KOBEL, H., LOOSLI, H. R., AND VOGES, R.: Contribution to the knowledge of the biosynthesis of cyclosporin A. Experientia **39:** 873–876, 1983.
13. KOBEL, H., AND TRABER, R.: Directed biosynthesis of cyclosporins. Eur. J. Appl. Microbiol. Biotechnol. **14:** 237–240, 1982.
14. LAUTZ, J., KESSLER, H., KAPTEIN, R., AND VAN CUNSTEREN, W. F.: Molecular dynamics simulation of cyclosporin A: the crystal structure and dynamic modelling of a structure in apolar solution based on NMR data. J. Computer-aided Mol. Design **1:** 219–241, 1987.
15. LIPMANN, F.: Attempts to map a process evolution of peptide biosynthesis. Science **173:** 875–884, 1971.

15a. PETCHER, T. J., WEBER, H. P., AND RÜEGGER, A.: Crystal and molecular structure of an iodo-derivative of the cyclic undecapeptide cyclosporin A. Helv. Chim. Acta **59:** 1480–1488, 1976.

16. QUESNIAUX, V. F. J.: The use of monoclonal antibodies to probe the surface of cyclosporines. Transplant. Proc. **18 (suppl. 5):** 111–114, 1986.
17. QUESNIAUX, V. F. J., HIMMELSPACH, K., AND VAN REGENMORTEL, M. H. V.: An enzyme immunoassay for the screening of monoclonal antibodies to cyclosporin. Immunol. Lett. **9:** 99–104, 1985.
18. QUESNIAUX, V. F. J., TEES, R., SCHREIER, M. H., WENGER, R. M., DONATSCH, P., AND VAN REGENMORTEL, M. H. V.: Monoclonal antibodies to cyclosporin. Prog. Allergy **38:** 108–122, 1986.
19. QUESNIAUX, V. F. J., TEES, R., SCHREIER, M. H., WENGER, R. M., AND VAN REGENMORTEL, M. H. V.: Fine specificity and cross-reactivity of monoclonal antibodies to cyclosporine. Mol. Immunol. **24:** 1159–1168, 1987.
20. QUESNIAUX, V. F. J., WENGER, R. M., SCHREIER, M. H., AND VAN REGENMORTEL, M. H. V.: Immunochemical study of cyclosporine conformation in aqueous medium. *In* Protides of the Biological Fluids, ed. by Peeters, vol. 35, pp. 507–510, Pergamon Press, Oxford, 1987.
21. QUESNIAUX, V. F. J., WENGER, R. M., SCHMITTER, D., AND VAN REGENMORTEL, M. H. V.: Study of the conformation of cyclosporine in aqueous medium by means of monoclonal antibodies. Int. J. Pept. Prot. Res. **31:** 173–185, 1988.

22. RAMA RAO, A. V., MURALI DHAR, T. G., CHAKRABORTY, T. K., AND GURJAR, M. K.: A stereospecific synthesis of (4R)-4[(E)-2-butenyl]-4,N-dimethyl-L-threonine. Tetrahedron Lett. **29:** 2069–2072, 1988.
23. RICH, D.: Presented at the Euchem Conference: New Trends in Peptide Synthesis, Port-Camargue, France, April 25–29, 1988.
24. RICH, D. H., AND TUNG, R. D: Total synthesis of the unusual cyclosporine amino acid MeBmt. Tetrahedron Lett. **28:** 1139–1142, 1987.
25. RICH, D. H., DHAON, M. K., DUNLAP, B., AND MILLER, S. P. F.: Synthesis and antimitogenic activities of four analogues of cyclosporin A modified in the 1-position. J. Med. Chem. **29:** 978–984, 1986.
25a. RÜEGGER, A., KUHN, M., LICHTI, H., LOOSLIE, H. R., HUGUENIN, R., QUIQUEREZ, C., AND VON WARTBURG, A.: Cyclosporin A, ein immunsuppressiv wirksamer Pilzmetabolit aus *Trichoderma polysporum* (Link ex Pers.) *Rifai.* Helv. Chim. Acta **59:** 1075–1092, 1976.
26. SCHMIDT, U., AND SIEGEL, W.: Synthesis of (4R)-4-(E)-2-butenyl-4,N-dimethyl-L-threonine (MeBmt), the characteristic amino acid of cyclosporine. Tetrahedron Lett. **28:** 2849–2852, 1987.
27. SEEBACH, D., JUARISTI, E., MILLER, D. D., SCHICKLI, C., AND WEBER, T.: Addition of chiral glycine, methionine, and vinylglycine enolate derivatives to aldehydes and ketones in the preparation of enantiomerically pure alpha-amino-beta-hydroxy acids. Helv. Chim. Acta **70:** 237–261, 1987.
28. SEEBACH, D., MURTIASHAW, W., NAEF, R., SHODA, S. I., KRIEGER, M., BOLLINGER, P., LEUTWILER, A., AND WENGER, R. M.: Aktive Cyclosporinderivate durch C-Alkylierung unter Ersatz von H-re der Sarkosin-Einheit. Erzeugung polylithiierter Peptide. Presented at the Swiss Chemical Society Meeting, Berne, Oct. 18, 1985.
29. TRABER, R., HOFMANN, H., LOOSLI, H. R., PONELLE, M., AND VON WARTBURG, A.: Neue Cyclosporine aus Tolypocladium inflatum. Die Cyclosporine A-Z. Helv. Chim. Acta **70:** 13–36, 1987.
30. VON WARTBURG, A., AND TRABER, R.: Chemistry of the natural cyclosporin metabolites. Prog. Allergy **38:** 28–45, 1986.
31. WEBER, H. P., LAUTZ, J., AND VAN GUNSTEREN, W.: Molecular dynamics of cyclosporin. Presented at the XXXVth Annual Coll.: Protides of the Biological Fluids, Brussels, Belgium, April 27–29, 1987.
32. WENGER, R. M.: Synthesis of enantiomerically pure (2S, 3R, 4R, 6E)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid starting from tartaric acid. Helv. Chim. Acta **66:** 2308–2321, 1983.
33. WENGER, R. M.: Total synthesis of cyclosporin A and cyclosporin H, two fungal metabolites isolated from the species Tolypocladium inflatum Gams. Helv. Chim. Acta **67:** 502–525, 1984.
34. WENGER, R. M.: Synthesis of cyclosporine and analogues: structural requirements for immunosuppressive activity. Angew. Chem. Int. Ed. Engl. **24:** 77–85, 1985.
35. WENGER, R. M.: Cyclosporine and analogues—isolation and synthesis—mechanism of action and structural requirements for pharmacological activity. Fortschr. Chem. Org. Naturst. **50:** 123–168, 1986.
36. WENGER, R. M.: Cyclosporine and analogues: structural requirements for immunosuppressive activity. Transplant. Proc. **28 (suppl. 5):** 213–218, 1986.
37. WENGER, R. M.: Methodologic advances in cyclosporine measurement. Transplant. Proc. **20:** 313–318, 1988.
38. ZOCHER, R., NIHIRA, T., PAUL, E., MADRY, N., PEETERS, H., KLEINKAUF, H., AND KELLER, U.: Biosynthesis of cyclosporin A: partial purification and properties of a multifunctional enzyme from Tolypecladium inflatum. Biochemistry **25:** 550–553, 1986.