Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 1, pp 187–192, 1997

PII: S0968-0896(96)00204-0
0968-0896/97 $17.00 + 0.00

Conformational Control of Cyclosporin through Substitution of the N-5 Position. A New Class of Cyclosporin Antagonists

Christos Papageorgiou,* Jörg Kallen, Julien France and Richard French†
Sandoz Pharma Ltd, Preclinical Research, CH-4002 Basel, Switzerland

Abstract—Cyclosporin A (CsA) can be regiospecifically alkylated at the NH of Val-5 with reactive bromides in the presence of phosphazene-base P_4-t-Bu to yield derivatives **2–5**. These are devoid of immunosuppressive activity in vitro but they have binding affinity for cyclophilin A (CypA) similar to that of CsA and thus represent a new class of cyclosporin antagonists. ^{1}H NMR (DMSO-d_6) studies have shown that the compounds exist in a single, all *trans* conformation. A comparison of this NMR data with X-ray crystallographic analysis of a CypA/CsA derivative complex demonstrates that the solution structure does not correspond to the bioactive conformation. Copyright © 1997 Elsevier Science Ltd

Introduction

Cyclosporin A (CsA, **1**) is a naturally occurring cyclic undecapeptide which has not only revolutionized organ transplantation (Sandimmun®, Neoral®) but has also allowed the elucidation of the signal transduction mechanism leading to T-cell activation and proliferation.[1] CsA elicits its cellular effects through allosteric regulation of calcineurin (CaN), a Ca^{2+} and calmodulin dependent Ser/Thr phosphatase.[2] To this effect, cyclosporin first binds to its soluble intracellular receptor cyclophilin (CypA), a member of the immunophilin protein family,[3] and subsequently the resulting complex interacts with CaN and inhibits its phosphatase activity.[4] An analogous mode of action has been attributed to the immunosuppressant FK-506, which binds to its immunophilin receptor FKBP12 and the binary complex formed interacts with calcineurin.[5] The detailed 3D architecture of the latter tertiary complex has been elucidated by X-ray crystallographic analysis,[6] however, such information is unavailable for the CsA/CypA/CaN complex. Nevertheless, the large amount of 3D data available on complexes of CypA with cyclosporin and derivatives thereof demonstrates that residues 1, 2, 9, 10 and 11 of **1** compose its cyclophilin 'binding domain', while the solvent-exposed residues 4, 5, 6 and 7 define its 'effector domain', which binds to calcineurin (Fig. 1).[7–9] With no information being available on the molecular interaction between CaN and the CsA/CypA complex, empirical approaches were adopted for its study. Although the influence of substitution of CsA at position 4,[10–14] position 6[15] and position 7[16,17] has already been investigated, there have been no such studies performed for position 5. In this paper we report the results of our investigations with cyclosporin derivatives substituted at the nitrogen atom of Val-5 of **1**.

Key words: regiospecific alkylation, 2D-NMR, conformation, crystallography, cyclosporin antagonists.
†Present address: Institut für Organische Chemie der Universität Basel, St. Johanns-Ring 19, CH-4056 Basel, Switzerland.

Synthetic and Structural Results

Regioselective alkylation of CsA was achieved using 4 equivalents of the sterically demanding base phosphazene-base P_4-t-Bu and reactive halides (Fig. 1).[18] Indeed, among the four NH groups of **1** which can be potentially alkylated only the NH of Val-5 underwent the reaction, albeit in low yields. The remaining crude product consisted mainly of starting material. The reaction could be initiated at −78 °C instead of the recommended −100 °C without affecting the yield of the desired product or influencing the by-product formation. No reaction took place with methallylchloride or ortho-substituted benzyl bromides. When employing MeI, a complex mixture of various mono- and disubstituted derivatives was obtained with the predominant component being (MeVal-5, MeAla-8) cyclosporin.[19,20]

In principle, any modification of CsA could influence the conformational flexibility of the peptide backbone and thus affect pre-binding equilibria.[11,21] Experimental evidence concerning these equilibria can be obtained on the basis of ^{1}H-NMR experiments with cyclosporin derivatives in polar solvents (D_2O or DMSO-d_6).[22] In DMSO-d_6, cyclosporin itself shows multiple sets of NMe resonances which are indicative of a conformational mixture, whereas derivatives substituted at the Sar-3 position of **1** are conformationally rigid, showing only one set of NMe signals.[14,17,23] Consequently, the ^{1}H NMR spectra of the obtained (*N*-alkyl)Val-5 cyclosporin derivatives **2–5** were recorded in $CDCl_3$ (routine) and in DMSO-d_6 (conformational information). Subsequent spectral analysis indicated that compounds **2–5** exist as a mixture of three major conformers in $CDCl_3$ but that they adopt a unique

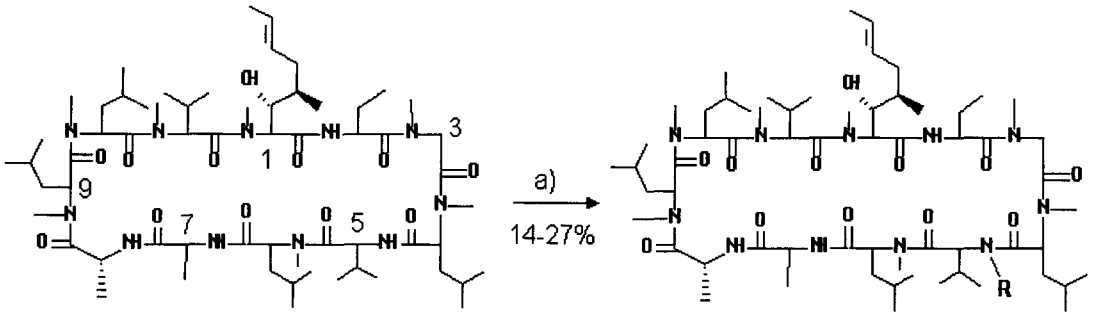

Figure 1. Synthesis and structures of N-5 substituted cyclosporin derivatives.

conformation in the relevant solvent (DMSO-d_6, Fig. 2). Therefore, the selective alkylation of the NH of Val-5 of CsA rigidified the molecule's backbone and suppressed the formation of alternate conformer populations. Moreover, both the differentiation of the chemical shifts of the $N\underline{CH_2}R$ diastereotopic protons, that of the corresponding coupling constants, as well as the low value (5.8 Hz) of some of the NH coupling constants, indicate that the conformation observed corresponds to very well structurally determined molecules.

To clarify if the unique conformation of the derivatives in DMSO-d_6 solution corresponds to their bioactive conformation, an analysis of the cyclophilin-bound structures of **2–5** was desirable. To this effect, large single crystals of **3** complexed with CypA were grown under conditions similar to those used for the CsA/CypA co-crystallization.[7] The CypA/**3** complex crystallized in the space group $P6_1$, instead of the usual $P2_12_12_1$, with $a=b=85.6$ Å, $c=53.8$ Å and one complex per asymmetric unit. These crystals allowed the high-resolution (1.9 Å) X-ray analysis of the structure to a crystallographic R-factor of 16.7%. The root-mean-square (rms) deviation between the C^α atoms of CypA (residues 2–164) in this complex and of those found in the CypA/CsA complex is 0.22 Å, showing that globally the conformation of the CypA backbone is unchanged. A comparison of the cyclosporin peptide backbone between bound **1** and bound **3** gave a rms difference of 0.27 Å for C, N, and C^α atoms, indicating that, overall, the backbone conformation of the two compounds is similar (Fig. 3). Variations can only be found for residues 4–6, with a maximum rms of 0.4 Å for N-5 (Table 1). The only noteworthy difference between **1** and **3** concerns the orientation of the MeLeu-4 side chain. Indeed, given the orientation of the bulky benzyl

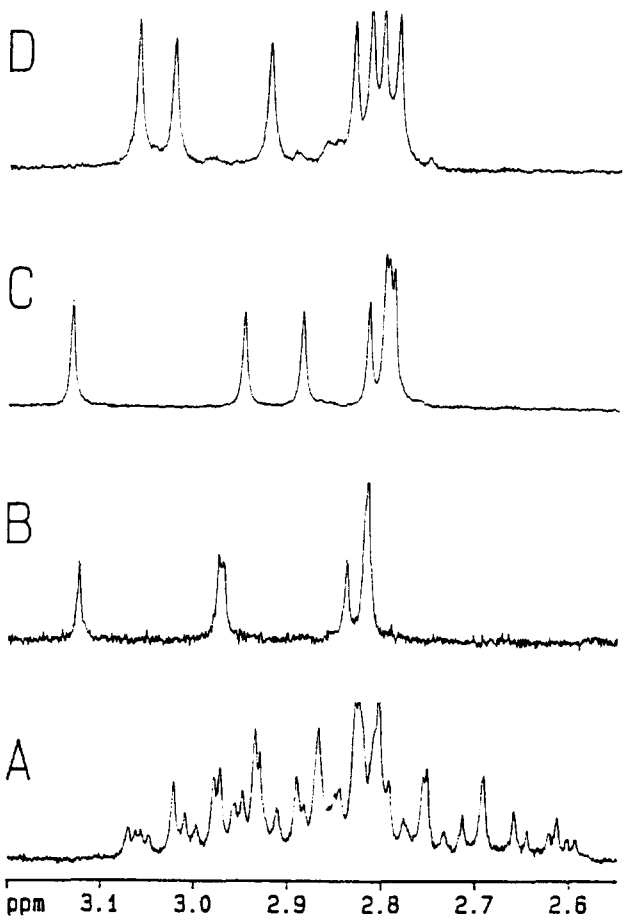

Figure 2. *N*-Methyl resonances of the ^{1}H NMR (DMSO-d_6) spectrum of **1**(A), **5**(B), **4**(C), **3**(D) . Compounds **2** and **3** display identical resonances in the 2.5–3.0 ppm region. One set of resonances, indicative of a unique conformation, is observed for the N-5 substituted compounds as compared to multiple sets for **1**.

group on N-5 in **3**, the isobutyl moiety of MeLeu-4 'turns away' from the *p*-fluorobenzyl moiety to minimize steric interactions. In particular, the χ^1- and χ^2-angles change from $-116°$ and $-53°$ in **1** to $-91°$ and 11° in **3**, respectively. Considering the mean cyclosporin plane, the p-fluorobenzyl group points to the same side as the side chains of MeBmt-1, MeLeu-4, MeLeu-6 and MeLeu-10. The values of the dihedral angles (C^α, N, CN, C^γ) and (N, CN, C^γ, $C^{\delta 1}$) are $-123°$ and $-138°$, respectively (CN, C^γ and $C^{\delta 1}$ are consecutive carbon atoms attached to the N of residue 5 in **3**).

To compare the X-ray-determined bioactive conformation of **3** with its solution conformation, the 2QF-COSY and ROESY spectra of **3** were measured in DMSO-d_6 and in a mixture DMSO-d_6/D_2O at 400 MHz. As expected, no differences either in chemical shifts or NOEs were observed. The important feature of all bioactive cyclosporin derivatives being the trans conformation of the 9,10-amide bond,[7-9,11,21-23] its conformation in **3** was investigated. The key experiment to answer this question, is the existence of NOE between the C^α-H of MeLeu-9 and of that of MeLeu-10. Due to the overlap of the resonances of the protons of interest, no such information could be obtained. Evidence for a *trans*-9,10-amide bond was extracted from the ^{1}H NMR spectrum of **3**, where no upfield resonances for the NMe groups were present. Indeed, based on the accumulated ^{1}H-NMR data with cyclosporin derivatives, it could be shown that the presence of *cis*-amide bonds leads invariably to NMe signals at ≤ 2.5 ppm. In the case of compounds **2–5**, the ensemble of the NMe resonances lay between 2.8 and 3.1 ppm (Fig. 2) indicating that they adopt an all-*trans* conformation. However, this conformation does not correspond to the bioactive one determined by X-ray crystallography. Indeed, in unbound-**3**, the presence of strong NOEs from NMe-1 and NMe-10 to the C^α-H of (N-benzyl)Val-5 and MeLeu-6, respectively as well as a NOE between NMe-10 and the NH of Ala-7 clearly indicate a different backbone conformation in the vicinity of amino acids 5–7 than the one observed in the CypA/**3** complex. Additional NOEs are found between the aromatic protons in **3** and NMe-1 suggesting that, in contrast to the bioactive conformation where the benzyl moiety points away from NMe-1, in the unbound conformation this N-5 substituent folds over the cyclosporin plane. The ensemble of the NOE experiments with **3** indicate that, in solution, residues 5–7 are close (2–4 Å) to residues 1, 10 and 11. It follows that, on approaching cyclophilin, the torsion angle of the N-5 substituent changes dramatically to allow the binding domain of the **3** to adopt its bioactive conformation.

Biological Results and Discussion

The in vitro biological activity of the synthesized compounds concerning both their binding affinity to CypA and their immunosuppressive activity is summarized in Table 1. The binding affinity to CypA was determined under competitive ELISA systems,[24] whereas the immunosuppressive activity was determined in the mouse mixed lymphocyte reaction (MLR), a standard model of cell-based immunity indicative of potential allograft rejection.[25] Furthermore, the direct interaction of the CypA/Cs derivative complex with calcineurin (CaN) was measured in a cell free system. This assay is based on the observation that the basal activity of CaN towards its classical RII phosphopeptide substrate increases in the presence of the CypA/CsA complex. The same effect is obtained when *p*-nitrophenyl phosphate (PNPP) is used as substrate. The UV-absorption of the resulting *p*-nitrophenol ($\lambda = 405$ nm) provides an easy read-out for the inhibitory activity of the CypA/**1–5** complex.[26]

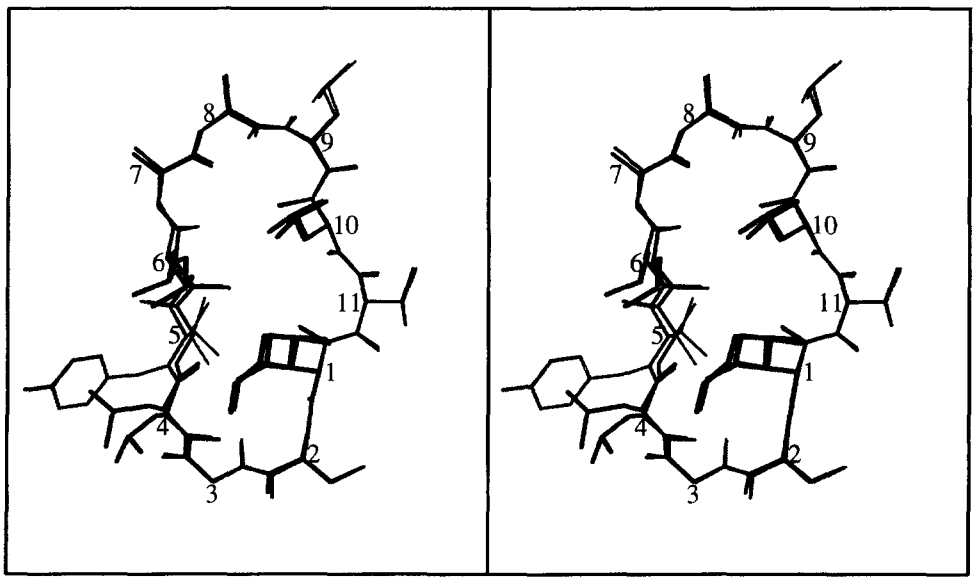

Figure 3. Stereoview of the superposition of CypA-bound CsA **1** (black) onto CypA-bound **3** (gray) obtained by making a least square fit for all atoms of **1**.

Table 1. In vitro biological activity of the compounds

R	No.	CypA[a,b] (rel IC_{50})[c]	MLR[a] (rel IC_{50})[c]	CaN[d] (%)[e]
H	**1**	190 (1.0)	10 (1.0)	0.97 (100)
$PhCH_2$	**2**	280 (1.5)	>1000 (>100)	0.20 (21)
(*p*-F)$PhCH_2$	**3**	420 (2.2)	>1000 (>100)	0.19 (21)
$CH_2{=}CHCH_2$	**4**	450 (2.3)	>1000 (>100)	0.12 (13)
$PhCH{=}CHCH_2$	**5**	350 (1.8)	>1000 (>100)	0.23 (25)

[a]Numbers indicate the mean IC_{50} values in nM of three independent experiments.
[b] Mean standard deviation is ±4.2nM.
[c] The relative IC_{50} value (rel IC_{50}) is the ratio of IC_{50} (compound)/IC_{50} CsA.
[d] UV-absorbance.
[e]Calcineurin activity compared to that of CsA (CsA set to 100%, total inhibition).

From the above results it is clear that cyclosporin derivatives **2–5** bind to their receptor with an affinity similar to that of the parent compound **1**. Their lack of immunosuppressive activity in the MLR together with their low inhibition (13–25%) of the CaN phosphatase activity, demonstrates that the substituents introduced perturb the interaction of the CypA/Cs-derivative complex with calcineurin. The molecular basis of this perturbation can not be conclusively assessed based on the available data. It can be due either to the unavailability of the NH-5 for H-bond formation with the protein or to the conformational change imposed at the MeLeu-4 side chain.

A rationale for the novel backbone conformation in **2–5** thanks to the N-alkylation of Val-5 can be found in the differences observed between the structures of CsA uncomplexed and CsA complexed with CypA. In the first case, the backbone of CsA is held together by four H-bond bridges[27] including the one between the CO of Abu-2 and the NH of Val-5 whereas, in the second, all H-bonds are absent.[7] Consequently, the unavailability of the H-bond donor at position 5 in **2–5** appears to be dentrimental for the whole intramolecular H-bonding pattern of the backbone in a zipper-like fashion. Nevertheless, although the unique solution conformation of the compounds is made-up exclusively of trans amide bonds, by extrapolation of the structural results obtained with bound-**3** and unbound-**3**, it can be concluded that their solution conformation does not correspond to their bioactive one. In spite of the apparent structural stabilization, the molecules are still flexible enough to alter their structure upon receptor binding.

Conclusion

Compared to the multiple conformations of CsA observable in polar solvents, only the substitution of Sar-3 of cyclosporins was known to stabilize a single solution conformation which corresponds to the bioactive conformation of CsA. The work described here shows that conformational stabilization can also be achieved through the substitution of the NH of Val-5. The removal of the intramolecular hydrogen bond between the carbonyl of Abu-2 and the NH of Val-5 is responsible for this phenomenon. Moreover, it has been demonstrated that together with cyclosporin residues 4 and 6, the NH-5 plays a critical role in CsA's calcineurin binding domain. Importantly, the two divergent derivation strategies (Sar-3 versus Val-5) allow the preparation of conformationally stable cyclosporin agonists or antagonists.

Experimental

Chemistry

General procedure for the synthesis of [(*N*-alkyl)Val]-5-cyclosporins (2–5). In a flame-dried round bottom flask which had been flushed with argon, cyclosporin A (**1**) (0.50 g, 0.41 mmol, 1.0 eq.) and the alkyl bromide (1.75 mmol, 4.2 eq.) were dissolved in 10 ml dry THF. The soln was cooled to −78 °C and the phosphazene-base P_4-t-Bu (1 N in hexane, Fluka) (1.75 mL, 1.75 mmol, 4.2 eq.) was added via syringe, such that the temperature did not exceed −70 °C. The reaction was allowed to reach −30 °C and then quenched with a 1 N aq solon of citric acid. THF was removed under vacuum and the aqueous residue extracted ×3 with EtOAc. The combined organic phases were dried over Na_2SO_4 and concd under vacuum. The crude product was filtered through a pad of silica gel and further purified by prep HPLC [NovaPack C_{18}, 60 Å, 6 μM, 19 mm × 300 mm; oven temperature 50 °C; flow rate 17 mL/min; eluent CH_3CN:H_2O, 70:30 (removal of unreacted CsA) then 75:25 (product isolation)].

Analytical data of N-5 substituted cyclosporin derivatives (Table 2): Characteristic ^{1}H NMR (360 MHz) resonances in DMSO-d_6 (see also Fig. 2):

Compound **2**: δ 4.3 (d, J=17 Hz, 1H, NCH_2Ph), 4.6 (d, J=17 Hz, 1H, NCH_2Ph), 6.3 (d, J=8.4 Hz, NH), 7.9 (d, J=6.6 Hz, NH), 8.5 (d, J=5.8 Hz, NH), 7.0 (d, J=6.6 Hz, 2H, Ar), 7.3 (m, 3H, Ar).

Compound **3**: δ 4.2 (d, J=17, 1H, NCH_2Ar), 4.6 (d, J=17 Hz, 1H, NCH_2Ar), 6.3 (d, J=8.5 Hz, NH), 7.9 (d, J=6.5 Hz, NH), 8.5 (d, J=5.8 Hz, NH), 7.0 (m, 2H, Ar), 7.1 (t, J=9.0 Hz, 2H, Ar)

Table 2. Yields and selected physical constants

R	No.	Yield (%)	$[\alpha]_D$ (MeOH)	c	R_t (min)[a]	MH^+[c]
$PhCH_2$	**2**	27	−169.4°	0.5	5.21[b]	1292
(*p*-F)$PhCH_2$	**3**	14	−215.7°	1.0	7.81	1310
$CH_2{=}CHCH_2$	**4**	21	−262.1°	0.5	6.72	1242
$PhCH{=}CHCH_2$	**5**	17	−184.3°	1.0	8.54	1318

[a]Analytical HPLC conditions: eluent CH_3CN:H_2O (70:30); flow rate 1.7 mL/min; column RP18 (3.9 mm × 300 mm, NovaPack); oven temperature 70°C.
[b]Eluted with CH_3CN/H_2O (75:25).
[c]Molecular ion detected in FAB mass spectrometry (Xe: 8 keV).

Compound **4**: δ 6.4 (d, J=8.2 Hz, NH), 7.9 (d, J=7.5 Hz, NH), 8.6 (d, J=6.5 Hz, NH)

Compound **5**: δ 6.3 (m, 1H, $CH_2C\underline{H}{=}CH$), 6.5 (d, J=15.0 Hz, 1H, $CH{=}C\underline{H}Ph$), 6.4 (d, J=8.6 Hz, NH), 7.9 (d, J=5.8 Hz, NH), 8.6 (d, J=6.1 Hz, NH), 7.2 (m, 1H, Ar), 7.3 (t, J=7.5 Hz, 2H, Ar), 7.4 (d, J=6.6 Hz, 2H, Ar)

X-Ray crystallographic analysis complex.

Crystal parameters	
Crystallization method	hanging drop[7]
Space group	$P6_1$
Cell dimensions	$a=b=85.6$ Å, $c=53.8$ Å $\alpha=\beta=90°$, $\gamma=120°$
Crystal size (mm)	$0.3\times0.3\times1.0$
Diffraction data:	
X-Ray source	rotating anode 40 kV, 80 mA, CuK_α
Apparatus	FAST television area detector
Unique h,k,l	16585 (94.3% complete to 1.9 Å)
No. of measurements, R_{merge}	98798, 9.5%
Phase determination and refinement	
Method	molecular replacement[28]
Model	monomeric complex without CsA[7]
Refinement	X-PLOR[28]
Quality of structure	
Resolution limits	8.0–1.9 Å
Final R-factor (all data)	0.167
No. of Cyp non-H atoms	1266
No. of **3** non-H atoms	93
No. of solvent molecules	181
Estimated error on coordinates	0.14 Å
Weighted rms deviations from ideality	
Bond length	0.014 Å
Bond angle	2.5°

Calcineurin binding assay (Dr M. Zurini). To a soln of 20 mM Hepes (pH 7.30), 1 mM $MnCl_2$, 100 μM $CaCl_2$, 1 μM cyclophilin A, 1 μM calmodulin, 1 ng/mL p-nitrophenyl phosphate and 0.5 μ of **1–5** was added bovine brain calcineurin (40 nM/ml). The reaction was stirred for 20 min at 37 °C and then a sample was monitored by UV spectroscopy (405 nm). The basal activity (no cyclosporin present) was taken as blank. For scoring of the various compounds, the value obtained for CsA was arbitrarily set to 100%.

Acknowledgments

The authors express their thanks to Dr V. Quesniaux for measuring the binding affinity of the compounds, to Dr G. Zenke for running the MLR assay and to Dr M. Zurini for measuring the calcineurin inhibition. The skillful synthetic chemistry contributions of Mr R. Haltiner and the NMR studies, necessary for the elucidation of the structure of (MeVal-5, MeAla-8)cyclosporin conducted by Mr L. Oberer are gratefully acknowledged.

References and Notes

1. Borel, J-F. *Pharmacol. Rev.* **1989**, *41*, 259.

2. Cardenas, M. E.; Muir, R. S.; Breuder, T.; Heitman, J. *EMBO J.* **1995**, *14*, 2772.

3. Fischer, G. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1415.

4. Bram, R. J.; Hung, D. T.; Martin, P. K.; Schreiber, S. L.; Crabtree, G. R. *Mol. Cell. Biol.* **1993**, *13*, 4760.

5. Etzkorn, F. A.; Stolz, L. A.; Chang, Z. Y.; Walsh, C. T. *Curr. Opin. Struct. Biol.* **1993**, *3*, 929.

6. Griffith, J. P.; Kim, J. L.; Kim, E. E.; Sintchak, M. D.; Thomson, J. A.; Fitzgibbon, M. J.; Fleming, M. A.; Caron, P. R.; Hsiao, K.; Navia, M. A. *Cell* **1995**, *82*, 507.

7. Mikol, V.; Kallen, J.; Walkinshaw, M. D. *J. Mol. Biol.* **1993**, *234*, 1119.

8. Mikol, V.; Kallen, J.; Walkinshaw, M. D. *Proc. Natl Acad. Sci. U.S.A.* **1994**, *91*, 5183.

9. Burkhardt, P.; Kallen, J.; Mikol, V.; Walkinshaw, M. In *Proceedings of the International Conference on Molecular Structural Biology*; Kungl, A., Andrew, P., Schreiber, H., Eds.; Gesellshaft Oesterreicher Chemiker, Vienna; 1995; pp 44–60.

10 Papageorgiou, C.; Borer, X.; French, R. R. *BioMed. Chem. Lett.* **1994**, *4*, 267.

11. Papageorgiou, C.; Florineth, A.; Mikol, V. *J. Med. Chem.* **1994**, *37*, 3674.

12. Rosenwirth, B.; Billich, A.; Datema, R.; Donatsch, P.; Hammerschmid, F.; Harrison, R.; Hiestand, P.; Jakse, H.; Mayer, P.; Peichl, P.; Quesniaux, V.; Schatz, F.; Schuurmann, H-J.; Traber, R.; Wenger, R.; Wolff, B.; Zenke, G.; Zurini, M. *Antimicrob. Agents Chemother.* 1994, *38*, 1763.

13. Fliri, H.; Baumann, G.; Enz, A.; Kallen, J.; Luyten, M.; Mikol, V.; Movva, R.; Quesniaux, V.; Schreier, M.; Walkinshaw, M.; Wenger, R.; Zenke, G.; Zurini, M. *Ann. N.Y. Acad. Sci.* **1993**, *696*, 47.

14. Papageorgiou, C.; Sanglier, J-J.; Traber, R. *BioMed. Chem. Lett.* **1996**, *6*, 23.

15. Bartz, S.R.; Hohenwalter, E.; Hu, M-K.; Rich, D.H.; Malkovsky, M. *Proc. Natl Acad. Sci. USA* **1995**, *92*, 5381.

16. Alberg, D. G.; Schreiber, S. L. *Science* **1993**, *262*, 248.

17. Hu, M-K.; Badger, A.; Rich, D. H. *J. Med. Chem.* **1995**, *38*, 4164.

18. For an application of this base in the benzylation of peptides, see: Pietzonka, T.; Seebach, D. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1481.

19. The structure was assigned based on COSY and ROESY NMR experiments. In addition to the signal for MeVal-5, a

second NMe signal appeared which belonged to an Ala and which had a sequential NOE with the α-H of an Ala.

20. The methylation of the Val-5 residue in low yield has already been described using BuLi and Me_2SO_4, see Seebach, D.; Beck, A. K.; Bossler, H. G.; Gerber, C.; Ko, S. Y.; Murtiashaw, W. C.; Naef, R.; Shoda, S.; Thaler, A.; Krieger, M.; Wenger, R. *Helv. Chim. Acta* **1993**, *76*, 1564.

21. Kofron, J. L; Kuzmic, P.; Kishore, V.; Gemmecker, G.; Fesik, S. W.; Rich, D. *J. Am. Chem. Soc.* **1992**, *114*, 2670.

22. Wenger, R. M.; France, J.; Bovermann, G.; Walliser, L.; Widmer, A.; Widmer, H. *FEBS Lett.* **1994**, *340*, 255.

23. Mikol, V.; Papageorgiou, C.; Borer, X. *J. Med. Chem.* **1995**, *38*, 3361.

(*Received in U.S.A.* 1 *April* 1996; *accepted* 10 *May* 1996)

24. Quesniaux, V.; Schreier, M. H.; Wenger, R.; Hiestand, P. C.; Harding, M. V.; Van Regenmortel, M. H. V. *Eur. J. Immunol.* **1987**, *17*, 1359.

25. Meo, T. The MLR test in the mouse. In *Immunological Methods*; Lefkovits, I., Pernis, B., Eds.; Academic Press: New York, 1979; pp 227–239.

26. Zurini, M. unpublished results.

27. Loosli, H-R.; Kessler, H.; Oschinat, H.; Weber, H-P.; Petcher, T. J.; Widmer, A. *Helv. Chim. Acta* **1985**, *68*, 682.

28. Brunger, A. T. *X-PLOR Version 3.1: A System for X-ray Crystallography and NMR*; Yale University Press: New Haven, CT, 1992.