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Expedited Articles

Cyclosporin Analogs Modified in the 3,7,8-Positions: Substituent Effects on Peptidylprolyl Isomerase Inhibition and Immunosuppressive Activity Are Nonadditive[1]

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Four analogs of cyclosporin A (CsA) were synthesized to determine if the biological activities of CsA analogs generated by multiple amino acid replacements are predictable from the effects on biological activity of analogs with single residue changes. CsA analogs [Phe^7]CsA (**8a**), [D-MeAla^3,Phe^7]CsA (**8b**), [D-Ser^8,Phe^7]CsA (**8c**), and [D-MeAla^3,Phe^7,D-Ser^8]CsA (**8d**) were designed by modification of positions 3, 7, and 8, which are adjacent to one effector region of the cyclophilin-bound CsA complex. The syntheses of CsA analogs **8a–d** were carried out by suitable modifications of the reported strategy. Each analog was characterized by NMR in deuterated chloroform and DMSO solutions, and their biological activities as inhibitors of *cis–trans*-peptidyl prolyl isomerase (PPIase), inhibitors of proliferation in BDF1 mouse spleen cells stimulated with concanavalin A (Con A), and inhibitors of IL-2 release stimulated with PMA/ionomycin by Jurkat cells were determined. Incorporation of the phenylalanine residue in position 7 diminished activities 5–8-fold. Substitution at position 3 decreased activity nearly 2-fold, and substitution at position 8 did not lower activities. However, when all three modifications (D-MeAla^3, Phe^7, and D-Ser^8) were incorporated into one molecule, the resulting analog, **8d**, was found to bind more tightly to cyclophilin than CsA (K_i = 3 ± 1.5 vs 6 ± 2 nM) and to produce the full immunosuppressive effect in the other assay systems. Our structure–activity results show that combinations of substitutions that individually lower PPIase or immunosuppressive activity produce fully active analogs when combined in a single compound. These results suggest that other, multimodified CsA derivatives may be discovered that possess excellent or improved immunosuppressive activities even though they contain a substitution that otherwise reduces immunosuppressive activity.

Cyclosporin A (CsA, **1**), an important therapeutic agent widely used to prevent rejection of transplanted human organs,[2] exerts its immunosuppressive effect by inhibiting T lymphocyte activation at a step preceding transcription of IL-2.[3] Cyclosporin binds to cyclophilin (CyP), a well-characterized cytosolic peptidylprolyl isomerase (PPIase),[4–7] and the resulting CsA–CyP complex binds to and inhibits the calcium/calmodulin-dependent protein phosphatase calcineurin,[8] a key enzyme in the T-cell signal transduction cascade.[9,10] These data support the hypothesis that inhibition of calcineurin by the CyP–CsA complex is the molecular basis for CsA-mediated immunosuppression.[4,5]

The conformations of CsA in solution and bound to cyclophilin[12–15] have been characterized by NMR and X-ray methods and reveal that CsA presents two distinct protein-binding surfaces. Amino acid residues 9–11 and 1–3 of CsA (Figure 1) bind to CyP, whereas amino acid residues 4–6, which do not interact closely with CyP, form a solvent-exposed surface that is pro-

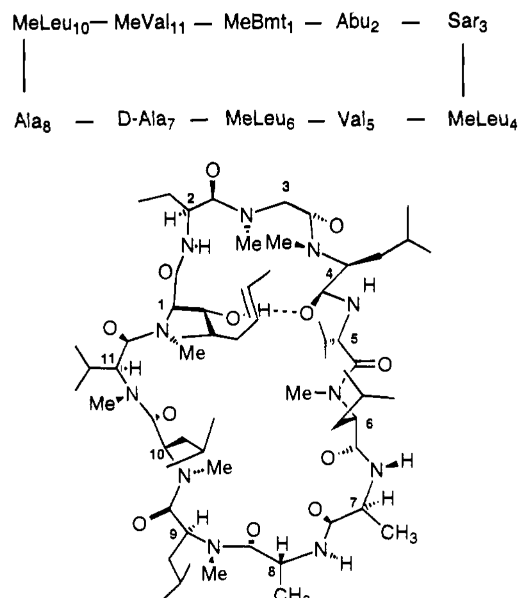

Figure 1. Cyclosporin A peptide sequence and nomenclature and schematic representation of the conformation of cyclosporin bound to cyclophilin.

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posed to interact with calcineurin.[16,17] The immunosuppressive properties of most cyclosporin analogues can be rationalized through these models.

Many natural and synthetic CsA analogs have been assayed for immunosuppressive activity in a search for analogs with diminished side effects,[11,18-26] but few analogs have been reported that possess improved therapeutic properties, and most have greatly reduced immunosuppressive activity.[27] These results have led to the prevailing impression that super active or more selective analogs of CsA may not be obtainable by modification of CsA. However, most synthetic CsA analogs prepared to date differ from the parent structure by only one amino acid residue. Although never tested rigorously, the results of these single-site modifications of CsA have been assumed to be good predictors of the effect that replacement might produce when combined with other modifications of CsA.

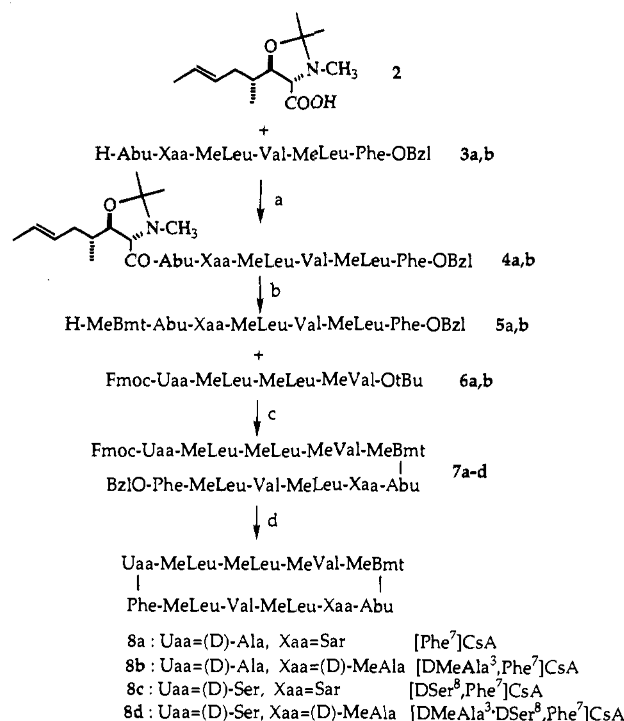
In this paper we report the first of our studies to determine if the biological activities of CsA analogs generated by multiple amino acid replacements are predictable from the effects on biological activity of analogs with single residue changes. We have designed and synthesized CsA analogs modified at positions adjacent to one effector region of the cyclophilin-bound CsA complex. Our results establish that the biological activities of multimodified CsA derivatives cannot always be predicted from the activities of the corresponding singly modified CsA derivatives.

Results

Synthesis. The synthesis of CsA analogs **8a–d** was carried out by suitable modifications of the reported strategy.[28-32] Hexapeptide fragments **3a,b**, obtained in 18% overall yield starting from H-PheOBzl, were condensed with *N,O*-isopropylidene-MeBmt (**2**) by use of DCC/1-HOBt to give the protected heptapeptides **4a,b** in 71–81% yield (Scheme 1). MeBmt was prepared according to the method of Weber and Evans[33] starting with (2*R*,4*E*)-2-methyl-4-hexenal, which was obtained by the method of Deyo et al.[34] Removal of the isopropylidene protecting group and purification of the resulting amino heptapeptides by flash chromatography (2–5% MeOH/CH_2Cl_2) gave **5a,b** in 49–68% yield, with the lower yields resulting from the formation of the methyl ester.[23,24] Tetrapeptides **6a,b** and amino heptapeptides **5a,b** were coupled by use of BOP/NMM for 3 days at room temperature.[32] The resulting undecapeptides **7a–d** were readily isolated by flash chromatography (10–40% acetone/hexane) in 42–52% yield. The final cyclization was also carried out by a modification of the method described by Wenger.[32] Saponification of the C-terminal benzyl ester and concomitant removal of the N-terminal Fmoc group of **7a–d** using ethanolic/aqueous NaOH gave crude deprotected undecapeptide intermediates. After workup, the free undecapeptides were immediately cyclized, by use of propylphosphonic anhydride/4-(dimethylamino)pyridine, in a dilute solution (2×10^{-4} M), to give CsA analogs **8a–d** in 30–70% yields. Physical constants for linear precursors **7a–d** and CsA derivatives **8a–d** are given in Table 1. High-resolution mass spectrometry data for each peptide are provided as supporting information.

Solution NMR studies of the conformations of the CsA analogs **8a–c** were carried out by using 1D and 2D NMR methods. Analogs **8a–d** are present in one

Scheme 1[a]



[a] (a) DCC/1-HOBt, NMM, 49–68%; (b) aqueous HCl/MeOH, 16 h, 79–81%; (c) BOP, NMM, 3 days, 42–52%; (d) (1) aqueous NaOH/EtOH, 5–12 h, (2) $(PrPO_2)_3$, DMAP, 2 days, 30–70%.

Table 1. Physical Properties of Linear Undecapeptides and Final CsA Analogs

product	TLC R_f (%)[a]	$[\alpha]_D$ (deg) (*c*, $CHCl_3$)	yield (%)	formula[b]
7a	0.47 (50)	−137.8 (0.52)	42	$C_{90}H_{133}N_{11}O_{15}$
7b	0.68 (55)	−124.5 (0.8)	45	$C_{91}H_{135}N_{11}O_{15}$
7c	0.50 (50)	−110.0 (0.02)	42	$C_{90}H_{133}N_{11}O_{16}$
7d	0.58 (50)	−70.9 (0.11)	53	$C_{91}H_{135}N_{11}O_{16}$
8a	0.41 (45)	−170.0 (0.03)	70	$C_{68}H_{115}N_{11}O_{12}$
8b	0.62 (60)	−216.0 (0.05)	30	$C_{69}H_{117}N_{11}O_{12}$
8c	0.38 (50)	−240.0 (0.02)	38	$C_{68}H_{115}N_{11}O_{13}$
8d	0.38 (40)	−180.0 (0.04)	31	$C_{69}H_{117}N_{11}O_{13}$

[a] Percent acetone/hexane in parentheses. [b] Exact mass was obtained by HR-FABMS as $[M + H]^+$.

predominant conformation (>95% with respect to the peptide backbone) in chloroform. NMR analysis indicates that the chemical shifts of the amide protons, the *N*-methyl protons, the α protons, and the carbonyl carbons in these [Phe[7]]CsA analogs are very similar to those for CsA (Table 2–4), indicating that all adopt a similar conformation in chloroform solution. These data also exclude the possibility that the purified products contained α-proton epimers, since epimeric analogs of these peptides have dramatically different chloroform spectra.

In comparison with the 1D ^{1}H NMR spectrum of CsA in chloroform, the [Phe[7]]CsA analogs do show some characteristic differences in that the α protons in residues 1 and 7 are shifted downfield (5.66–5.76 vs 5.47 ppm and 4.71–4.74 vs 4.52 ppm, respectively). Also, 2D correlation spectroscopy experiments establish that the upfield doublet resonances near 0.54–0.60 ppm arise from one of the two methyl groups in the Val-5 isopropyl side chain, which in CsA are found close to 0.82 ppm. These differences are due to the added phenyl group in position 7, which shields the methyl at Val-5 due to the close proximity of the 5- and 7-positions

Table 2. Chemical Shifts of Protons in CsA and Analogs

	NH				N-CH_3						
compd	2	5	7	8	1	3	4	6	9	10	11
CsA	7.96	7.48	7.68	7.17	3.51	3.40	3.11	3.25	3.12	2.70	2.71
8a	7.83	7.53	7.61	7.03	3.54	3.39	3.09	3.15	3.13	2.71	2.74
8b	8.00	7.55	7.62	7.03	3.55	3.24	3.07	3.17	3.13	2.70	2.75
8c	7.98	7.51	7.88	7.47	3.55	3.45	3.09	3.27	3.19	2.72	2.75
8d	7.96	7.52	7.88	7.46	3.56	3.27	3.07	3.26	3.18	2.71	2.75

Table 3. Chemical Shifts of Protons in CsA and Analogs

	α-H												C_β-H
			3										
compd	1	2	*re*-H	*si*-H	4	5	6	7	8	9	10	11	1
CsA	5.47	5.03	3.23	4.76	5.34	4.66	5.02	4.52	4.83	5.70	5.10	5.14	3.82
8a	5.72	5.05	3.22	4.73	5.29	4.51	5.09	4.72	4.85	5.68	5.12	5.18	3.81
8b	5.76	5.03		4.94	5.25	4.48	5.07	4.73	4.85	5.68	5.11	5.17	3.76
8c	5.66	5.02	3.21	4.73	5.14	4.55	5.05	4.71	4.87	5.69	5.08	5.17	3.88
8d	5.73	5.06		4.94	5.26	4.51	5.05	4.74	4.86	5.68	5.08	5.16	3.82

Table 4. Chemical Shifts of Carbonyls in CsA and Analogs

	carbonyl of amino acid residue										
compd	1	2	3	4	5	6	7	8	9	10	11
CsA	169.7	173.0	169.4	173.1	170.4	170.9	170.4	172.9	169.8	169.5	172.9
8a	170.3	173.6	171.2	169.9	173.9	172.1	170.8	173.3	170.3	170.0	173.2
8b	170.3	175.5	172.2	169.8	174.6	173.3	170.8	173.7	170.3	170.1	173.4
8c	170.2	173.7	171.6	170.1	173.9	172.4	171.2	173.5	170.7	170.1	172.6
8d	170.2	175.4	172.3	170.2	174.5	172.4	171.4	173.7	170.7	170.0	173.4

Table 5. Comparison of PPIase Inhibition and Immunosuppressive Activity *in vitro* of CsA Analogs

activity (nM)	CsA	8a	8b	8c	8d
PPIase (K_i)	6 ± 1	33 ± 7	6 ± 2	6 ± 2	3 ± 1.5
proliferation of BDF1 (IC_{50})	40 ± 5	248 ± 26	142 ± 27	105 ± 20	20 ± 5
IL-2 release (IC_{50})	12 ± 4	107 ± 39	62 ± 29	21 ± 12	20 ± 9

in the chloroform conformation. The phenyl group also deshields the α proton in residue 7 due to its proximity.

Biological Activities (Table 5). Inhibition of the PPIase activity of CyP by compounds **8a–d** was determined by using the previously described assay procedures.[35,36] Compound **8a** is a good inhibitor of cyclophilin (K_i = 33 ± 7 nM), whereas analogs **8b,c** are excellent PPIase inhibitors (K_i = 6 ± 2 nM), comparable to CsA itself (K_i = 6 ± 1 nM). Analog **8d**, which contains both structural modifications found in analogs **8b,c**, is a stronger PPIase inhibitor (K_i = 3 ± 1.5 nM) than CsA or any of the singly substituted compounds. In repeated side-by-side comparisons, **8d** is always a stronger PPIase inhibitor than **8c**.

In vitro immunosuppressive activities of compounds **8a–d** were determined as previously described.[22] Data for inhibition of proliferation in BDF1 mouse spleen cells stimulated with concanavalin A (Con A) and of IL-2 release stimulated with PMA/ionomycin by Jurkat cells are summarized in Table 5, which show an excellent correlation between PPIase inhibition and immunosuppressive activity for **8a–d**.

Discussion

Our structure–activity results show that combinations of substitutions that individually lower PPIase or immunosuppressive activity produce fully active analogs when combined in a single compound. We chose to modify the amino acid in position7 (Ala[7]) since very few analogs of CsA have been reported at this position. Replacement of alanine by phenylalanine produces [Phe[7]]CsA (**8a**), which has 5–6-fold diminished CyP binding and immunosuppressive activity relative to CsA. Diminished activities of this order of magnitude are not uncommon when single amino acid residues are changed in CsA. For example, Seebach and co-workers reported that [D-MeAla[3]]CsA[19,21] showed 81% of CyP binding and 61% of IL-2 release inhibition of CsA. On the other hand, [D-Ser[8]]CsA, obtained by precursor-directed biosynthesis,[39] retains full immunosuppressive activity.[38] Residues 7 and 8 are thought to border but not to participate in the critical binding to the CsA–cyclophilin and CsA–calcineurin interfaces. In contrast, residues 1, 4, and 6 of CsA are critical to immunosuppressive activity, since their removal diminishes immunosuppressive activity more than 100-fold.[42,43]

Surprisingly, when these 3- and 8-position replacements were combined with the activity-depressing Phe-7 substitution, full restoration of biological activity was obtained (Table 5). Addition of a methyl group at position 3 to form [D-MeAla[3],Phe[7]]CsA (**8b**), or addition of a polar hydroxy group at the 8-position to form [D-Ser[8],Phe[7]]CsA (**8c**), restored much of the CyP binding (after extension of preincubation time)[40] and T-cell inhibition properties of the analogs, but when all three modifications (D-MeAla[3], Phe[7], and D-Ser[8]) were incorporated, the resulting analog **8d** was found to bind more tightly to CyP than CsA (3 ± 1.5 vs 6 ± 2 nM) and to produce the full immunosuppressive effect in both assay systems (Table 5).

These results clearly show that the lost activity caused by a single substitution (Phe-7) can be restored by multiple modifications of additional amino acids. What is most striking is that neither the 3-substituent nor the 8-substituent by itself increases the activity of the resulting analog, yet together they more than

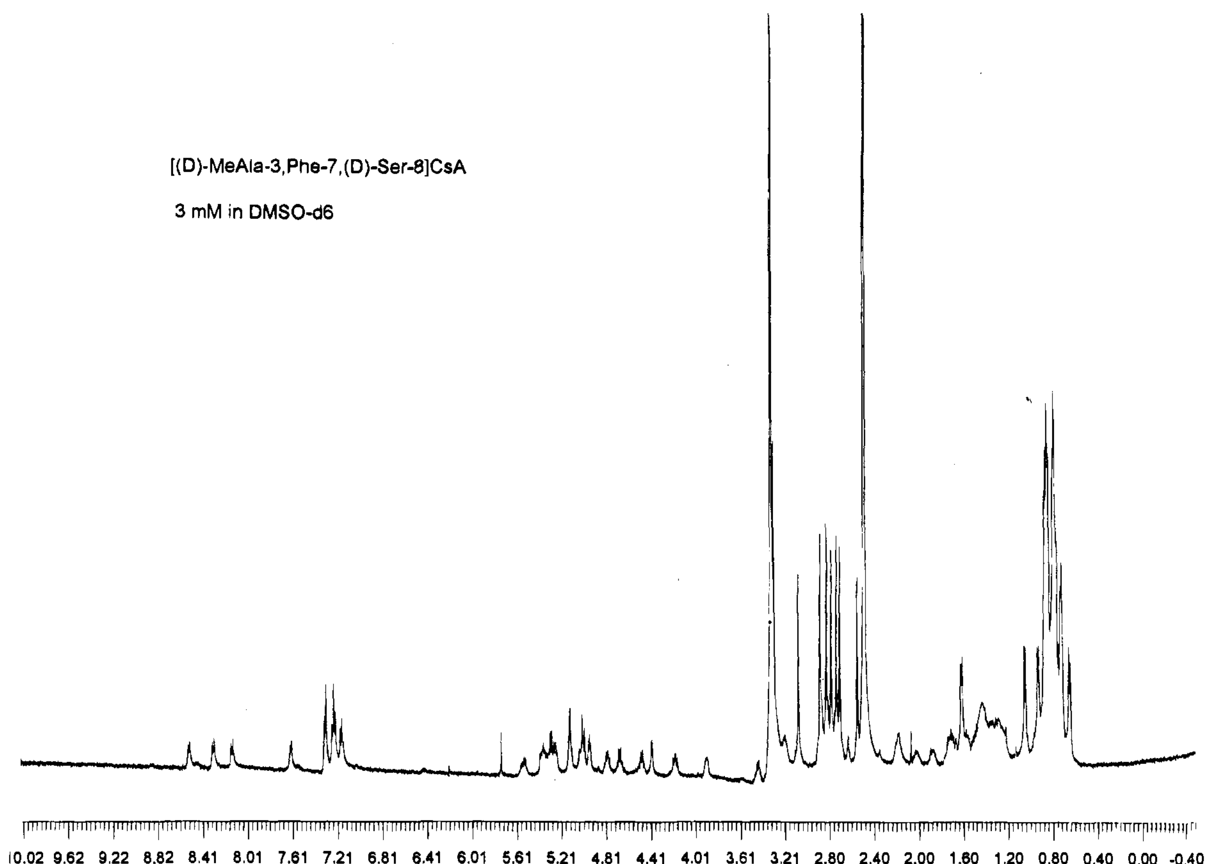


Figure 2. ^{1}H NMR (500 MHz) of [D-MeAla^3,Phe^7,D-Ser^8]CsA (**8d**) in DMSO-d_6, concentration 3 mM, at 20 °C. Peaks at 2.45 and 3.25 ppm are from DMSO and H_2O.

compensate for the lost activity when combined with the Phe-7 substitution. These results suggest that other, multimodified CsA derivatives may be discovered that possess excellent or improved immunosuppressive activities even though they contain a substitution otherwise known to dramatically reduce immunosuppressive activity. Although many CsA analogs have been synthesized and isolated, they represent only a tiny fraction of potential analogs. The discovery that nonadditive effects are possible with multiple substitutions in the CsA system may lead to an increased interest in the synthesis of CsA derivatives, especially since some derivatives can be synthesized by solid phase methods.[41] The strong inhibition of PPIase activity shown by **8d** is of special interest since nonimmunosuppressive CsA analogs have been shown to inhibit HIV replication in newly infected cells,[42,43] and this remarkable activity correlates with tight binding to cyclophilin.[44]

In an attempt to see if conformational differences between analogs **8a–d** and CsA might explain the restored activities, we compared the conformations of these compounds as determined by NMR in DMSO. Wenger and co-workers have shown that the bioactive conformation of CsA bound to CyP is nearly identical with the conformation of a water-soluble CsA derivative, [D-MeSer^3,D-Ser(O-Gly)^8]CsA, in water and in a DMSO solution that contained a small amount of water.[45] The Sandoz work showed that the substitution of D-MeSer for Sar in position 3 of CsA stabilizes a single solution conformation compared to multiple conformations of CsA observable in polar solvents. Their results confirmed that the bioactive conformation of CsA is formed upon dissolution in water, as had been anticipated on the basis of enzyme inhibition kinetic studies[36,46] and X-ray crystallography of a CsA–FAB complex.[47] Since the bioactive conformation of 3-substituted CsA derivatives is also formed in their DMSO solution, solvation of amide NH bonds plays a major role in inducing the new conformation, which may be partially stabilized by hydrophobic clustering.[46] Our NMR data (Figure 2) show that [D-MeAla^3,Phe^7,D-Ser^8]CsA (**8d**) also adopts predominately a single conformation in DMSO-d_6. The spectrum for **8d** is remarkably similar to the spectrum of the [D-MeSer^3,D-Ser^8]CsA derivative reported by Wenger et al.,[45] and the spectra for both analogs are very different from the DMSO spectrum of CsA, which shows signals for about seven conformers. Consequently, we conclude that the peptide backbone conformation of **8d** is closely similar to that determined for [D-MeSer^3,D-Ser^8]CsA. Presumably the added methyl group in D-MeAla at position 3 of **8d** stabilizes the conformation of the peptide ring system.

NMR spectra of CsA analogs dissolved in DMSO also provide a convenient way to screen CsA derivatives that are not soluble in water to see if they adopt stabilized bioactive conformations. Since the NMR chemical shifts and coupling constant data obtained for [D-MeAla^3,Phe^7]-CsA and [D-MeAla^3,Phe^7,D-Ser^8]CsA in DMSO are closely related to each other (data not shown) and to those reported for Sandoz's [D-MeSer^3,D-Ser(O-Gly)^8]CsA,[45] the stronger PPIase inhibition activity of **8d** vs **8b** cannot be due solely to conformational stabilization. Both derivatives contain the conformationally stabilizing substitutent at position 3. It follows that the enhanced PPIase inhibition, relative to **8b**, obtained with [D-MeAla^3,Phe^7,D-Ser^8]CsA (**8d**) is due either to an increased preorganization of the bioactive conformation that is not detectable by NMR or to additional but subtle

interactions between residues flanking the effector regions and cyclophilin. In either case, it is clear that the structure–activity relationships of cyclosporin analogs with multiple substitutions will be more complex than predicted by extrapolating data obtained from analogs with single substitutions. CsA analogs with improved CyP binding and/or immunosuppressive activities remain a possibility.

Experimental Section

I. Synthesis Procedures.[23,29] **General Procedure A: Synthesis of Heptapeptides by Coupling of Acetonide-Protected MeBmt with Hexapeptide Amine Analogs (H-Abu-Xaa-MeLeu-Val-MeLeu-Phe-OBzl). 1. Acetonide Formation of MeBmt.** A suspension of MeBmt (0.2 mmol, 1 equiv) in freshly distilled acetone (60 mL) was heated to reflux under N_2 for 24 h until an almost clear solution appeared. The solution was concentrated in vacuo down to 1.5 mL which was directly used for the next coupling reaction without further purification.

2. Coupling Reaction. A solution of freshly prepared acetonide-protected amino acid **2** (0.2 mmol, 1 equiv) in acetone (1.5 mL) was added to 3 mL of THF, *N*-methylmorpholine (0.22 mmol, 1.1 equiv), 1-hydroxybenzotriazole (0.44 mmol, 2.2 equiv), and hexapeptide amines **3a,b** (0.22 mmol, 1.1 equiv). The resulting mixture was cooled to 0 °C, and DCC (0.22 mmol, 1.1 equiv) was added. The mixture was allowed to warm up to room temperature and stirred under N_2 for 20 h, after which time the precipitated DCU was removed by filtration and washed with a small portion of methylene chloride. The combined filtrate was washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$, and concentrated in vacuo. The residue was dissolved in ethyl acetate and filtered to remove more DCU. The residue obtained after the second filtration was purified by chromatography with 10–40% acetone in freshly distilled *n*-hexane to give the title compounds **4a,b**.

General Procedure B: Removal of the *N,O*-Isopropylidene Protecting Group of Heptapeptides (Residues 1–7). A solution of *N,O*-isopropylidene heptapeptide **4a** or **4b** (0.156 mmol) in 3 mL of MeOH was stirred with 1 N HCl aqueous solution (0.6 mmol, 4 equiv) at room temperature for 15 h. The reaction mixture was treated with $NaHCO_3$ (2 mmol) and concentrated in vacuo to a white solid. The residue was taken up into 2% MeOH in CH_2Cl_2 and flash-chromatographed with 2–4% MeOH in CH_2Cl_2 to give title compound **5a** or **5b**.

General Procedure C: Synthesis of Linear Undecapeptides. A solution of amino heptapeptide **5a** or **5b** (residues 1–7) (0.1 mmol) and N-protected tetrapeptide[30] **6a** or **6b** (residues 8–11) (0.15 mmol, 1.5 equiv) in CH_2Cl_2 (2 mL) was treated sequentially with *N*-methylmorpholine (0.2 mmol) and BOP reagent. The reaction mixture was sealed tightly and stirred at room temperature under N_2 for 3 days. The mixture was then diluted with CH_2Cl_2 (15 mL) and water (10 mL). The aqueous layer was extracted with additional CH_2Cl_2 (3 × 10 mL), and the combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The resulting residue was purified by flash chromatography on silica gel with 10–40% acetone in freshly distilled *n*-hexane to give pure fully protected undecapeptides **7a–d**. Some impurities with higher R_f values, possibly another undecapeptide epimer or unreacted substrates, were usually removed during the chromatographic process.

General Procedure D: Synthesis of Cyclosporin Analogs. 1. Removal of Fmoc and OBzl Ester Protecting Groups. A solution of the protected undecapeptides **7a–d** (0.05 mmol) in EtOH (2 mL) was flushed with N_2 and cooled to 0 °C. The mixture was treated with 0.2 N aqueous NaOH solution (0.5 mL) and stirred for 1.5 h; an additional portion of 0.2N NaOH solution (0.25 mL) was added, and stirring was continued at 0 °C for 3.5–12 h. The reaction mixture was then neutralized to pH 6 with 0.2 N aqueous HCl solution (0.75 mL) and washed with brine (10 mL) and CH_2Cl_2 (20 mL). The aqueous layer was then extracted with additional CH_2Cl_2 (4 × 10 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo to dryness to give a clear oil which was used directly in the next reaction.

2. Cyclization to Cyclosporin Analogs. The oily residue (0.05 mmol) was dissolved in CH_2Cl_2 (200 mL) and treated sequentially with DMAP (0.25 mmol) and propylphosphonic anhydride (50% w/v solution in CH_2Cl_2; Fluka) according to the method of Wenger.[32] The reaction mixture was stirred at room temperature under N_2 for 2 days, concentrated down to 1–2 mL, and applied directly to a silica gel column. Flash chromatography with 10–40% acetone in freshly distilled *n*-hexane gave pure cyclic undecapeptide compounds **8a–d**.

II. Physical properties of linear undecapeptides **7a–d** and final CsA analogs **8a–d** are summarized in Table 1. The ^1H NMR (500 MHz, $CDCl_3$) spectra of **8a–d** and high-resolution of FAB data are provided as supporting information.

III. Biological Assays. Cyclophilin-Binding Test. PPIase Assays. A stock solution of CsA and analogs **8a–d** (1–2 mM) was prepared in dried THF and serially diluted to give secondary stock solutions 10 times more concentrated than the concentration desired in the enzyme assay (between 5 and 50 nM). The final 1:10 dilution was performed with dried THF. The substrate Suc-Ala-Ala-Pro-Phe-pNA was dissolved in 0.47 M LiCl in THF. The population of the cis-trans Ala-Pro conformer of the substrate was determined by a previously reported method[35,36] (typically 30–40% cis). A stock solution of cyclophilin (18 μM, 50 μL) was added to the thermally equilibrated buffer at 0 °C (1 mL, 50 mM Hepes-NaOH, 100 mM NaCl, pH 8.0, 0 °C). A serially diluted solution of the inhibitor (final concentration 100 μM–50 nM) was then added, and the components were mixed. The resulting aliquots of the enzyme–inhibitor mixture were withdrawn (each time 890 μL) at 4 and 24 h preincubation and assayed for PPIase inhibition after addition of the substrate (10 μL) and chymotrypsin (100 μL) as described elsewhere.[35,36]

Immunosuppressive Activity Test. Reagents: Concanavalin A (Con A) was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and dissolved in RPMI-1640 medium (Flow Laboratories, Rockville, MD) that was supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (Grand Island Biological Co., Grand Island, NY) and 10% heat inactivated (56 °C, 30 min) bovine calf serum. This medium is referred to as RPMI-10.

Con A-Stimulated Activation. Spleen cells from BDF1 mice were established in RPMI-10 at 5 × 10^6/mL, and 100 μL aliquots were dispensed into 96-well round-bottomed microtiter plates (Linbro, Flow Laboratory). Cyclosporin A and analogs were added at varying dilutions, and Con A (5 μg/mL) was added as mitogen. The final volume in the microtiter wells was adjusted to 200 μL with RPMI-10.

Cell cultures were incubated for 72 h at 37 °C in 5% CO_2 atmosphere and pulsed with 0.5 μCi [^3H]thymidine for the last 16 h of culture. The cells were harvested on an automated multiple sample harvester, and cell-associated radioactivity was counted in a Beckman liquid scintillation counter. The results are expressed as the mean values derived from quadruplicate measurements. Cell viability was determined by trypan blue exclusion after 72 h of incubation. Data are plotted in Figure 5 (supporting information) and IC_{50} values given in Table 5.

IL-2 Assays. Jurkat cells were established in 96-well flat-bottomed microtiter plates (Linbro) at 2 × 10^5/mL. Compounds to be tested were suspended in RPMI-10 and added at various dilutions. Phorbol myristate acetate (PMA) at 1000 ng/mL and ionomycin at 125 ng/mL were then added, and the volume in the wells was adjusted to 200 μL. Then the cultures were allowed to incubate overnight, and the IL-2 level in the supernatants was determined by an ELISA assay (IL-2 EIA Kit, Advanced Magnetics, Cambridge, MA). Data are plotted in Figure 6 (supporting information) and IC_{50} values given in Table 5.

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Supporting Information Available: ^{1}H NMR spectra for CsA analogs **8a–d** in chloroform and biological data (10 pages). Ordering information is given on any current masthead page.

References

(1) Nomenclature and symbols of amino acids and peptides generally follow the recommendations of the IUPAC-IUB Joint Commission of Biological Nomenclature (*Pure Appl. Chem.* **1984**, *56*, 595.) Additional abbreviations: BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; CyP, cyclophilin; DCC, dicyclohexylcarbodiimide; FABMS, fast atom bombardment mass spectrometry; Fmoc, [(9-fluorenylmethyl)-oxy]carbonyl; HOBt, hydroxybenzotriazole; MeBmt, *N*-methyl-(4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine; NMM, *N*-methylmorpholine; PPIase, peptidylprolyl cis–trans isomerase.

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