Synthesis, Conformation, and Immunosuppressive Activity of a Conformationally Restricted Cyclosporine Lactam Analogue[1]

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Cyclosporine A (CsA, 1), an immunosuppressive cyclic undecapeptide, in apolar solvents adopts a II′ β-turn at the Sar[3]-MeLeu[4] residues. [D-Proline[3]]Cs has been reported to be a nonimmunosuppressive analogue in which the II′ β-turn is retained. In order to determine if this loss of activity is caused by steric hindrance between the Cs analogue and its receptor or is caused by a change in the peptide conformation, an analogue that stabilizes a II′ β-turn has been synthesized, [lactam[3,4]]Cs (4). We also have studied the solution conformation of two other analogues, [D-MeAla[3]]Cs (2) and [L-MeAla[3]]Cs (3). The conformations have been established by 1D difference NOE and 2D (NOESY or ROESY) NMR. The conformations of [lactam[3,4]]Cs (4) and [D-MeAla[3]]Cs are indistinguishable from that of CsA in solution. [L-MeAla[3]]Cs (3) was found to adopt a conformation with a cis amide bond between Sar[3] and MeLeu[4]. The inhibition of concanavalin A stimulated thymocytes by CsA (1), [D-MeAla[3]]Cs (2), [L-MeAla[3]]Cs (3), and [lactam[3,4]]Cs (4) gave IC_{50} values (nM) of 5, 6, 100, and 100, respectively. The weak immunosuppressive activity of [lactam[3,4]]Cs (4) possessing the II′ β-turn suggests that the loss of activity for 4 is due to steric hindrance with the Cs receptor.

Cyclosporine A (CsA, 1),[2a,b] an unusually effective immunosuppressive agent[2c] first isolated from *Tolypocladium inflatum gams*,[3] is a cyclic undecapeptide marketed as Sandimmune that is used to suppress rejection of transplanted human organs.[4] In addition, CsA has been reported to have biological activities that might be useful for treatment of other human diseases,[5] although the nephrotoxicity of cyclosporin has limited its use in other applications.[6] Thus, new cyclosporines with improved biological specificities could prove to be useful agents.

We report herein our first attempt to determine the bioactive conformation of this important drug by means of synthesizing conformationally constrained analogues of cyclosporine. The strategy of constraining the conformation of biologically active peptide hormones, notably somatostatin, α-MSH, LH-RH, vasopressin, and certain enzyme inhibitors, e.g., renin inhibitors,[7a-g] has provided important information about the permissible conformations of each of these peptides when it is bound to the target receptors, and has been used to guide the design of new analogues.

The structure of CsA (1) was established by chemical degradation of the natural peptide[8] and confirmed by X-ray crystallography of both an iodocyclosporine derivative and CsA itself.[9,11] In addition, Wenger, in an elegant series of papers,[10a-f] has described the total synthesis of cyclosporine and has provided most of the structure–activity information available to date. A solution conformation of CsA has been determined by Kessler and colleagues,[11] who found close similarities between the solution and solid-state conformations with respect to the conformation of the 33-membered peptide ring system. Much of the published structure–activity data has been rationalized in terms of this conformational model.

While the chloroform solution and solid-state conformations of CsA are very similar, hydrogen-bonding solvents easily alter the conformation of the 33-membered ring system by disrupting intramolecular hydrogen bonds. Our preliminary studies established that a few percent of DMSO added to a chloroform solution of CsA led to multiple NMR resonances that were consistent with multiple solution conformations of CsA. Since any of these uncharacterized conformations might be the conformation acting at the receptor (the bioactive conformation), we set out to find conformationally constrained analogues of CsA that might clarify whether the solution conformation of CsA persisted at the CsA receptor or receptors.

One distinguishing feature of the CsA conformation is the presence, both in solution and in the crystal structure,

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(1) IUPAC–IUB Joint Commission on Biochemical Nomenclature. Nomenclature and symbolism for amino acids and peptides, recommendation 1983. *Eur. J. Biochem.* **1986**, *138*, g. Additional abbreviations used are DMSO, dimethyl sulfoxide; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; THF, tetrahydrofuran; pTos-Cl, *p*-toluenesulfonyl chloride; TFA, trifluoroacetic acid; DMF, *N,N*-di-methylformamide; DEAD, diethyl azodicarboxylate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; α-MSH, α-melanotropin stimulating hormone; LH-RH, luteinizing hormone-releasing hormone; NMR, nuclear magnetic resonance.

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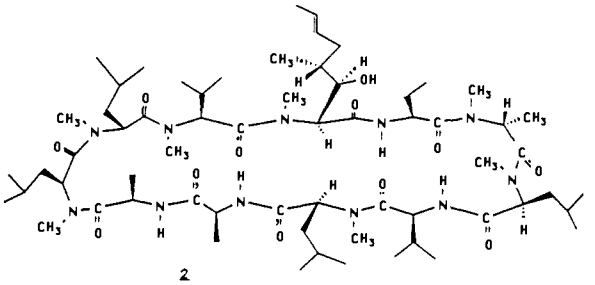
of a type II′ β-turn involving residues 2–5 (Figure 1). The second position of a type II′ β-turn (Sar, sarcosine in this case) can accommodate a D-amino acid but not an L-amino acid, the latter being destabilized by a steric interaction between the added side chain and the following *N*-methyl group.[12] Wenger[10b] had found that [D-MeAla^3]Cs (**2**) retained good immunosuppressive activity while the [L-MeAla^3]Cs (**3**) derivative was much less potent. Surprisingly, [D-Pro^3]Cs, which can also adopt a type II′ β-turn conformation, was inactive, suggesting either that the type II′ β-turn was not retained in the bioactive conformation of CsA or else that the extra carbon in the proline ring relative to D-MeAla was attenuating the biological response, presumably due to steric hindrance between [D-Pro^3]Cs and the receptor.



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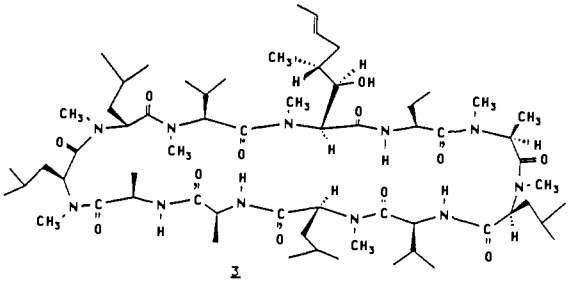

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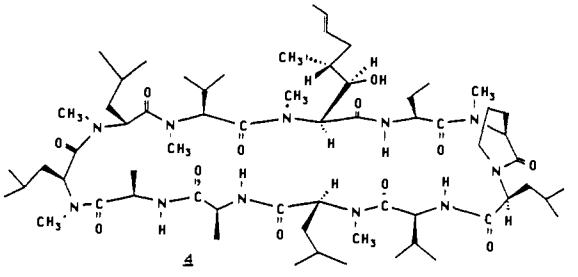

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To differentiate between these possibilities, we have developed a new synthesis of a 6-membered lactam, dipeptide isostere (**8**), which corresponds to a conformationally constrained analogue of L-MeAla-L-MeLeu. Lactam **8** has been incorporated into a new cyclosporine derivative, [lactam^{3,4}]Cs (**4**). We have also studied the conformations of [D-MeAla^3]Cs (**2**), [L-MeAla^3]Cs (**3**), and [lactam^{3,4}]Cs (**4**) in order to help correlate the biological activities of these analogues with the primary structure and solution conformations.

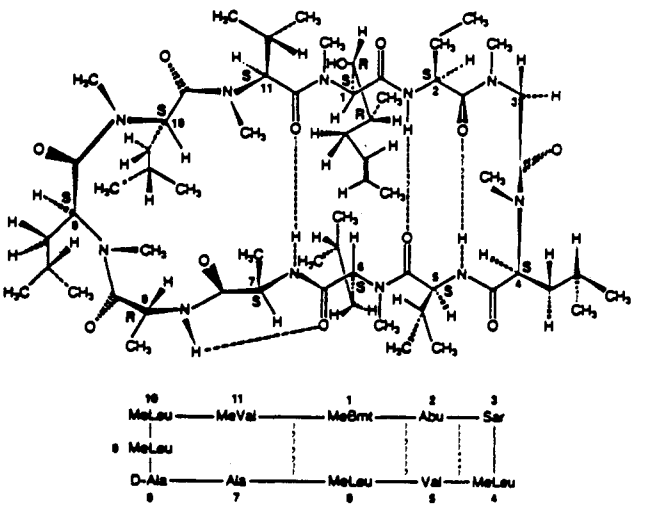
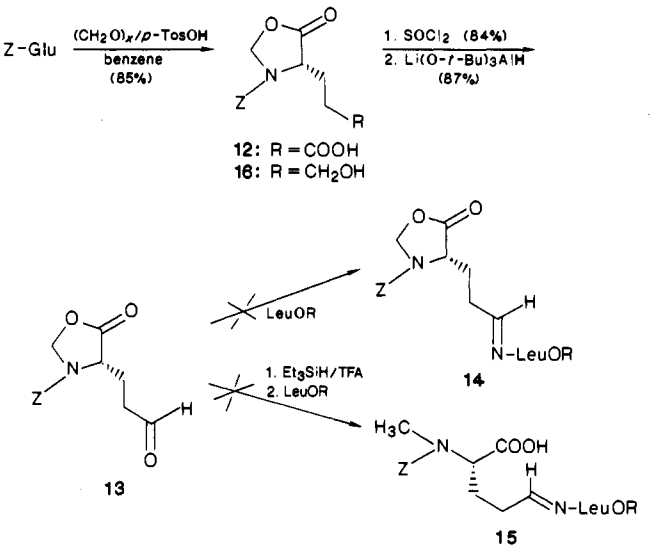


Figure 1. Schematic representation of the structure of cyclosporin A (CsA, **1**). MeBmt is an abbreviation for 2-*N*-methyl-(*R*)-4-((*E*)-2-butenyl)-4-methyl-L-threonine. The configuration of L-threonine is 2*S*,3*R*. Conventions used to number amino acid positions and side-chain functionality follow that suggested by Wenger.[10a] The three-dimensional conformation is derived from Kessler et al.[11a]

Scheme I



Results

Synthesis of [Lactam^{3,4}]Cs (4). The synthesis of the peptide portion of [lactam^{3,4}]Cs (**4**) closely followed the strategy employed by Wenger[10e] for synthesis of CsA (Figure 2). The tetra- and heptapeptide fragments **6** and **7** were assembled by the stepwise coupling strategy shown by the arrows (Figure 2) and coupled together to give the undecapeptide **5**. The 6-membered lactam dipeptide moiety **8** was synthesized separately (vide infra) and then coupled first with Boc-Abu to form a tripeptidyl analogue **9** that corresponds to residues 2–4 of CsA. This fragment was attached to the tripeptide **10** to give the desired hexapeptide derivative **11**. Syntheses of heptapeptide **7**, undecapeptide **5**, and [lactam^{3,4}]Cs (**4**) were achieved by methods derived from reported procedures.[10e,f,13,14]

Several approaches to the stereospecific synthesis of the optically pure N-methylated dipeptidyl lactam **8** were

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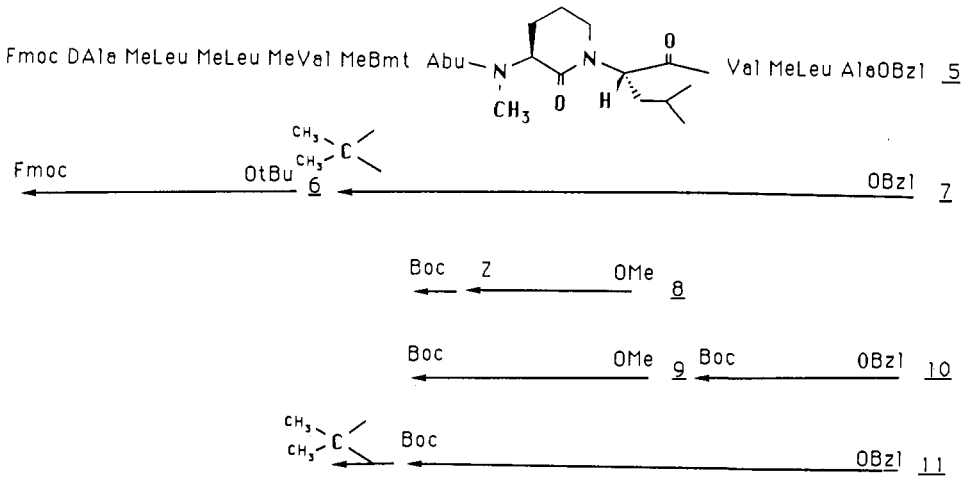


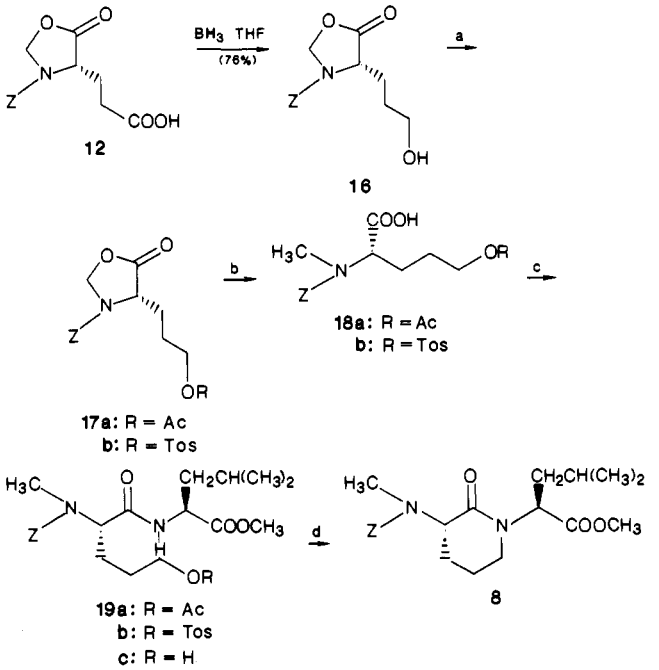
Figure 2. Strategy used for the synthesis of linear peptide [lactam^{3,4}]Cs precursor **5**.

evaluated. The synthesis of optically pure 5-membered lactam-bridged dipeptide, where both amino acids are chiral, has been described by Freidinger.[15] However, because preliminary molecular modeling studies (data not shown) suggested that the 6-membered lactam would be tolerated by the type II′ β-turn in CsA, we decided to synthesize the corresponding 6-membered lactam **8**. Several attempts to use a variation of the strategy of Freidinger to synthesize **8** were unsuccessful (Scheme I). Because the alcohol **16b**[16,17] is known to be unstable (apparently the 6-membered lactone is formed by intramolecular cyclization), we synthesized the aldehyde **13**, which is known to form an oxime with *O*-benzylhydroxylamine.[16] Several attempts to synthesize imine **14** by reaction of **13** with leucine methyl ester were unsuccessful, so that the planned reduction of the oxazolidinone to the N-methylated amino acid and reduction of the imine to the amine followed by cyclization to the required protected dipeptide **8** could not be attempted. The alternate route involving reduction of the oxazolidinone **12** to the N-methylated amino acid[17] and subsequent formation of the imine **15** did not work (Scheme I).

Lactam **8** was successfully prepared by the route shown in Scheme II. Careful reduction of acid **12** at −30 °C with diborane and quenching at −30 °C with 10% methanolic acetic acid gave alcohol **16**, which could be utilized for the dipeptide synthesis. Alcohol **16** was protected by acetylation (**17a**). Reduction of the oxazolidinone gave the protected *N*-methyl derivative **18a**, which was coupled to leucine methyl ester to give dipeptide **19a** in good yield (58%, beginning from **12**).

Selective removal of the acetate (1 equiv of NaOH in MeOH) was successful, but various attempts to activate alcohol **19c** failed (see Scheme II, method A). Fortunately, the tosylated alcohol **17b**, prepared from **16** under special conditions (see the Experimental Section), survived the conditions needed for reduction of the oxazolidinone (→ **18b**) and the condensation with leucine methyl ester (→ **19b**). The latter reaction, coupling with BOP-Cl/ethyldiisopropylamine **19**, gave some side products, but because of the lability of *p*-tosyl dipeptide **19b** formed, the side products were not removed nor were the reaction conditions optimized. The dipeptide **19b** was cyclized in 30–40% yield, beginning from **18b**, to give the required

Scheme II[a]



[a] Method A. R = Ac: (a) Ac_2O/py/DMAP (quantitative); (b) Et_3SiH/TFA; (c) BOP-Cl/HCl-$LeuOCH_3$ (58% from **17a**); (d) cyclization failed. 1. NaOH/MeOH (86%). 2. DEAD/Ph_3P, Ph_3P/CCl_4Et_3N, PH_3P/CBr_4/DBU, $SOCl_2$, BOP-Cl/(*i*-Pr)$_2$EtN. Method B. R = Tos: (a) TosCl/py; (b) Et_3SiH/TFA (58% from **12**); (c) 1. BOP-Cl/HCl·$LeuOCH_3$, 2. NaH in CH_2Cl_2/DMF 30–40% from **18b**.

protected dipeptide **8** (92% diastereomericly pure). The yield of this reaction (peptide coupling → **18b**, cyclization → **19b**) is acceptable and provided enough lactam **8** to enable us to carry out the synthesis of the corresponding CsA analogue. In view of the low biological activity of the final product, no further attempts to optimize the yield by changing the base and using lower temperatures were carried out.

NMR. In order to establish the conformations of the 3–4-position-modified cyclosporine analogues **2–4**, complete assignment of the protons and carbons was accomplished at 500 MHz by a combination of 1D and 2D NMR techniques. Each 1D ^1H NMR spectrum displayed at 23 °C only one or a major conformation in the $CDCl_3$ spectra (Figure 3A). The proton chemical shifts were assigned by using the same NMR methods described by Kessler et al. in his elegant study of the conformation of cyclosporine A.[11] The direct couplings were determined from the ho-

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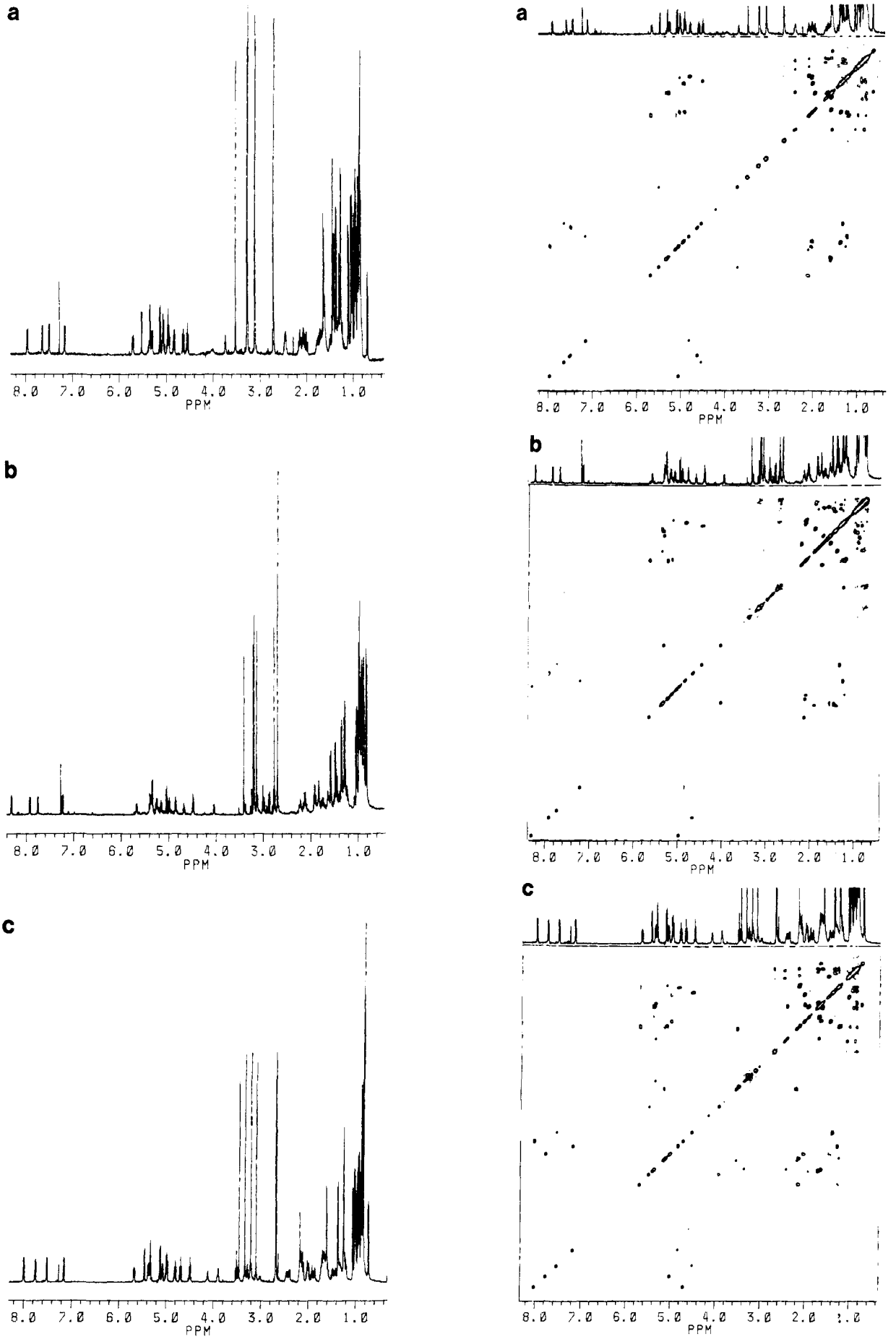
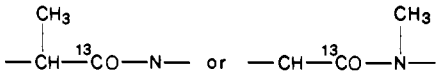


Figure 3. (A, left) 1D NMR spectra in chloroform for three cyclosporin analogues: (a) [D-MeAla^3]Cs (**2**), (b) [L-MeAla^3]Cs (**3**), (c) [lactam^{3,4}]Cs (**4**). (B, right) COSY spectra in chloroform for (a) **2**, (b) **3**, and (c) **4**.

Scheme III



monuclear 2D correlation COSY[20] shown in Figure 3B. A second COSY (COSY-long range)[21] was carried out in order to observe specifically the small couplings in the molecule (data not shown). This experiment allowed us to assign each of the NCH_3 resonances from the coupling between the α-protons and the *N*-methyl protons of the same and/or the following residue.

The chemical shifts of the amide protons, α-protons, and NMe protons are reported in Table I. The assignments for the protons in the amino acid side chains are not reported here.

The assignment of carbon resonances in the cyclic peptides was carried out at 75 MHz by using a DEPT[22] experiment to determine the multiplicity of the carbons and a heteronuclear correlation was used to identify the protonated carbons. The assignment of the carbonyl resonances was achieved by use of a COLOC[23] experiment which highlights the ^3J couplings between the carbonyl and a methyl group located on a neighboring atom (Scheme III). Because of the limited supply of the various CsA analogues available to us (8 mg of each sample), coupling to the α-proton was not observed for the three analogues we studied in contrast to the results obtained with CsA.[11] All resonances of carbonyls bonded to a NCH_3 group and/or adjacent to a $CHCH_3$ group have been characterized. In the case of the [lactam^{3,4}]Cs (**4**), coupling between the MeBmt^1-CO and the MeBmt^1-αCH permitted the identification of the MeBmt^1 carbonyl carbon. For the two cases where no coupling to a methyl was possible, (MeLeu^6-CO and MeLeu^4-CO), the assignments were made by analogy with the literature assignments for CsA. Table II lists the assignments of the chemical shifts for the carbonyl carbons, α-carbons, and NCH_3 carbon resonances.

Conformational Analysis. General Methods. The variations in the chemical shifts and coupling constants of the amide protons as a function of the temperature were determined (Table III) in order to identify changes either in hydrogen bonding to these protons or in the conformation of the peptide ring system. In order to determine if the ring system conformation of the analogues was invariant with temperature, the coupling constants were measured at two different temperatures (Table IV). Only one significant change in the coupling constant of the amide protons was observed at low or room temperature, that for the Abu^2-NH, which appears as a broad singlet at 243 K. We conclude that variations in temperature did not significantly alter the overall cyclic peptide conformations of the analogues.

It is known that the variation of the chemical shift of an amide proton as a function of the temperature gives information on the presence and strength of the hydrogen bonds involving this proton. It has been reported that the amide protons of CsA have a different behavior at low and high temperature.[11] Therefore, we measured the tem-

Table I. Chemical Shifts for Cyclosporin and Cyclosporin Analogues ($CDCl_3$, 23 °C)

	NH				α-CH									NMe						
	2	7	5	8	1	2	H_{Re}	H_{Si}	4	5	6	7	8	9	10	11	1	3	4	6	9	10	11
CSA[11]	7.96	7.68	7.48	7.17	5.47	5.03	3.23	4.76	5.34	4.66	5.02	4.52	4.83	5.70	5.10	5.14	3.51	3.40	3.11	3.25	3.12	2.70	2.71
[Me-D-Ala^3]Cs	7.90	7.62	7.45	7.12	5.48	5.03		4.92	5.26	4.60	4.92	4.50	4.79	5.67	5.03	5.10	3.48	3.22	3.06	3.24	3.08	2.66	2.67
[Me-L-Ala^3]Cs	7.89	7.72	8.29	7.20	5.29	4.63	5.29		5.29	4.92	5.19	4.43	4.80	5.62	5.11	5.01	3.36	3.15	2.71	3.07	3.16	2.65	2.64
[lactam^{3,4}]Cs	7.70	7.47	7.95	7.11	5.42	4.96	3.48		5.35	4.66	4.96	4.46	4.80	5.64	5.04	5.11	3.43	3.31		3.18	3.07	2.65	2.65

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Table II. Carbon Assignments for Ring System Atoms of Cs Analogues

	carbonyls				α-C				NCH_3			
	lactam	D-Me-Ala	Me-L-Ala	CsA[11]	lactam	Me-D-Ala	Me-L-Ala	CsA[11]	lactam	Me-D-Ala	Me-L-Ala	CsA[11]
1	170.2	170.3	169.8	169.65	58.8	59.0	58.15	58.75	33.7	34.1	33.7	34
2	172.6	176.3	171.7	173.0	49.1	49.6	51.7	48.8				
3	169.9	175.6	172.4	170.5	62.6	51.1	48.2	50.4	3.95	31.6	30.2	39.4
4	169.2	169.9	169.3	169.35	54.1	55.2	57.5	55.5		30.8	29.4	31.3
5	173.7	173.7	172.8	173.1	55.2	55.5	54	55.4				
6	171.5	171.6	171.5	170.9	55.2	55.4	54.7	55.3	31.4	31.5	31.3	31.5
7	171.1	171.1	171.0	170.4	48.8	48.5	48.4	48.7				
8	173.3	173.35	173.6	172.9	45.2	45.1	44.8	45.2				
9	170.1	170.2	170.6	169.75	48.3	48.2	48.2	48.3	29.5	29.6	29.3	29.65
10	169.8	170.0	170.4	169.45	57.6	57.6	57.3	57.5	29.5	29.7	29.7	29.8
11	173.0	173.5	173.1	172.85	57.8	57.8	58.4	57.9	29.7	29.7	29.9	29.8

Table III. Effect of Temperature on the Chemical Shifts of Amide Protons in CsA and CsA Analogues

$10^{-3} \times (\Delta\delta/\Delta T)$	243–263 K				216–256 K:	300–310 K				
	lactam	Me-D-Ala	Me-L-Ala	CsA	CsA[11]	lactam	Me-D-Ala[3]	Me-L-Ala[3]	CsA	CsA[11]
Abu[2]NH	4.2	4	1.9	1.6	1.4	3	3.7	4.1	2.9	0.7
Val[5]NH	3	1.8	1.3	0.05	0.7	2.8	2.0	0.8	1.5	0.5
Ala[7]NH	9.9	7.6	10.3	10.2	9.7	4	3.5	7.8	4	0.3
Ala[8]NH	1.1	0.7	−0.7	0.2	0.7	1.8	1.1	3	1	0.3

Table IV. Effect of Temperature on the Amide Proton Coupling Constants (Hz)

	[lactam[3,4]]Cs		[Me-D-Ala[3]]Cs		[Me-L-Ala[3]]Cs		CsA	
	243 K	305 K	243 K	305 K	243 K	305 K	214 K	298 K
Abu[2]NH	*a*	9.2	9.5	9.8	7.5	8.9	8.6	8.6
Val[5]NH	8.1	8.5	7.8	7.7	7.50	7.9	7.6	8
Ala[7]NH	7.4	7.2	7.9	8.3	8.8	7.1	6.0	8
Ala[8]NH	7.7	7.7	7.75	7.6	9.8	7.7	6.8	8

[a] Broad singlet.

perature coefficients of the amides for the three analogues both at low and high temperature. The variations at low temperature of the chemical shifts of Abu[2]-NH, Ala[7]-NH, and Ala[8]-NH are similar to those reported for CsA. On the other hand, the temperature coefficient for the Val[5]-NH chemical shift is larger for the three CsA analogues than for CsA itself, suggesting that the modification introduced at position 3 for each of the CsA analogues disturbs the hydrogen bonds within the molecule. The rate of exchange of amide protons in D_2O was determined to see if the hydrogen bonds were retained in the three analogues. The slow exchange of the NH protons in D_2O for all three analogues suggests that the hydrogen bonds are still present. Because we have observed different temperature coefficients between 300 and 310 K than those reported by Kessler et al. for CsA,[11] we remeasured the temperature coefficients for the amide protons of CsA. The values that we obtained are higher than the reported values and are in the same range found for the three analogues described here.

Conformational Analysis of [D-MeAla[3]]Cs (2). The four amide protons of [D-MeAla[3]]Cs have chemical shifts close to the reported values for the four amide protons of CsA. The ^{13}C spectrum of **2** displays five downfield carbonyl resonances instead of four for CsA **1** (D-MeAla[3]-CO, Abu[2]-CO, Val[5]-CO, Ala[8]-CO, and MeVal[11]-CO). The unusual chemical shift of D-MeAla[3]-CO can be explained by the β effect induced by the new methyl group and is not directly correlated with a modification of the hydrogen bonds. The positions of the *N*-methyl group in MeLeu[4] relative to D-MeAla[3] were determined by NOE experiments. An NOE effect between the *N*-methyl of MeLeu[4] and the α-proton of D-MeAla[3] clearly indicates that the NCH_3 group is oriented above the ring system adjacent to the D-MeAla α-proton and that the amide bond is trans. Thus, the amide bond between positions 3 and 4 of [D-MeAla[3]]Cs has the same conformation that is found for CsA. Most of the remaining NOE effects for the amide protons, α-protons, and *N*-methyl groups are qualitatively similar to those reported for CsA. Thus, the ring system conformation of [D-MeAla[3]]Cs is very close to that of CsA.

[L-MeAla[3]]Cs (3). The chemical shifts of the four amide protons of [L-MeAla[3]]Cs are somewhat different from those of CsA. The Val[5]NH is more deshielded while the Abu[2]NH resonates at a frequency close to that observed for CsA (see Table I). The temperature coefficients for the amide protons indicate that the hydrogen bond between the NH of Abu[2] and the CO of Val[5] is weaker than that in CsA at high temperature.

The Val[5]-NH has an extremely downfield shifted resonance. This may be the result of an increase in the strength of a hydrogen bond Val[5]NH-Abu[2]CO or the introduction of a new, as yet uncharacterized hydrogen bond or a modification of the β II′ turn. This is also suggested by the small temperature coefficient of the Val[5]-NH at high temperature.

An important conformational feature of L-MeAla[3] can be deduced from the NOE data. For the most part, the NOE data are identical with those reported for CsA except for one very important difference, no NOE was observed (either at 270 or 500 MHz) between the *N*-methyl of MeLeu[4] and the α-proton of L-MeAla[3] or the methyl of L-MeAla[3]. Thus, a cis amide bond appears to be present between L-MeAla and MeLeu. In addition, the large upfield shift of the *N*-methyl protons on MeLeu[4] (2.71 ppm vs an average of 3.08 for the other analogues) shows that the shielding environment of the *N*-methyl group is very different in this analogue as would be expected for the cis amide bond.[24] Furthermore, the shape of the MeLeu[4] α-H

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resonance changes from a doublet of doublets observed for CsA and the other analogues (J = 3 Hz, 12 Hz) to a triplet (J = 7.2 Hz), also suggesting a modification of the β II′ turn. Thus, changing the configuration of the amino acid in position 3 of CsA from D to L appears to cause the amide bond between residues 3 and 4 to flip from trans to cis. As a result, the type II′ β-turn structure is lost. The peptide ring conformation for residues 5–11 is probably similar to that of CsA, as seen from the NOE data.

[Lactam^{3,4}]Cs (4). The chemical shift and coupling constant data for the lactam analogue of cyclosporine appear to be very similar to the corresponding data for CsA, apart from the obvious differences brought on by replacing the Sar^3-L-MeLeu^4 unit with the lactam **8**. In addition, the NOE experiments carried out at 270 MHz by using difference spectroscopy show that the NOE spectra for **4** are very similar to that obtained for CsA except for Val^{5}NH for which no NOE is observed with the MeLeu^4 α-CH or with any proton, suggesting a local perturbation of the conformation about this residue. The NOE experiments were carried out under several conditions and field strengths to establish the absence of the interaction between these two centers. The chemical shift data for Val^5-NH (7.95 ppm) vs CsA (7.48 ppm) suggest that the hydrogen bond system involving this residue has been altered. We tried to identify which carbonyl group was being paired with the NH of Val^5. The four deshielded carbonyl carbons in [lactam^{3,4}]Cs (**4**) and CsA are Val^5-CO, Abu^2-CO, Ala^8-CO, and MeVal^{11}-CO, with the most shielded of these four assigned to Abu^2-CO. These values have to be correlated with the variation in the chemical shifts of the amide protons as a function of the temperature. The Val^5 amide proton of [lactam^{3,4}]Cs is more sensitive to the variations in temperature than the other analogues or CsA. These changes in the NMR spectra indicate that the presence of the lactam ring in **4** could have decreased the strength of the hydrogen bond between Abu^2-CO/Val^5-NH ($\Delta\delta/\Delta T$ NH higher; δ(C=O) is upfield with respect to this hydrogen bond in CsA). In order to explain the downfield chemical shift of Val^5-NH in analogue **4**, we must suspect that this amide proton is now part of a new hydrogen bond system.

The slightly downfield shifted carbon resonance for the Val^5 carbonyl carbon suggests that a bifurcated hydrogen bond involving Val^5-NH/Val^5-CO and Abu^2-NH/Val^5-CO is possible. The new hydrogen bonding system would be Val^{5}NH-Val^{5}CO and the two hydrogen bonds Val^{5}NH·Abu^{2}CO and Val^{5}CO·Abu^{2}NH would still be present but weaker than in CsA. Such a system would also explain the upfield chemical shift of Abu^{2}NH and its large temperature coefficient (due to the weaker hydrogen bond). Nevertheless, these are minor modifications in hydrogen bonding about the amino acid residues 2 and 5 and are not expected to change the overall conformation of the cyclic undecapeptide ring system.

Biological. The biological activities of CsA analogues **2–4** vs CsA (**1**) were determined using inhibition of Con-A stimulated thymocytes as previously described.[25,26] The results are presented in Figure 4. Previous reports for the biological activities of [L-MeAla^3]Cs and [D-MeAla^3]Cs have indicated that [D-MeAla^3]Cs is nearly as potent as CsA whereas [L-MeAla^3]Cs is much less potent.[10b] Our results in the murine thymocyte assay are consistent with these data but provide a quantitative estimate of the relative potencies of the analogues.

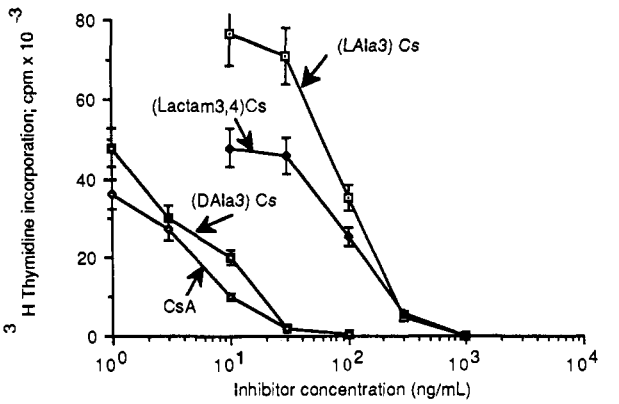

Figure 4. Inhibition of concanavalin A stimulated thymocyte mitogenesis as a function of increased concentration of the cyclosporin analogue. Error bars represent ±SEM. Two additional experiments gave similar results. (a) CsA (**1**), (b) [D-MeAla^3]Cs (**2**), (c) [L-MeAla^3]Cs (**3**), (d) [lactam^{3,4}]Cs (**4**).

Discussion

This work was stimulated by the report of Wenger that [D-Pro^3]Cs has low immunosuppressive activity whereas the [D-MeAla^3]Cs analogue, which differs only by an added methylene group, has high immunosuppressive activity that is nearly equipotent with that of CsA.[10b] Both compounds adopt closely related conformations in chloroform,[10b] which preserve the type II′ β-turn structure found in CsA in solution and in the solid state.[11a] The low immunosuppressive activity of the [D-Pro^3]Cs derivative could be caused by steric hindrance between the added methylene and the receptor when analogue **3** binds to the receptor as has been previously suggested.[10b] Alternatively, [D-MeAla^3]Cs might adopt a new conformation by rotating the ψ torsion angle in D-MeAla (to adopt, for example, the type I β-turn), a change that cannot occur with the D-Pro analogue due to the constraint of the 5-membered ring.

The idea to constrain the conformation of CsA follows closely the general strategy proposed by Freidinger et al., who first prepared cyclic lactam replacements for amide bonds in LH-RH and other peptides.[7a,b,d] Both the 5-membered and 6-membered ring systems were considered. Preliminary molecular modeling studies suggested that a lactam could be formed in the type II′ β-turn between L-MeAla^3 and L-MeLeu^4 without severely destabilizing the overall conformation of the 33-membered peptide ring system found in CsA. The synthesis and biological activity of the 5-membered lactam analogue will be reported later.

The cyclic lactam **8** was synthesized by following the route outlined in Scheme II. Both asymmetric centers were introduced by starting with the chiral amino acids, L-glutamic acid and L-leucine, and employing reaction conditions that are not likely to cause epimerization of α centers. However, the last step in the synthesis utilizes sodium hydride to catalyze the ring closure, conditions that have the potential to cause epimerization of either α center. Analysis of the product mixture by NMR is further complicated by the presence of the tertiary amide bond in **8**, which can exist in two amide bond conformations, cis and trans, each with its own sets of resonances in the NMR. Analysis of the NMR spectrum of the optically active product by 200-MHz NMR showed the presence of two major components; these were shown to be different amide bond conformations because the ratios changed when the NMR solvent systems were changed. At 500 MHz, four components were detected in ratios of 67:26:5:3. We did not study the effect of solvent on these ratios so that the

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two minor components present in 5% and 3% respectively could be cis-trans amide conformations of a minor diastereomer or they could be two minor diastereomers. In either case, the diastereomeric excess of the cyclic lactam 8 is at least 92%. It should be noted that the ratios of the four components in 8 were the same in the crude product as well as in purified samples of 8 so that enrichment of the major product did not take place during the purification procedures. The configurations of the lactam are assigned *S,S* on the basis of the minimal racemization detected in the synthetic product and because the conformation of the final CsA derivative 4 was very similar to that of CsA. Changing the configuration at the α-carbon in MeAla^3 dramatically alters the conformation of the cyclic CsA analogue; thus, incorrect lactam 8 diastereomers would be expected to greatly alter the conformation of CsA analogue 4. The remaining steps used to synthesize 4 closely followed reported procedures.[25,13] MeBmt was synthesized by the method of Aebi et al.[14] and coupled as the acetonide[10e] by means of DCC/HOBt. The resulting heptapeptide 5 was deprotected and coupled with the Fmoc-protected tetrapeptide 6[13] by using the fragment coupling procedure developed by Wenger.[10e] Double deprotection[27] and cyclization with propylphosphorus anhydride[10e,28] gave [lactam^{3,4}]Cs (4) in 51% yield.

The biological activity of [lactam^{3,4}]Cs (4) was determined in an assay that measures the inhibition of concanavalin A stimulated thymocytes[25] (Figure 4). For comparison we also assayed the activities of the [MeAla^3]Cs analogues 2 and 3. The biological activities of both 2 and 3 have been reported previously,[10b] but the relative potencies of 2 and 3 vs CsA have been reported only in qualitative terms; the D-MeAla^3 analogue was described as having immunosuppressive activity comparable to that of CsA while the [L-MeAla^3]Cs derivative was reported as being much less potent.[10b] Our data are consistent with these rankings; the [D-MeAla^3]Cs has about 50–80% of the activity of CsA whereas the [L-MeAla^3]Cs analogue has about 6–8% of the activity of CsA. The conformationally constrained analogue 4 is a weak inhibitor with a biological activity in this assay of about 4–9% or about comparable with the activity of the L-MeAla^3 derivative 3. Thus, introduction of the 6-membered lactam between the 3–4-position of CsA decreases biological activity by about 20-fold.

In an attempt to determine whether the low biological activity of 4 was caused by a major perturbation of the 33-membered peptide ring system brought about by introduction of the lactam group, we carried out a conformational analysis by using 500- and 270-MHz 1D and 300- or 500-MHz 2D techniques (NOESY[29] and ROESY[30]). The conformations of 2 and 3 were also studied in order to provide standards for comparing the biological activity and conformations of these systems. The [D-MeAla^3]Cs analogue 2 has been reported to retain the ring system conformation found for CsA in solution while the [L-MeAla^3]Cs analogue has been reported briefly to have a very different conformation, although supporting data for either conformation have not yet been published. These two analogues plus CsA itself were characterized by NMR by using the methods described by Kessler and his colleagues in their analysis of the CsA conformation.[11] Together, the model peptides and the NMR methods provided a solid basis for evaluating the conformation of the lactam analogue 4.

The NMR data establish quite clearly that the 33-membered ring system in the [D-MeAla^3]Cs analogue 2 and the [lactam^{3,4}]Cs analogue 4 are very close to that of CsA (1). The chemical shifts and coupling constants for all three derivatives are remarkably close as are the hydrogen-bonding patterns for these compounds. While minor differences between each peptide are detectable, the overall conformation of the lactam analogue 4 appears to be very close to that of CsA (1) and [D-MeAla^3]Cs (2). Thus, the low biological activity of 4 cannot be ascribed to an altered solution conformation of the peptide ring system. Instead, it seems likely that either the C-3′ atom (as in [L-MeAla^3]Cs) or the extra atoms between amino acids 3 and 4 needed to form the lactam structure (atoms 3′, 4′, 5′) are encountering steric hindrance with the receptor. Unfortunately, one of the limitations of the constrained analogue approach to determining bioactive conformation is that only positive results can be interpreted unambiguously. Our data do not enable us to differentiate between the loss of biological activity arising because the type II′ β-turn is not the bioactive conformation or a loss arising because of steric hindrance between lactam atoms (3′, 4′, 5′) of the CsA analogue and the receptor. Thus, analogues constrained between the 3- and 4-position amino acids in CsA that are not as sterically hindered are being prepared to differentiate between these possibilities.

It is probable that the low biological activity of the [L-MeAla^3]Cs (3) arises because this modification alters the ring system conformation in a major way. More conformations are detected by the NMR and the NMR data are consistent with a major conformation with a cis amide bond between L-MeAla^3 and L-MeLeu^4, in contrast to the trans amide bond present in CsA. The presence of a cis amide bond between MeAla^3 and MeLeu^4 is difficult to prove by NMR because we are detecting the *absence* of an NOE between these residues rather than the presence of an NOE as is found with the trans amide bond. However, three major changes in the NMR spectra for 4 are consistent with the cis amide bond. These are the unusual chemical shift of the MeLeu^4 amide methyl group, the unusual chemical shift of the MeAla^3 α-proton, and the absence of the NOE between the MeLeu^4-NMe and any proton of MeAla^3. A ROESY[30] experiment at −10 °C in chloroform was used to detect an NOE between the α-protons on L-MeAla^3 and L-MeLeu^4. This temperature was selected from temperatures between −40 and 50 °C as giving the best resolution between the two peaks. An apparent cross-peak was observed, which would have confirmed the presence of the cis amide bond if the peak could be verified. However, the chemical shifts of those two α-protons are so close to each other (δ(CH α Ala^3) 5.35 ppm, δ(CH α MeLeu^4) 5.41 ppm; they superimpose at room temperature) that an unambiguous assignment of the cross-peak was not possible because it lies so close to the diagonal. In addition, the significant differences in the chemical shifts and hydrogen-bonding patterns for the Val^5-NH and the chemical shifts of the α-protons on MeBmt^1 and Abu^2 and especially the α-CH of L-MeAla^3 suggest that the conformation of the 33-membered peptide ring system has been altered at several sites. In view of the ease with which immunosuppressive activity can be diminished upon relatively subtle modifications of the primary structure of CsA, the loss in biological activity of

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3 occurring with the isomerization of a trans amide bond to a cis amide bond is not surprising.

The incorporation of the cyclic lactam dipeptidyl replacement in place of L-MeAla[3]-L-MeLeu[4] accomplished the objective that we set for it, to stabilize the trans amide conformation for the sequential L,L amino acids in residues 3 and 4 of CsA. As was reported earlier[10b] and we confirm here, placement of a L side chain at position 3 of CsA severely destabilizes the conformation of CsA because of the interaction between the adjacent methyl groups, forcing the analogue to adopt the cis amide conformation. The lactam group 8 not only removes this unfavorable interaction but also restricts the amide bond to the trans amide conformation. Thus, [lactam[3,4]]Cs (4) retains the trans amide bond between the third and fourth residue in spite of the L configuration of the amino acid at position 3 of CsA. The synthesis of lactam 8 thus provides a chiral dipeptide unit that can be used to probe the conformational space about type II' β-turns in other biologically active peptides.

Experimental Section

General Procedures. A mixture of [L-MeAla[3]]Cs and [D-MeAla[3]]Cs was received from Prof. D. Seebach and was purified by flash chromatography (1–1.5% MeOH in ether). The purified products were characterized by 500-MHz NMR. The NMR spectra were recorded on degased samples (8 mg in 0.5 mL of $CDCl_3$). The 1D experiments were carried out on a Bruker WP-200 or WP-270 instrument. The other experiments were done on a Bruker AM 500 or AC 300 spectrometer equipped with an Aspect 3000 computer. For the NOE difference spectra, 800 acquisitions were recorded for each on and off resonance. The delays, decoupling times, and power were identical with those reported for CsA.[11a,b]

All nonaqueous reactions were carried out under a dry argon atmosphere in oven dried (140 °C, 12 h) glassware.

Typically, flash chromatography was effected by starting with a less polar solvent system and progressing to more polar systems, indicated by (*X*–*Y*%).

TLCs were run on Merck Kieselgel 60-F_{254} with fluorescent indicator visualized with 7% phosphomolybdic acid (PMA) in ethanol.

Because of their instability, several intermediates were not purified and fully characterized. After ^{1}H NMR assignment of the product, the compound was directly used in the next reaction. If the weight recovery was over 100%, the conversion is said to be quantitative.

(4*S*)-3-(Benzyloxycarbonyl)-4-(3-hydroxypropyl)-1,3-oxazolidin-5-one (16).[18] A solution of acid **12** (11.53 g, 39.31 mmol)[31] in THF (75 mL) was cooled (−30 ± 5 °C) and treated with BH_3·THF (86.5 mL, 1 M THF solution, 2.2 equiv) dropwise over 20 min. To initiate the reaction, 2 mL of diborane was added and the reaction solution was removed from the cooling bath. Once bubbling was observed, the rest of the diborane was added at −30 °C. The solution was warmed up to −25 °C for 1 h and stirred afterword for 4 h at 0 °C. The reaction was carefully acidified at −30 °C with 10% methanolic acetic acid (30 mL, added over 20 min). The solution was warmed to room temperature, evaporated in vacuo and dissolved in EtOAc (60 mL). The organic phase was washed with 40 mL each of 1 N HCl (0 °C), H_2O, 1 N NH_4HCO_3, and saturated NaCl solution. Each aqueous phase was washed twice with EtOAc (2 × 50 mL). The combined organic phases were dried ($MgSO_4$) and evaporated in vacuo to give 11.0 g (quant) of crude alcohol **16**: ^{1}H NMR ($CDCl_3$, 90 MHz) δ 1.40–2.30 (m, 5 H, CH_2CH_2,OH), 3.60 (t, J = 6 Hz, 2 H, CH_2OH), 4.36 (t, J = 5.5 Hz, 1 H, C_4-H), 5.10–5.26 (m, 3 H, C_2-H, benzyl H), 5.51 (d, J = 4.8, 1 H, C_2-H), 7.34 (s, 5 H, arom H). For the 200-MHz ^{1}H NMR spectrum, the coalescence temperature is ca. 25 °C.

(4*S*)-3-(Benzyloxycarbonyl)-4-[3-(*p*-tolylsulfonyl)-propyl]-1,3-oxazolidin-5-one (17b).[32] To a cooled (0 °C) solution of crude alcohol **16** (8.20 g, 29.30 mmol) in 30 mL of CH_2Cl_2 was added pyridine (4.73 mL, 58.6 mmol) and *p*-toluenesulfonyl chloride (8.38 g, 43.9 mmol) in four portions over 20 min. Stirring was continued overnight (warming up to room temperature in the water bath). Ether (60 mL) and water (20 mL) were added, and the organic layer was washed successively with 1 N HCl (0 °C), 5% $NaHCO_3$, and water (each 40 mL). The aqueous phases were washed a second time with ether (100 mL). The organic phase was dried (Na_2SO_4) and evaporated to give 13.35 g (quant) of tosylate **17b**: ^{1}H NMR ($CDCl_3$, 90 MHz) δ 1.40–2.12 (m, 4 H, CH_2CH_2), 2.41 (s, 3 H, *p*-CH_3-tosyl), 4.02 (t, J = 6 Hz, CH_2O*p*Tos), 4.16–4.46 (m, 1 H, C_4-H), 5.11–5.27 (m, 3 H, benzyl H, C_2-H), 7.26–7.88 (AA'BB' system, 4 H, arom H), 7.38 (s, 5 H, arom H). For the 200-MHz ^{1}H NMR spectrum, the coalescence temperature is ca. 25 °C.

(2*S*)-2-[*N*-(Benzyloxycarbonyl)-*N*-methylamino]-5-(*p*-tolylsulfonyl)pentanoic Acid (18b).[17] The crude tosylate **17b** (13.35 g, 29.3 mmol) was dissolved in 80 mL of CH_2Cl_2 and 80 mL of TFA. After the addition of triethylsilane (14.0 mL, 87.9 mmol), the solution was stirred for 22 h (room temperature). The solvents were evaporated in vacuo, and the residue was dissolved in CH_2Cl_2 (3 × 60 mL), evaporated, and dried (1 Torr/room temperature). The oil was dissolved in 10 mL of CH_2Cl_2 and suspended in ether (60 mL). After extraction with 5% $NaHCO_3$ (solid $NaHCO_3$ had to be added to keep pH >8) (2 × 40 mL), the aqueous phase was cooled (0 °C), acidified (6 N HCl → pH ~2) and extracted with EtOAc (3 × 50 mL). The organics were dried (Na_2SO_4) and evaporated to give 7.41 g (58%) of the acid **18b**: ^{1}H NMR ($CDCl_3$, 200 MHz, two rotamers 3:1) δ 1.53–2.16 (m, 4 H, CH_2Cl_2), 2.42 (s, 3 H, *p*-CH_3-tosyl), 2.84, *2.86* (s, 3 H, NCH_3), 3.94–4.10 (m, 2 H, CH_2O*p*Tos), 4.56 (dd, J = 5, 5.5 Hz, 1/4 H, C_2-H), 4.67 (dd, J = 5, 5.5 Hz, 3/4 H, C_2-H), 5.12 (s, 2 H, benzyl H), 7.26–7.42 (m, 7 H, 7 arom H), 7.68–7.82 (m, 2 H, arom H).

(2*S*)-2-[*N*-(Benzyloxycarbonyl)-*N*-methylamino]-5-(*p*-tolylsulfonyl)pentanoyl-L-leucine Methyl Ester (19b).[13] A solution of tosylate **18b** (7.41 g, 17.02 mmol) and leucine methyl ester hydrochloride (3.40 g, 18.71 mmol) in CH_2Cl_2 (100 mL) was cooled (0 °C). After the addition of diisopropylethylamine (10.77 mL, 62.90 mmol) and of BOP-Cl (5.00 g, 19.64 mmol), the reaction mixture was warmed up overnight to room temperature. The solution was evaporated in vacuo, and the residue was dissolved in EtOAc (50 mL) and washed with 30 mL each of 10% $KHSO_4$ (0 °C), saturated $NaHCO_3$ (0 °C), and water. The aqueous phases were extracted a second time with EtOAc (50 mL). The organics were dried (Na_2SO_4) and evaported in vacuo to give 8.57 g (95%) of crude dipeptide **19b**: ^{1}H NMR ($CDCl_3$, 200 MHz) (to describe the ^{1}H NMR, the peptide numbering system used by Wenger[10e] was adopted) δ 0.84–0.92 (m, 6 H, 2 CH_3-C(4^2)), 1.35–2.23 (m, 7 H, 2H-C(3^1), 2H-C(4^1), 2H-C(3^2), H-C(4^2)), 2.41 (s, 3 H, *p*-CH_3-tosyl), 2.80 (s, 3 H, CH_3-N^1), 3.72* (s, 3 H, CH_3O^2), 3.90–4.75* (m, 4 H, H-C(2^1), 2H-C(5^1), H-C(2^2)), 5.16 (br s, 2 H, benzyl H), 6.40 (br s, 1 H, H-N^2), 7.26–7.45 and 7.70–7.82 (AA'BB' system, 4 H, arom H), 7.38 (s, 5 H, arom H). [(*) Because of a major side product and rotamers, the assignment of the protons is not clear.]

(2*S*)-2-[(3*S*)-3-[*N*-(Benzyloxycarbonyl)-*N*-methylamino]-2-oxo-1-piperidinyl]-4-methylpentanoic Acid Methyl Ester (8).[15] The amide dipeptide **19b** (3.19 g, 6.0 mmol) was dissolved in 75 mL of a 1:1 DMF/CH_2Cl_2 solution, cooled (0 °C), and treated with NaH (0.15 g, 6.32 mmol, 1.05 equiv of 97% NaH). The mixture was stirred at 0 °C for 3 h. EtOAc (20 mL) followed by water (9 mL) was added to the solution. The resulting mixture was stirred 1 h (room temperature). After evaporation in vacuo (1 Torr/35 °C), the residue was partitioned between water (40 mL) and CH_2Cl_2 (50 mL). The water phase was extracted with CH_2Cl_2 (2 × 40 mL). The organics were dried ($MgSO_4$) and evaporated to give 1.78 g (74%) of crude lactam **8**. Purification by flash chromatography using 10–40% EtOAc in hexane as eluant gave 0.88 g (38% from **18b**) of the pure lactam **8**: R_f 0.27 (50% EtOAc/hexane); $[\alpha]^{rt}_D$ −32.9° (*c* 0.90, $CHCl_3$); IR ($CHCl_3$) 2950, 1731, 1692, 1654, 1435, 1346, 1305 cm^{-1}. ^{1}H NMR: the dipeptide shows two conformers in the 200-MHz ^{1}H NMR spectrum in a ratio of 2:1 in $CDCl_3$ and in a ratio 3:1 in CD_3OD. The 500-MHz spectrum (in $CDCl_3$) showed two sets (probably) of conformers in a ratio of 67:26:5:3. We did not prove (by changing to another solvent) that the two minor components (5:3) are conformers and not epimers. ^{1}H NMR ($CDCl_3$, 500 MHz, 2:1) δ 0.88–0.96 (m, 6

(31) Itoh, M. *Chem. Pharm. Bull.* **1969**, *17*, 1679.

H, 2 CH_3-C(4^2)), 1.45 (m, 1 H, H-C(4^2)), 1.62–1.71 and 1.81–2.11 (2 m, 6 H, 2 H-C(4^1), 2H-C(5^1), 2H-C(3^2)), 2.84, *2.87* (s, 3 H, CH_3-N^1), 3.15–3.23 and 3.24–3.31 (2 m, 2 H, 2H-C(6^1), 3.69, *3.71* (s, 3 H, CH_3O^2), 4.54–4.60 (m, 1/3 H, H-C(3^1)), 4.81 (dd, J = 7, 12 Hz, 2/3 H, H-C(3^1)), 5.11–5.18 (m, 2 H, $PhCH_2OC$), 5.31 (dd, J = 7, 10 Hz, 1 H, H-C(2^2)), 7.27–7.38 (m, 5 H, arom H); MS, m/e 391 (M^+ + 1, 3), 390 (M^+, 9), 359 (13), 358 (25), 331 (14), 227 (28), 191 (20), 138 (27), 127 (27), 91 (100). Anal. Calcd for $C_{21}H_{30}N_2O_4$: C, 64.60; H, 7.74; N, 7.17. Found: C, 64.49; H, 7.84; N, 7.07.

(2*S*)-2-[(3*S*)-3-(*N*-Methylamino)-2-oxo-1-piperidinyl]-4-methylpentanoic Acid Methyl Ester (20). A solution of dipeptide **8** (2.43 g, 6.23 mmol) in 50 mL of MeOH was hydrogenated for 9 h at room temperature with 0.25 g of 10% Pd/C. The suspension was filtered through Celite, the filtrate evaporated, and the residue dried under high vacuum to yield 1.48 (93%) of the free amine **20**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.91 (d, J = 5.5 Hz, 3 H, CH_3-C(4^2)), 0.94 (d, J = 5.5 Hz, 3 H, CH_3-C(4^2)), 1.36–2.22 (m, 7, 2H-C(4^1), 2H-C(5^1), 2H-C(3^2), H-C(4^2)), 2.42 (s, 3 H, CH_3-N^1), 2.56 (br s, 1 H, H-N^1), 3.08–3.38 (m, 3 H, H-C(3^1), 2H-C6^1)), 3.69 (s, 3 H, CH_3O^2), 5.26 (m, H-C(2^2)).

***N*-(*tert*-Butyloxycarbonyl)-L-2-aminobutyryl-(2*S*)-2-[(3*S*)-3-(*N*-methylamino)-2-oxo-1-piperidinyl]-4-methylpentanoic Acid Methyl Ester (9).**[13] To a cold (0 °C) solution of deprotected dipeptide **20** (1.48 g, 5.77 mmol) and Boc-Abu (1.41 g, 6.93 mmol, 1.2 equiv) in CH_2Cl_2 (25 mL) were added $Et(i\text{-}Pr)_2N$ (2.37 mL, 13.85 mmol, 2.4 equiv) and BOP-Cl (1.76 g, 6.93 mmol, 1.2 equiv). The mixture was stirred for 5 h at 0 °C and warmed up overnight to 6 °C. The solution was evaporated in vacuo. The resulting oil was dissolved in EtOAc (60 mL) and washed with 30 mL each of 10% $NaHSO_4$ (0 °C), saturated $NaHCO_3$ (0 °C), and water. The aqueous phases were extracted with EtOAc (2 × 60 mL). The organics were dried ($MgSO_4$) and evaporated to give 2.53 g (99%) of crude tripeptide **9**. Purification by flash chromatography using 25–50% acetone in hexane as eluant gave 2.32 g (91%) of pure tripeptide **9**: R_f 0.44 (40% acetone/hexane); $[\alpha]^{rt}_D$ −23.8° (*c* 1.14, $CHCl_3$); IR ($CHCl_3$) 2955, 1735, 1700, 1646, 1485, 1460, 1410, 1365 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.80–1.05 (m, 9 H, CH_3-C(4^1), 2 CH_3-C(4^3)), 1.34–2.12 (m, 9 H, 2H-C(3^1), 2H-C(4^2), 2H-C(5^2), 2H-C(3^3), H-C(4^3)), 1.42 (s, 9 H, O-*t*-Bu), 2.94 (s, 3 H, CH_3-N^2), 3.14–3.36 (m, 2 H, 2 H-C(6^2)), 3.70 (s, 3 H, CH_3O^3), 4.56 (m, 1 H, H-C(2^1)), 5.16–5.45 (m, 3 H, H-N^1, H-C(3^2), H-C(2^3)); MS, m/e 442 (M^+ + 1, 5), 441 (M^+, 3), 342 (12), 227 (23), 168 (12), 137 (10), 102 (24), 84 (26), 83 (21), 70 (48), 58 (100), 57 (78). Anal. Calcd for $C_{22}H_{39}N_3O_6$: C, 59.84; H, 8.90; N, 9.52. Found: C, 60.01; H, 8.96; N, 9.35.

***N*-(*tert*-Butyloxycarbonyl)-L-2-aminobutyryl-(2*S*)-2-[(3*S*)-3-(*N*-methylamino)-2-oxo-1-piperidinyl]-4-methylpentanoic Acid (21).** A solution of tripeptide **9** (0.69 g, 1.56 mmol) in 5 mL of MeOH was treated at 0 °C with 1 N NaOH (2.03 mL). After 1 h at 0 °C and 2.5 h at room temperature, the solution was evaporated in vacuo (room temperature). The resulting oil was dissolved in 30 mL of CH_2Cl_2/30 mL of 1 N HCl (0 °C). The aqueous phase was extracted with CH_2Cl_2 (2 × 30 mL). The organics were dried ($MgSO_4$) and evaporated to give 0.66 g (99%) of the acid **21**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.83–1.04 (m, 9 H, CH_3-C(3^1), 2 CH_3-C(4^3)), 1.44 (s, 9 H, O-*t*-Bu), 1.50–2.16 (m, H, 2H-C(3^1), 2H-C(4^2), 2H-C(5^2), 2H-C(3^3), H-C(4^3)), 3.01 (s, 3 H, CH_3N^2), 3.18–3.34 (m, 2 H, 2H-C(6^2)), 4.62 (m, 1 H, H-C(2^1)), 4.80 (m, 1 H, H-C(2^3)), 5.34 (dd, J = 10.5, 5.5 Hz, 1 H, H-C(2^2)), 5.48 (d, J = 9.5 Hz, 1 H, H-N^1) (CO_2H > 12 ppm).

L-Valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (22).[10e,33] At −20 °C, the protected tripeptide **10** (1.11 g, 2.20 mmol) (precooled to −20 °C) was dissolved in 5 mL of TFA (precooled to −20 °C) and stirred for 6 h at −15 to −20 °C. The solution was poured into a mixture of ice/saturated $NaHCO_3$ (+5 g of $NaHCO_3$)/CH_2Cl_2 (40/60 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL). The organics were dried (Na_2SO_4) and evaporated to give 0.90 g (quantitative) of deprotected tripeptide **22**.

***N*-(*tert*-Butyloxycarbonyl)-L-2-aminobutyryl-(2*S*)-[(3*S*)-3-(*N*-methylamino)-2-oxo-1-piperidinyl]-4-methyl-2-pentanyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (11).**[10e] At −20 °C, pivaloyl chloride (0.20 mL, 1.62 mmol, 1.05 equiv) and *N*-methylmorpholine (0.31 mL, 2.78 mmol, 1.8 equiv) were added to a solution of lactam **21** (0.66 g, 1.54 mmol) in 13 mL of CH_2Cl_2 (precooled to −20 °C), and the mixture was stirred at −15 to −20 °C for 4 h. Then, tripeptide **22** (0.90 g, 2.2 mol, 1.4 equiv with 5% of Boc tripeptide **8** as an impurity) in 6 mL of CH_2Cl_2 was added. The solution was stirred overnight at −15 °C. The resulting solution was warmed up to room temperature (1 h), diluted with CH_2Cl_2 (30 mL), and washed with 1 N HCl (30 mL) and saturated $NaHCO_3$ (2 × 40 mL). The aqueous phases were washed with CH_2Cl_2 (2 × 40 mL). The organics were dried (Na_2SO_4) and evaporated in vacuo to give 1.41 g of crude product. Purification by flash chromatography using 20–35% acetone in hexane gave 0.81 g (64%) of pure hexapeptide **11**: R_f 0.23 (30% acetone/hexane); $[\alpha]^{rt}_D$ −161.9° (*c* 1.20, $CHCl_3$); IR ($CHCl_3$) 3310, 2955, 1740, 1700–1650 (shoulder), 1635 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz, room temperature; ca. two conformers) δ 0.72–1.11 (m, 21 H, CH_3), 1.32, 1.33 (d, J = 9.5 Hz, 3 H, $CH_3C(2^6)$), 1.40 (s, 9 H, O-*t*-Bu), 1.45–2.45 (m, 13 H, CH_2, CH), 2.80, 3.00, 3.09, 3.15, 3.10–3.35 (s, 8 H, CH_3-N, CH_2-N), 4.40–5.50 (m, 9 H, α-CH, benzyl H, H-N), 6.53 (d, J = 6.5 Hz, 1 H, H-N), 7.29–7.38 (m, 5 H, arom H), 7.80 (d, J = 6 Hz, 1 H, H-N); MS, m/e 716 (M^+ + 2 − Boc, 7), 715 (M^+ + 1 − Boc, 11), 509 (18), 282 (19), 234 (18), 195 (22), 100 (100), 91 (37), 72 (64). Anal. Calcd for $C_{43}H_{70}N_6O_9$: C, 63.37; H, 8.66; N, 10.31. Found: C, 63.29; H, 8.87; N, 10.03.

L-2-Aminobutyryl-(2*S*)-[(3*S*)-3-(*N*-methylamino)-2-oxo-1-piperidinyl]-4-methyl-2-pentanyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (23).[10e] At −20 °C, the Boc-hexapeptide **11** (0.42 g, 0.52 mmol) (precooled to −20 °C) was dissolved in 2.5 mL of TFA (precooled to −20 °C to prevent crystallization 0.2 mL of CH_2Cl_2 was added). The light heterogeneous mixture was stirred 16 h at −15 °C. The clear solution was poured into a mixture of ice/saturated $NaHCO_3$ (2.6 g $NaHCO_3$)/CH_2Cl_2 (20/30 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 30 mL). The organics were dried (Na_2SO_4) and evaporated in vacuo to give 0.42 g of the deprotected hexapeptide **23**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.72–1.10 (m, 2 H, CH_3), 1.39 (d, J = 8.5 Hz, CH_3-C(6^2)), 1.40–2.35 (m, 13 H, CH_2, CH), 2.76, 3.12, 3.16–3.36 (s, 8 H, CH_3-N, CH_2-N), 4.21 (t, J = 4 Hz, α-CH), 4.44 (d, J = 8 Hz, α-CH), 4.65 (t, J = 9.5 Hz, α-CH), 4.86–5.42 (m, 5 H, α-CH, benzyl H), 6.70 (s, 3 H, H_2N, H-N), 7.29–7.40 (m, 5 H, arom H), 7.98 (d, J = 7 Hz, H-N).

[(4*S*,5*R*,1′*R*,3′*E*)-2,2,3-Trimethyl-5-(1′-methyl-3′-pentenyl)-4-oxazolidinylcarbonyl]-L-2-aminobutyryl-(2*S*)-[(3*S*)-3-(*N*-methylamino)-2-oxo-1-piperidinyl]-4-methyl-2-pentanyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (7).[10e] **(4*S*,5*R*,1′*R*,3′*E*)-2,2,3-Trimethyl-5-(1′-methyl-3′-pentenyl)-4-oxazolidinecarboxylic Acid (24).**[10e] A suspension of (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoic acid[14] (0.103 g, 0.53 mmol) in 160 mL of anhydrous acetone was heated at reflux for 24 h until a clear solution was obtained. The acetone was concentrated to 3–5 mL under vacuum and the remaining mixture of **24** used directly for synthetic purposes without further purification.

7: A mixture of freshly prepared *N*,*O*-isopropylidene-MeBmt **24** (0.53 mmol) in 3.5 mL of acetone was diluted with 2 mL of THF, and *N*-methylmorpholine (0.06 mL, 0.57 mmol) was immediately added. Then *N*-hydroxybenzotriazole (HOBt·H_2O; 0.16 g, 1.04 mmol) was dehydrated (by azeotropic distillation of H_2O with two 25-mL portions of toluene) and added to the solution together with crude hexapeptide **23** (0.42 g, 0.52 mmol) diluted in 1.8 mL of THF. The resulting mixture was cooled (0 °C) and DCC (0.11 g, 0.52 mmol) was added. The mixture was allowed to warm to room temperature and stirred for 27 h at room temperature. The mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (30 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 40 mL). The organics were dried (Na_2SO_4) and evaporated. The residue was suspended in 10 mL of EtOAc and filtered. After evaporation of the solvent, the oil was flash chromatographed on 30 g of silica gel with 10–20% acetone in hexane to give 0.24 g (50%) of the heptapeptide **7**: R_f 0.29 (40% acetone/hexane); $[\alpha]^{rt}_D$ −139.0° (*c* 1.01, $CHCl_3$); IR ($CHCl_3$) 2955, 1740, 1670 (shoulder), 1638, 1508 cm^{-1}; ^{1}H NMR

(32) For optimization of tosylation of alcohols, see: Kabalka, G. W.; Varma, R. S. *J. Org. Chem.* **1986**, *51*, 2386. See also tosylation of a homoserine derivative: Ozinskas, A. J.; Rosenthal, G. A. *J. Org. Chem.* **1986**, *51*, 5047.

(33) Petrie, J.; Tung, R. D., unpublished data.

($CDCl_3$, 200 MHz, at room temperature at least two conformers) δ 0.76–1.10 (m, 24 H, CH_3), 1.04–1.47 (m, 9 H, 2 CH_3-C(2^1), CH_3-C(2^7)), 1.66 (d, J = 4.5, CH_3-C(4^1)), 1.50–2.20 (m, 15 H, CH_2, CH), 2.31, 2.33 (s, 3 H, CH_3-N^1), 2.79, 3.03 (s, 6 H, CH_3-N), 3.05–3.35 (m, 2 H, CH_2-N^2), 3.74 (m, 1 H, H-C(5^1)), 4.38–5.50 (m, 11 H, benzyl, olefin, α-C-H), 6.48 (d, J = 7.5 Hz, 1 H, H-N), 6.82 (br s, H-N), 7.35 (s, 5 H, benzyl H), 7.90 (d, J = 9.5 Hz, 1 H, H-N).

[(2*S*,3*R*,4*R*,6*E*)-3-Hydroxy-4-methyl-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-(2*S*)-[(3*S*)-3-(methylamino)-2-oxo-1-piperidinyl]-4-methyl-2-pentanyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (25).[10e] A solution of *N,O*-isopropylidene **7** (0.24 g, 0.26 mmol) in 2.5 mL of MeOH was stirred for 24 h at room temperature in the presence of 0.26 mL of 1 N HCl. The acid in the reaction medium was neutralized with $NaHCO_3$ (0.16 g, 1.32 mmol) and the solvent evaporated completely, taking care that the temperature does not rise above 30 °C. The residue was taken up in 2% MeOH/CH_2Cl_2 and flash chromatographed on 25 g of silica gel with 2–6% MeOH/CH_2Cl_2 as eluant to give 0.17 g (74%) of **25**. During the chromatography of the deprotected MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl with 30–40% acetone in hexane, reformation of the *N,O*-isopropylidene-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl was observed. **25**: R_f 0.09 (6% MeOH/CH_2Cl_2); ^1H NMR ($CDCl_3$, 200 MHz at least two conformers at room temperature) δ 0.75–1.14 (m, 24 H, CH_3), 1.37, 1.38 (d, J = 7 Hz, CH_3-C(2^7)), 1.45–1.50 (m, 19 H, CH_3-C(4^1), CH_2, CH), 2.39, 2.40 (s, 3 H, CH_3-N^1), 2.80, 3.16, 3.24, 3.30 (s, 8 H, CH_3-N, CH_2-N), 3.45–3.75 (m, 2 H, OH, H-C(5^1)), 4.38–4.72 and 4.90–5.50 (m, 11 H, benzyl, olefin, α-C-H), 7.30–7.41 (m, 5 H, arom H), 7.85 (d, J = 7 Hz, 1 H, H-N), 8.08 (d, J = 5.5 Hz, 1 H, H-N).

[[(9-Fluorenylmethyl)oxy]carbonyl]-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl-[(2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-(2*S*)-[(3*S*)-3-(*N*-methylamino)-2-oxo-1-piperidinyl]-4-methyl-2-pentenyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (5).[28b] **Fmoc-D-Ala-MeLeu-MeLeu-MeVal-OH (26).**[13,33] At −20 °C, Fmoc-D-Ala-MeLeu-MeLeu-MeVal-O-*t*-Bu (**6**) (0.35 g, 0.48 mmol) (precooled to −20 °C) was dissolved in 2.6 mL of TFA (precooled to −20 °C) and stirred for 9 h at −15 °C. The reaction mixture was poured into a slurry of ice/saturated $NaHCO_3$ (2.5 g of $NaHCO_3$)/EtOAc (40/50 mL). The aqueous phase was extracted with EtOAc (2 × 50 mL). The organics were washed with 40 mL each of 5% $KHSO_4$ (0 °C) and H_2O, then dried ($MgSO_4$), and evaporated in vacuo to give 0.36 g of crude tetrapeptide **26**: ^1H NMR ($CDCl_3$, 200 MHz, two rotamers 1:1) δ 0.81–1.11 (m, 18 H, 2CH_3-C(4^2), 2CH_3-C(4^3), 2CH_3-C(3^4)), 1.38 (d, J = 7 Hz, 3 H, CH_3-C(2^1)), 1.21–1.89 (m, 6 H, 2H-C(3^2), H-C(4^2), 2H-C(3^3), H-C(4^3)), 2.30 (m, 1 H, H-C(3^4)), 2.79, 2.82, 2.99, 3.04, 3.05, 3.06 (s, 9 H, CH_3-N^2, CH_3-N^3, CH_3-N^4), 4.02–4.53 (m, 4 H, OCH_2C*H*, H-C(2^4)), 4.71 (m, H-C(2^1)), 5.13 (s, 1 H, HOOC^4), 5.49 (m, 2 H, H-C(2^2), H-C(2^3)), 5.91, 6.48 (d, J = 8 Hz, H-N^1), 7.29–7.81 (m, 5 H, arom H).

5:[28b] At room temperature heptapeptide **25** (0.17 g, 0.19 mmol) and tetrapeptide **26** (0.14 g, 0.21 mmol) were dissolved in 2 mL of CH_2Cl_2. Then *N*-methylmorpholine (0.045 mL, 0.40 mmol, 1.95 equiv) and BOP reagent (0.14 g, 0.31 mmol, 1.5 equiv) were added to the solution, and the mixture was stirred for 2.5 days at room temperature. The solution was diluted with CH_2Cl_2 (30 mL) and washed with saturated $NaHCO_3$ (30 mL). The water phase was extracted with CH_2Cl_2 (2 × 30 mL). The organics were dried (Na_2SO_4) and evaporated. The residue was flash chromatographed on 25 g of silica gel with 10–20% acetone in hexane as eluant, and 0.19 g (64%) of pure undecapeptide **5** was isolated. Probably another diastereomeric undecapeptide with a larger R_f value (0.32, 40% acetone/hexane) was also isolated (0.04 g (14%)).

5: R_f 0.26 (40% acetone/hexane); $[\alpha]^{rt}_D$ −160.3° (*c* 0.93, $CHCl_3$); IR ($CHCl_3$) 3300, 2955, 1710, 1670 (shoulder), 1632, 1540–1460 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz, room temperature, at least two rotamers) δ 0.49, 0.60 (d, J = 5 Hz, 3 H, CH_3-C(4^5)), 0.70–1.10 (m, 39 H, CH_3), 1.20–2.40 (m, 32 H, CH_3-C(2^1), CH_3-C(7^5), CH_3-C(2^{11}), CH_2CH), 3.01, 3.16, 3.28, 3.33, 3.46 (s, 18 H, CH_3-N), 3.15–3.34 (m, 2 H, CH_2-N^7), 3.72 (m, 1 H, H-C(3^5)), 4.12–5.82 (m, 17 H, H-N^1, 2 benzyl H, OCH_2C*H*, olef H, α-CH), 7.29–7.80 (m, 12 H, H-N, arom H). Anal. Calcd for $C_{86}H_{131}N_{11}O_{15}\cdot{}^1/_2H_2O$: C, 65.87; H, 8.48; N, 9.83. Found: C, 65.84; H, 8.53; N, 9.46.

***cyclo*[[(2*S*,3*R*,4*R*,6*E*)-3-Hydroxy-4-methyl-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-(2*S*)-[(3*S*)-3-(methylamino)-2-oxo-1-piperidinyl]-4-methyl-2-pentanyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl] (4).**[10e,28b] A solution of fully protected linear undecapeptide **5** (0.125 g, 8.04 10^{-5} mol) in 3 mL of absolute ethanol (0 °C) was treated with 0.8 mL of 0.2 N aqueous NaOH and stirred at 0 °C for 1 h. At that time, an additional 0.4 mL of 0.2 N aqueous NaOH was added and stirring was continued at 0 °C. After 5.5 h, essentially no starting material was left (TLC 40% acetone in hexane).[27] The mixture was acidified with 1.2 mL of 0.2 N aqueous HCl (pH → 6) and treated with 10 mL of saturated NaCl/30 mL of CH_2Cl_2. The layers were separated, and the aqueous portion was extracted with CH_2Cl_2 (4 × 20 mL). The combined organics were dried (Na_2SO_4) and concentrated to a pale yellow oil (0.10 g), which was carefully dried and dissolved in 300 mL of CH_2Cl_2 and treated with vigorous stirring with DMAP (0.049 g, 4.02 × 10^{-4} mol, 5 equiv) and $(PrPO_2)_3$ (0.052 mL of a 50% w/w solution in CH_2Cl_2 (Fluka), 3.21 × 10^{-4} mol, 4 equiv). This solution was stirred at room temperature for 28 h, then concentrated to 1 mL, and directly applied to 25 g of silica gel. Flash chromatography with 10–30% acetone in hexane as eluant gave 0.050 g (51%) of pure **4**. For analytical purposes (lactam^{3,4})Cs (**4**) was precipitated from ether with pentane: R_f 0.21 (40% acetone/hexane); $[\alpha]^{rt}_D$ −260.0° (*c* 0.25, $CHCl_3$); mp 135.0–138.0 °C; IR ($CHCl_3$) 3310, 2955, 1670 (shoulder), 1630; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.70 (d, J = 6 Hz, 3 H, CH_3-C(4^1)), 0.75–1.08 (m, 39 H, CH_3-C(3^2), 2CH_3-C(4^4), 2CH_3-C(3^5), 2CH_3-C(4^6), 2CH_3-C(4^9), 2CH_3-C(4^{10}), 2CH_3-C(3^{11})), 1.11–1.72 and 2.80–2.19 (2 m, 21 H, H-C(4^1), H-C(5^1), 2H-C(3^2), 2H-C(5^3), 2H-C(4^3), 2H-C(3^4), H-C(4^4), 2H-C(3^6), H-C(4^6), 2H-C(3^9), H-C(4^9), 2H-C(3^{10}), H-C(4^{10}), H-C(3^{11}), 1.21 (m, 3 H, CH_3-C(2^8)), 1.33 (d, J = 7 Hz, 3 H, CH_3-C(2^7)), 1.57 (m, 3 H, CH_3-C(7^1)), 2.31–2.47 (2 m, 2 H, H-C(5^1), H-C(3^5)), 2.64 (s, 3 H, CH_3-N^{11}), 2.65 (s, 3 H, CH_3-N^{10}), 3.07 (s, 3 H, CH_3-N^9), 3.14–3.30 (m, 2 H, 2H-C(6^3)), 3.18 (s, 3 H, CH_3-N^6), 3.31 (s, 3 H, CH_3-N^3), 3.43 (s, 3 H, CH_3-N^1), 3.48 (t, J = 9 Hz, H-C(3^3)), 3.88 (m, 2 H, H-C(3^1), HO-C(3^7)), 4.46 (m, 1 H, H-C(2^7)), 4.66 (t, J = 8 Hz, 1 H, H-C(2^5)), 4.80 (m, 1 H, H-C(2^8)), 4.96 (m, 2 H, H-C(2^2), H-C(2^6)), 5.04 (t, J = 7 Hz, 1 H, H-C(2^{10})), 5.11 (d, J = 11 Hz, 1 H, H-C(2^{11})), 5.30–5.37 (m, 3 H, H-C(6^1), H-C(7^1), H-C(2^4)), 5.42 (d, J = 6 Hz, 1 H, H-C(2^1)), 5.64 (dd, J = 10.5, 4 Hz, 1 H, H-C(2^9), 7.11 (d, J = 7.7 Hz, 1 H, H-N^8), 7.47 (d, J = 7.2 Hz, 1 H, H-N^7), 7.70 (d, J = 9.2 Hz, 1 H, H-N^2), 7.95 (d, J = 8.5 Hz, 1 H, H-N^5); mass spectrum, exact mass calcd for $C_{64}H_{114}N_{11}O_{12}$ 1228.8648, found 1228.8610.

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Registry No. **2**, 108466-41-7; **3**, 115224-89-0; **4**, 115141-85-0; **5**, 115160-84-4; **6**, 103478-71-3; **7**, 115160-85-5; **8**, 115141-86-1; **9**, 115141-87-2; **10**, 81135-26-4; **11**, 115141-88-3; **12**, 23632-67-9; **12** (acid chloride), 58456-26-1; **13**, 89969-26-6; **16**, 115141-89-4; **17a**, 115141-97-4; **17b**, 115141-90-7; **18a**, 115141-98-5; **18b**, 115141-91-8; **19a**, 115141-99-6; **19b**, 115141-92-9; **19c**, 115142-00-2; **20**, 115141-93-0; **21**, 115141-94-1; **22**, 81135-27-5; **23**, 115160-86-6; **24**, 81135-31-1; **25**, 115141-95-2; **26**, 115141-96-3; H-Leu-OMe·HCl, 7517-19-3; BOC-Abu-OH, 34306-42-8; H-MeBmt-OH, 59865-23-5; $(CH_3)_3CCOCl$, 3282-30-2.