I_{50}, defined here as the concentration of drug that inhibits hydrolysis of 1 μM substrate by 50%, were determined by using the xanthine analogues at concentrations between 1 and 100 μM. Due to limited solubility or limited degree of inhibition, I_{50} values could not be established for some analogues, and the levels of inhibition at 25 or 50 μM were determined. The presence or absence of calcium + calmodulin did not affect the level of inhibition of the peak I enzyme by the xanthine analogues. None of the analogues affected the 5'-nucleotidase or subsequent steps of the assay. IBMX inhibited the activity of peaks I and II with I_{50} values of 6.6 and 13.3 μM, respectively, which are similar to values reported previously.[4]

Acknowledgment. We thank the National Institute of General Medical Sciences of the National Institutes of Health for their generous support of this work through Grants GM-21220 and GM-07628 to the Vanderbilt group. We also appreciate the technical assistance of Barbara Duemler and the initial synthetic work in this area by Dr. William J. Christ during his Ph.D. studies at the University of South Florida.

Registry No. **2a**, 101031-51-0; **2b**, 101031-52-1; **2c**, 101031-53-2; **2d**, 101031-54-3; **2e**, 101031-55-4; **2f**, 101031-56-5; **3a**, 101031-57-6; **3b**, 101031-58-7; **3c**, 101031-59-8; **3d**, 101031-60-1; **3e**, 101031-61-2; **3f**, 101031-62-3; **4**, 101031-63-4; **5a**, 101031-64-5; **5b**, 101031-65-6; **5c**, 101031-66-7; **5d**, 101031-67-8; **6a**, 101031-68-9; **6b**, 101031-69-0; **6c**, 101031-70-3; **6d**, 101031-71-4; **7**, 101031-72-5; **8a**, 101031-73-6; **8b**, 101031-74-7; **8c**, 101031-75-8; **8d**, 101031-76-9; **9a**, 101031-77-0; **9b**, 101031-78-1; **9c**, 101031-79-2; **9d**, 101031-80-5; trimethylacetyl chloride, 3282-30-2; isoamyl isocyanate, 1611-65-0; 4-methylvaleryl chloride, 38136-29-7; methyl 2-amino-4-chlorobenzoate, 5900-58-3; 1-iodo-2-methyl propane, 513-38-2; methyl 2-acetamido-6-chlorobenzoate, 70625-65-9.

Synthesis and Antimitogenic Activities of Four Analogues of Cyclosporin A Modified in the 1-Position

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Cyclosporin A (CSA, 1), an immunosuppressive cyclic undecapeptide, contains a unique N-methylated amino acid, (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(*N*-methylamino)-6-octenoic acid, called both C-9-ene and MeBmt [(4*R*)-*N*-methyl-4-butenyl-4-methylthreonine] that may be essential for the biological activity of CSA. In order to determine the minimal portion of MeBmt needed for antimitogenic activity, four analogues of CSA specifically modified in the 1-position have been synthesized. These are (MeThr^1)CSA (**4**), (MeAbu^1)CSA (**5**), (MeAbu^1,Sar^{10})CSA (**6**), and [(MeLeu(3-OH)^1)]CSA (**7**). The synthesis of analogues was carried out by forming a linear undecapeptide that was cyclized at the two non-N-methylated amino acids. The structure of cyclic analogues **4**–**7** and their corresponding precursors were established unequivocally by ^{1}H NMR, FAB mass spectrometry, elemental analysis, and HPLC. The inhibition of Con A stimulated thymocytes by CSA (1), DH-CSA (**2**), **7**, **4**, **5**, and **6** gave IC_{50}'s (nM) of 4, 10, 600, 8×10^3, 15×10^3, and 40×10^3, respectively. The increase in IC_{50} by modification of the side chain in MeBmt suggested the importance of this amino acid in the 1-position of CSA for full antimitogenic activity.

Cyclosporin A (CSA) 1,[1] a unique and unusually effective immunosuppressive (IS) agent[2] isolated from the fungal species *Tolypocladium inflatum Gams*,[3] is a neutral, homodetic, hydrophobic cyclic peptide. The structure of CSA, established by chemical degradation[4] as well as by X-ray crystallographic analysis of an iodo derivative,[5] contains 11 amino acids, seven of which are N-methylated (Figure 1a). All amino acids have the 2*S* configuration except for the D-Ala at position 8 which has the *R* configuration. All cyclosporins isolated to date contain a unique N-methylated amino acid at position 1, (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(*N*-methylamino)-6-octenoic acid, initially called C-9-ene and more recently MeBmt [(4*R*)-*N*-methyl-4-butenyl-4-methyl-L-threonine]. X-ray crystallographic analysis of an iodo derivative of CSA revealed that a major portion of the molecule (residues 1–6) adopts an antiparallel β-pleated sheet conformation that contains three transannular hydrogen bonds; these include the NH of Abu hydrogen bonded to the C=O of Val, the NH of Val to the C=O of Abu, and the NH of Ala-7 to the C=O of MeVal. A fourth hydrogen bond is found as a γ-turn between the NH of D-Ala-8 to the C=O of MeLeu-6. Residues 7–11 form an open-loop featuring a cis amide bond between the MeLeu residues 9 and 10.[5a] The structural features are also found in the solution and X-ray structures of CSA.[5b]

Wenger has reported detailed synthetic procedures for the syntheses of MeBmt[6] and CSA[7] and biological data for several analogues of CSA in which positions 1, 2, 3, and 11 are modified (see Figure 1b).[8] Of particular interest were the effects on biological activity of modifying the MeBmt residue. While dihydro CSA (**2**, DH-CSA), formed by hydrogenation of the double bond, retains high biological activity, the deshydroxy analogue **3** and the (MeThr^1)CSA **4** analogues do not, so that a major portion of the unusual amino acid MeBmt appears to be essential for high biological activity. In order to further evaluate

(1) Borel, J. F. In *Cyclosporine A*; White, D. J. G., Ed.; Elsevier Biomedical: Amsterdam, 1982; p 5.
(2) Kahan, B. D.; Borel, J. F. *Transplant Proc.* **1983**, *15*, Suppl. 1.
(3) Dreyfuss, M.; Härri, E.; Hofmann, H.; Kobel, H.; Pache, W.; Tscherter E. *J. Appl. Microbiol.* **1976**, *3*, 125.
(4) Rüegger, A.; Kuhn, M.; Lichti, H.; Loosli, H. R.; Huguenin, R.; Quiquerez, C.; von Wartburg, A. *Helv. Chim. Acta* **1976**, *59*, 1072.
(5) (a) Petcher, T. J.; Weiber, H. P.; Rüegger, A. *Helv. Chim. Acta* **1976**, *59*, 1480. (b) Kessler, H.; Looshi, H.-R.; Oschkinat, H. *Helv. Chim. Acta* **1985**, *68*, 661–681. Loosli, H.-R.; Kessler, H.; Oschkinat, H.; Weber, H.-P.; Petcher, T. J.; Widmer, A. *Helv. Chim. Acta* **1985**, *68*, 682–704.
(6) Wenger, R. M. *Helv. Chim. Acta* **1983**, *66*, 2308.
(7) Wenger, R. M. *Helv. Chim. Acta* **1983**, *66*, 2672; *Helv. Chim. Acta* **1984**, *67*, 502 and references cited therein.
(8) Wenger, R. M. *Angew. Chem., Int. Ed.* Engl. **1985**, *24*, 77.
(9) McDermott, J. R.; Benoiton, M. L. *Can. J. Chem.* **1973**, *51*, 1915. Cheung, S. T.; Benoiton, N. L. *Ibid.* **1977**, *55*, 906.

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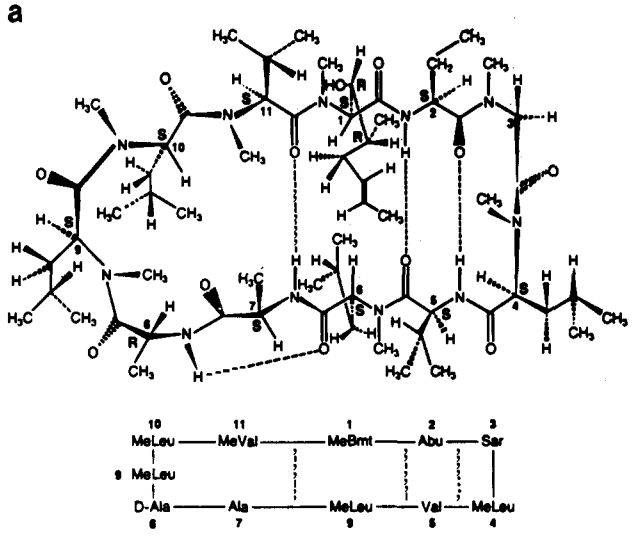
a



b

Cyclo (D-Ala-MeLeu-Y-MeVal-N-CHCO-Abu-Sar-MeLeu-Val-MeLeu-Ala)

R' = Me

1 R' = -CH(OH)-CH(Me)-CH2-CH=CH-CH3 (R,R); Y = MeLeu, Cyclosporin A (CSA)

2 R' = -CH(OH)-CH(Me)-CH2-CH2-CH2-CH3 (R,R); Y = MeLeu, Dihydro-CSA (DH-CSA)

3 R' = -CH2-CH(Me)-CH2-CH2-CH2-CH3 (R); Y = MeLeu, Deshydroxy-dihydro-CSA

4 R' = -CH(OH)-CH3 (R); Y = MeLeu, (MeThr[1]) CSA

5 R' = -CH2-CH3; Y = MeLeu, (MeAbu[1]) CSA

6 R' = -CH(OH)-CH2-CH3; Y = Sar, (MeAbu[1], Sar[10]) CSA

7 R' = -CH(OH)-CH(CH3)2; Y = MeLeu, [MeLeu(3-OH)[1]] CSA

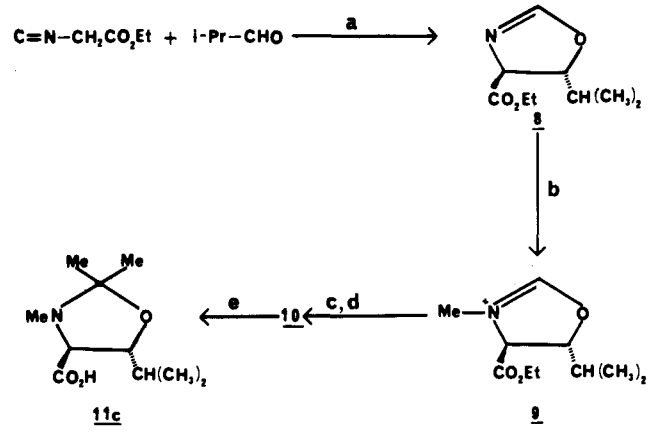
Figure 1. (a) Schematic structure of cyclosporin A. (b) Representative structures of cyclosporins.[7,8]

the structural requirements of MeBmt needed for full biological activity, we have synthesized four analogues of CSA modified in the MeBmt position (Figure 1b). The amino acid MeLeu[10] in analogue **5** was replaced by Sar. This replacement was expected to disrupt the cis amide linkage between MeLeu residues 9 and 10 and thereby affect the conformation and biological activity of analogue **6**.

Results

Chemistry. 1-Position Replacements. Boc-*N*-methyl-2-aminobutyric acid (Boc-MeAbu, **11a**) was synthesized from Boc-Abu by the method of McDermott and Benoiton.[9] Boc-*N*-methyl-*O*-benzyl-L-threonine (Boc-MeThr(Bzl), **11b**) was synthesized from Boc-Thr(Bzl) by N-methylation in dimethoxyethane (DME), followed by crystallization of Boc-MeThr(Bzl) as the cyclohexylamine salt. The cation solvating ability of DME suppresses β-elimination. This is especially evident in the preparation of Boc-MeSer(Bzl), which was synthesized in 85% yield via this modified procedure.[10]

Scheme I. Synthesis of (±)-*threo*-*N*-Methyl-3-hydroxyleucine[a]



[a] Key: (a) NaCN, EtOH, 0 °C, 50 min; (b) $CH_3OSO_2CF_3$, Et_2O, room temperature, 1 h; (c) 2 N HCl, Δ, 15 h; (d) Dowex 1, 0.2 N AcOH; (e) $(CH_3)_2CO$, Δ, 24 h.

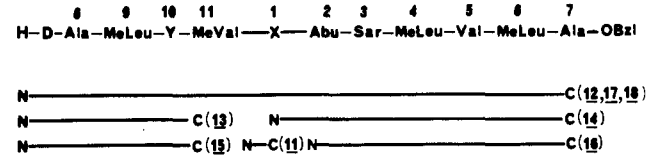


Figure 2. Strategy used for the synthesis of linear peptide cyclosporine precursors.[7]

The naturally occurring [11] β-hydroxyleucine has been synthesized in both racemic[12] and optically active forms.[13] The *N*-methyl derivative has not been reported. To obtain MeLeu(3-OH) of defined configuration, we adapted the procedure developed by Schöllkopf[14] for the synthesis of β-hydroxy amino acids (Scheme I). The reaction of ethyl isocyanoacetate with isobutyraldehyde in the presence of NaCN as base gave the thermodynamically stable *trans*-oxazoline **8** as the major product (78%) along with a minor component characterized as *N*-formyl-3-hydroxy-*threo*-leucine (4%). To obtain the *N*-methyl amino acid derivative, the oxazoline **8** was reacted with methyl triflate at room temperature to give the N-methylated imidate **9**. Hydrolysis of **9** with dilute HCl followed by ion-exchange chromatography of the amino acid (Dowex-1, hydroxide form) afforded 64% of (±)-*threo*-*N*-methyl-β-hydroxyleucine [MeLeu(3-OH) **10**]. This was converted to the dimethyloxazolidine **11c** by refluxing in acetone.

Synthesis of Peptides. The synthesis of the analogues reported here closely followed the strategy successfully employed by Wenger[7] for the synthesis of CSA (Figure 2). The undecapeptides were synthesized so that D-Ala[8] was at the N-terminus and Ala[7] at the C-terminus. With this strategy, cyclization is effected at the only consecutive pair of non-N-methylated amino acids. The undecapeptides **12** were synthesized following the strategy of Wenger by

(10) The use of DME in place of THF gives much higher yield of Boc-MeSer(OBzl)-OH due to decreased β-elimination. Use of lower reaction temperatures (0–5 °C) and no excess sodium hydride limited the dehydro amino acid formation to less than 7%. The cyclohexylamine salts of Boc-MeSer(Bzl) and Boc-MeThr(Bzl) derivatives crystallize more readily than the dicyclohexylamine salts.

(11) Kenner, G. W.; Sheppard, R. C. *Nature* **1958**, *181*, 48.
(12) Liwschitz, Y.; Rabensohn, Y.; Perera, D. *J. Chem. Soc.* **1962**, 116. Marchand, J.; Pais, M.; Jarreau, F. X. *Bull. Soc. Chim. Fr.* **1971**, 3742.
(13) Schöllkopf, U. *Tetrahedron* **1983**, *39*, 2085. Schöllkopf, U.; Goth, U.; Gull M. R.; Nozulak, J. *Liebigs Ann. Chem.* **1983**, 1133.
(14) Hoppe, D.; Schöllkopf, U. *Angew. Chem., Int. Ed. Engl.* **1970**, *9*, 300. Schöllkopf, U. *Angew. Chem., Int. Ed. Engl.* **1977**, *16*, 339.

Table I. Physical Properties of Tetrapeptide and Undecapeptide Intermediates[a]

compd	mp, °C	TLC: R_f (solv)	$[\alpha]^{24}_D$ (c)	yield, %	formula	anal.
14a	61–62	0.52 (A)	−144 (1.05)	80	$C_{46}H_{77}N_7O_{10}$	C, H, N
14b	65–67	0.57 (A)	−120 (1.5)	80	$C_{53}H_{82}N_7O_{11}$	C, H, N
14c	oil	0.68 (A)		73	$C_{47}H_{78}N_7O_9$	C, H, N
12a	74–75	0.49 (A)	−192 (0.95)	69	$C_{69}H_{119}N_{11}O_{14}$	C, H, N
12b	84–85	0.52 (A)	−134 (1.2)	67	$C_{76}H_{125}N_{11}O_{15}$	C, H, N
12c	88–90	0.46 (A)	−82 (0.2)	68	$C_{71}H_{123}N_{11}O_{15}\cdot 2H_2O$	C, H, N
12d	89–90	0.45 (A)	−183 (1)	66	$C_{65}H_{111}N_{11}O_{14}\cdot H_2O$	C, H, N

[a] Yield, melting point, and R_f (solvent) of C-deprotected undecapeptides: (**17a**) 92, 113–115, 0.45 (B); (**17b**) 75, 121–122, 0.36 (B); (**17c**) 89, 117–119, 0.43 (B); (**17d**) 91, 116–118, 0.37 (B).

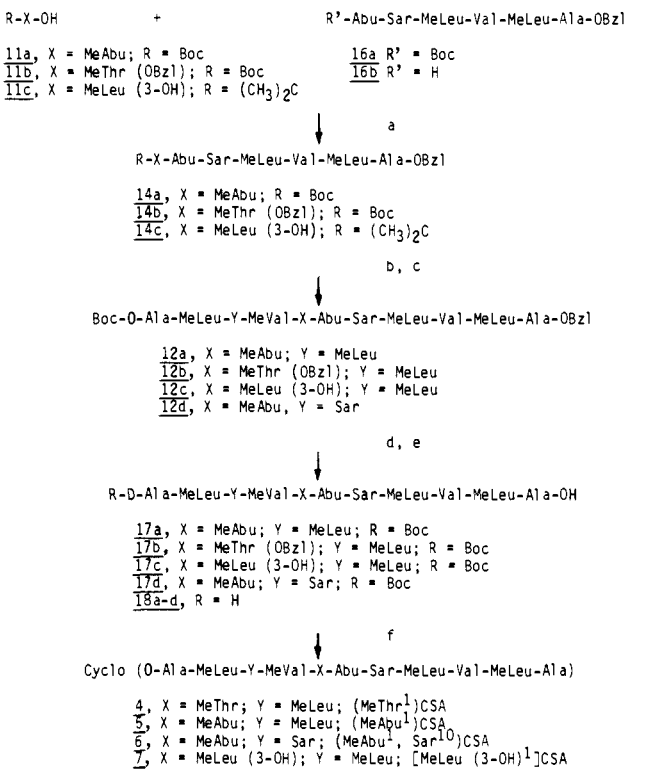
condensing tetrapeptides **13** with the N-deprotected heptapeptide analogues of **14**. The tetra- and heptapeptide fragments were built up in the direction shown by the arrows in Figure 2. In order to prevent racemization of *N*-methyl amino acids, the heptapeptide, X-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (**14**), was synthesized by stepwise addition of protected amino acids to Ala-OBzl.[7] In contrast, the tetrapeptide Boc-D-Ala-MeLeu-Y-MeVal-OH (**13**) was synthesized beginning with Boc-D-Ala-OH by stepwise addition toward the C-terminus, as has been described by Wenger.[7,23]

The synthesis of the different heptapeptides employed in the present study (Scheme II) was achieved by the coupling of the appropriate Boc *N*-methyl amino acids **11a** and **11b** with hexapeptide **16**[7] (DCC, HOBT,[15] NMM, CH_2Cl_2, 0 °C 5 h, 35 h at room temperature, column chromatography) in a yield of 70–80%. MeLeu(3-OH)OH was incorporated as the dimethyloxazolidine-protected derivative **11c**. The synthesis of tetrapeptide **15a** (benzyl ester) was carried out by the procedure of Wenger.[7] Removal of the benzyl ester group from **15a** gave Boc-D-Ala-MeLeu-MeLeu-MeVal-OH (**13a**) in 91% yield. The tetrapeptide Boc-D-Ala-MeLeu-Sar-MeVal-OH (**13b**) was synthesized in an analogous fashion.

The Boc group was removed from the heptapeptides **14a** and **14b** (Scheme II) with TFA–CH_2Cl_2 (0 °C, 2 h), whereas the isopropylidene group of heptapeptide **14c** was removed with MeOH–1 N HCl (15 h, N_2 atmosphere) followed by $NaHCO_3$ solution treatment. The coupling of tetrapeptides **13** with the N-deprotected heptapeptides **14a–14c** was most conveniently achieved by the use of the BOP reagent, [(benzotriazol-1-yl)oxy]tris(dimethylamino)-phosphonium hexafluorophosphate,[16] in the presence of 1 equiv of *N*-methylmorpholine to give the undecapeptides **12a–12c** in yields of 65–70%. Undecapeptide **12d** was prepared by deprotecting and coupling **14a** with **13b**.

The benzyl ester group in undecapeptides **12a**, **12c** and **12d** was removed by catalytic hydrogenolysis (H_2, 10% Pd–C, *i*-PrOH, 3 h, room temperature to give the undecapeptide as the C-terminal acid (Scheme II). The benzyl ether and benzyl ester groups from **12b** could be cleaved best by using phase-transfer catalytic hydrogenation procedure (10% Pd–C, 3:1 AcOH–*i*-PrOH, ammonium formate, 4 h).[17] The free undecapeptides **18a–18d** could be obtained by treating the Boc-protected peptides with TFA–CH_2Cl_2 (−20 °C, 3 h, followed by cold $NaHCO_3$ solution). It is important to note that the removal of the Boc group at higher temperatures gave multiple products that did not cyclize to identifiable cyclic undecapeptides, suggesting that racemization or another rearrangement of some *N*-methyl amino acid residue occurs in the undecapeptide that does not occur in either the tetra- or heptapeptide. The unprotected peptides **18a–18d** were cyclized at high dilution using BOP as the dehydrating reagent (4 equiv of BOP, 5 equiv of DMAP, CH_2Cl_2, 36 h), conditions developed by Wenger for CSA.[7] Cyclic analogues **4–7** were isolated in 50–70% yield after column chromatography.

Scheme II[a]



[a] Key: (a) DCC, 1-HOBT, NMM, CH_2Cl_2; (b) TFA, CH_2Cl_2, 0 °C; $NaHCO_3$ solution for **14a** and **14b**, MeOH–1 N HCl; $NaHCO_3$ solution for 14c; (c) Boc-D-Ala-MeLeu-Y-MeVal-OH, BOP, CH_2Cl_2, NMM; (d) H_2, 10% Pd–C, EtOH; (e) TFA, CH_2Cl_2, −20 °C, $NaHCO_3$ solution; (f) BOP, DMAP, CH_2Cl_2 (high dilution).

The ^1H NMR data of various intermediate peptides in $CDCl_3$ and Me_2SO-d_6 at room temperature showed the presence of multiple conformers, which is consistent with Wenger's observation of multiple conformations for CSA intermediates. The cyclic undecapeptides **4**, **5**, and **7** showed the presence of only one major conformer in $CDCl_3$, but the MeAbu[1],Sar[10] analogue **6** clearly showed multiple conformations in this solvent system. This is probably due to disruption of the cis amide bond between residues 9 and 10.

The chemical shifts of various NH protons of cyclic analogues were found to differ from the corresponding amide protons in CSA (Table III). However, the chemical shifts of nonexchangeable protons in synthetic analogues **4–7** correlated well with ^1H NMR spectra for CSA recorded in both $CDCl_3$ and C_6D_6. The most notable were the

(15) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788.
(16) Castro, B.; Dormoy, J. R.; Evin, J. G.; Selve, C. *Tetrahedron Lett.* **1975**, *14*, 1219.
(17) Anwer, K. M.; Spatola, A. F. *Synthesis* **1980**, 929.

Table II. Physical Properties of CSA Analogues

compd	mp, °C	TLC: R_f (solv)	cyclizn yield, %	HPLC:[a] ret time, min	formula	FAB MS: M^+
(MeThr^1)CSA (**4**)	95–96	0.56 (A)	61	6	$C_{64}H_{109}N_{11}O_{12}$	1134.78
(MeAbu^1)CSA (**5**)	124–125	0.64 (A)	71	10.4	$C_{57}H_{103}N_{11}O_{11}$	1118.79
(MeAbu^1, Sar^{10})CSA (**6**)	96–97	0.46 (A)	57	4.3	$C_{53}H_{95}N_{11}O_{11}$	1062.73
[(MeLeu(3-OH)^1]CSA (**7**)	121–122	0.55 (A)	58	8.9	$C_{59}H_{107}N_{11}O_{12}$	1162.82

[a] HPLC run on a reversed phase Bondapak C_{18} column (Waters) using a gradient of acetonitrile–water (65–80% acetonitrile in 20 min) and detected at 214 nM. **4**: ^{1}H NMR ($CDCl_3$) δ 0.7–1.10 (m, 39 H, CH_3 of Abu, MeLeu, Val, MeVal), 1.16 (d, J = 6 Hz, 3 H, CH_3 D-Ala), 1.26 (d, J = 15z, 3 H, MeThr-CH_3), 1.25 (d, J = 6 Hz, 3 H, CH_3 Ala), 1.40–1.80 (m, 10 H, β-CH_2 of Abu, MeLeu), 1.82–2.23 (m, 5 H, β-CH of MeVal and γ-CH of MeLeu), 2.30–2.60 (m, 1 H, Val β-CH), 2.67, 2.70, 3.09, 3.15, 3.27, 3.37, 3.46 (7 s, 21 H, N-CH_3), 3.22 (d, 1 H, Sar α-CH), 4.20 (m, 2 H, β-CH and OH of MeThr), 4.40–5.35 (m, 9 H, α-CH), 5.46 (d, 1 H, α-CH), 5.67 (m, 1 H, α-CH), 7.22 (d, 1 H, NH), 7.50 (d, 1 H, NH), 7.83 (d, 1 H, NH), 8.27 (d, 1 H, NH). **5**: ^{1}H NMR ($CDCl_3$) δ 0.7–1.14 (m, 42 H, CH_3 of Abu, MeAbu, MeLeu, Val, MeVal), 1.27 (d, J = 6 Hz, 3 H, CH_3 D-Ala), 1.35 (d, J = 6 Hz, 3 H, CH_3 Ala), 1.40–1.80 (m, 12 H, β-CH_2 of Abu, Me Abu, MeLeu), 1.84–2.25 (m, 5 H, β-CH of MeVal and γ-CH of MeLeu), 2.30–2.50 (m, 1 H, Val β-CH), 2.68, 2.70, 3.10, 3.17, 3.29, 3.36, 3.41 (7 s, 21 H, N-CH_3), 3.16 (d, 1 H, Sar α-CH), 4.43 (m, 1 H, α-CH), 4.65–5.38 (m, 8 H, α-CH), 5.62–5.72 (m, 2 H, α-CH), 7.37–7.50 (m, 2 H, NH), 8.02 (d, 1 H, NH), 8.40 (d, 1 H, NH). **6**: ^{1}H NMR ($CDCl_3$) (multiple conformers) δ 0.70–1.09 (m, 36 H, CH_3 of Abu, MeAbu, Val, MeVal), 1.12–1.39 (m, 6 H, CH_3 Ala), 1.42–2.10 (m, 14 H, β-CH_2 of Abu, MeAbu, MeLeu, β-CH of MeVal and γ-CH MeLeu), 2.20–2.40 (m, 1 H, β-CH of Val), 2.63, 2.74, 2.78, 2.88, 2.94, 3.02, 3.06, 3.20, 3.27, 3.35 (series of s, 21 H, N-CH_3), 3.11, 3.15 (2 d, 2 H, Sar α-CH), 3.65–5.55 (series of m, 12 H, α-CH), 6.68, 7.12, 7.26, 7.34, 7.85, 8.0, 8.11, 8.25 (no. of d, 4 H, NH). **7**: ^{1}H NMR ($CDCl_3$) δ 0.74 (d, 3 H, CH_3 of β-OH-Leu), 0.78 (d, 3 H, CH_3 of β-OH-Leu), 0.80–1.10 (m, 39 H, CH_3 of Abu, MeLeu, Val, MeVal) 1.27 (d, J = 6 Hz, 3 H, CH_3 D-Ala), 1.33 (d, J = 6 Hz, 3 H, CH_3 Ala), 1.40–1.88 (m, 10 H, β-CH_2 of Abu and MeLeu), 1.92–2.23 (m, 6 H, β-CH of MeVal, γ-CH of MeLeu and β-OH-Leu), 2.32–2.51 (m, 1 H, Val β-CH), 2.67, 2.68, 3.1, 3.16, 3.30, 3.44, 3.5 (7 s, 21 H, N-CH_3), 3.20, (d, 1 H, Sar α-CH), 3.87 (m, 2 H, β-CH and OH of β-OH-Leu), 4.49 (t, 1 H, α-CH), 4.60–5.15 (m, 8 H, α-CH), 5.28 (d, 1 H, α-CH), 5.34 (dd, 1 H, α-CH), 5.70 (dd, 1 H, α-CH), 7.37 (d, 1H, NH), 7.63 (d, 1 H, NH), 7.90 (d, 1 H, NH), 8.32 (d, 1 H, NH).

Table III. ^{1}H Chemical Shifts (δ) of Selected Cyclosporin Analogue Protons in $CDCl_3$ and C_6D_6

	1 (CSA)[a]		**5** [(MeAbu^1)CSA]		4 [(MeThr^1)CSA]		**7** [MeLeu(3-OH)^1]CSA	
proton	$CDCl_3$	C_6D_6	$CDCl_3$	C_6D_6	$CDCl_3$	C_6D_6	$CDCl_3$	C_6C_6
NH	(D-Ala) 7.15	7.63	7.37–7.50 (2 NH)	7.48	7.22	7.54	7.37	7.60
	(Val) 7.48	7.46		7.91	7.50	7.67	7.63	7.76
	(Ala) 7.65	8.01	8.02	8.30	7.83	8.18	7.90	8.13
	(Abu) 7.97	8.26	8.40	8.54	8.27	8.48	8.32	8.50
N-CH_3	(^1N-CH_3) 3.51	3.73	3.41[b]	3.46[b]	3.46	3.73	3.50[b]	3.61
	(^3N-CH_3) 3.40	3.08	3.36	3.04	3.37	3.05	3.44	3.14
	(^4N-CH_3) 3.11	2.60	3.10	2.67	3.09	2.59	3.10	2.61
	(^6N-CH_3) 3.25	3.24	3.29	3.29	3.27	3.24	3.30	3.30
	(^9N-CH_3) 3.12	2.93	3.17	3.0	3.15		3.16	2.91
	(^{10}N-CH_3) 2.69	2.84	2.68	2.81	2.67	2.86	2.67	2.80
	(^{11}N-CH_3) 2.71	2.98	2.70	3.15	2.70	2.96 (2 N-CH_3)	2.68	3.0
Ala^7-CH_3	1.36	1.68	1.35	1.62	1.35	1.61	1.33	1.65
Ala^8-CH_3	1.26	1.13	1.27	1.13	1.16		1.27	1.17
Sar α-CH	4.74	4.01	4.72	4.01	4.70	4.02	4.73	4.08
MeVal^{11}-γ-CH_3	0.86	0.66	0.86	0.64	0.86	0.65	0.87	0.67

[a] Assignments provided by Kessler and Loosli (see ref 5b). [b] N-Methyl signals are assigned by analogy.

changes in chemical shift of the Ala^7,D-Ala^8 methyl groups, and the Sar α-CH and the MeVal γ-methyl protons in two deuterated solvents (Table III). The high upfield shift of the γ-CH_3 of MeVal^{11} when the NMR was recorded in deuteriobenzene is very similar to that observed for CSA and suggested that chiral integrity of the amino acid was retained during the 4 + 7 coupling to form the undecapeptide, especially that of MeVal^{11}. In addition, the purity of the various intermediates and cyclic peptides was established by reversed-phase HPLC system (Waters, μ-Bondapak, C_{18} column) using acetonitrile–water as the gradient system with detection at 214 nM. The retention times of the cyclic analogues are reported in Table II.

Discussion

The structure of the immunosuppresive agent CSA is characterized by two novel features, the highly N-methylated cyclic undecapeptide ring system and the presence of the unique amino acid (4R)-4-[(*E*)-2-butenyl]-4,N-dimethyl-L-threonine (MeBmt) in the 1-position. Previous work by Wenger and collaborators had led to the successful syntheses of both MeBmt and CSA that have been reported in detail. In addition, the biological activities of several synthetic analogues modified in the 1-position have been reported.[8] These results had established that the double bond in MeBmt is not absolutely essential for biological activity because DH-CSA is about half as active as CSA. In addition, the (MeThr^1)CSA analogue was reported to be inactive as an immunosuppressive agent, although no synthetic details have been published previously. Since the configurations of C-2 and C-3 in N-MeThr are the same (2S,3R) as in (4R)-4-[(*E*)-2-butenyl]-4,N-dimethyl-L-threonine, this latter result suggested that some portion of the five atoms deleted from MeBmt were contributing in a major way to the biological activity of cyclosporine. This result was remarkable in view of the fact that the deletion corresponds to the removal of only five carbon atoms in a molecule with a molecular weight of 1202.

We therefore set out to evaluate the effect that replacing some of the missing atoms in the 1-position of (MeThr^1)CSA would have on the biological activity of the analogue. At the start of this work, the (MeThr^1)CSA analogue had not been reported, and the (MeAbu^1)CSA

was prepared as a standard for comparison with CSA in the biological assay. (MeThr[1])CSA and (MeAbu[1])CSA were synthesized via a modification of the procedure reported for the synthesis of cyclosporine. (MeAbu[1],Sar[10])CSA was synthesized in a similar way to evaluate the nature of the cis amide linkage between the two adjacent MeLeu residues 9 and 10 in cyclosporine. The structures of the analogues were established by microanalysis, FAB mass spectrometry, and high-field FT ^{1}H NMR spectrometry. In addition, the physical properties of the two intermediates **16** and **15** were essentially identical with those reported by Wenger. The possibility that the *N*-MeVal[11] residue racemized during coupling was excluded by the analysis of the ^{1}H NMR spectrum of analogues 4–7 in deuteriobenzene. In that solvent system the chemical shift of the γ-methyl of L-MeVal[11] (in CSA) is shifted upfield to 0.65 ppm in deuteriobenzene whereas for (D-MeVal[11])CSA these protons are not shifted. In addition, significant differences are evident between the NMR spectra of the two diastereomers.[5b,7] All synthetic analogues reported as having the L configuration in the 11-position showed the upfield shift (Table II). The *N*-MeLeu(3-OH) group was introduced into heptapeptide **14c** as the threo racemate. Separation of the diastereomers at either the heptapeptide or undecapeptide stages did not appear to occur during chromatography so that the mixture of diastereomers was cyclized together. The product obtained by this route clearly is the (2*S*,3*R*)-[MeLeu(3-OH)[1]]CSA on the basis of the NMR data that showed the presence of only one diastereomer with chemical shift and coupling constants consistent only with the 2*S*,3*R* diastereomer (Table II).

We have utilized the inhibition of Con A (conconavalin A) stimulated murine thymocytes as our assay of the relative biological activities of the various CSA analogues. A variety of in vitro assays have been used to measure the T-cell inhibitory activity of CSA, e.g., inhibition of allogeneically stimulated murine[18] and human[18] lymphocytes, inhibition of murine splenocyte[19] and rat splenocyte,[21] and human PBL (peripheral blood lymphocytes)[19] mitogen stimulation. The IC_{50} (concentration producing 50% inhibition) in these assays depends on the species used and the nature of the mitogenic stimulus. We have found that the inhibition of Con A stimulated murine thymocytes is a very sensitive assay, comparing favorably with the IC_{50} for inhibition of murine MLC[18] (mixed lymphocyte culture), and more sensitive than inhibition of Con A stimulated splenocytes[20] or human PBL's.[19] This sensitivity has thus enabled us to measure the activity of analogues with a wide range of IC_{50}'s.

As shown in Figure 3, the dose–response curves for each of the analogues are essentially parallel over a wide concentration range. The IC_{50} of CSA is in the range reported by Wang et al.[18], and the activities of the CSA analogues compare favorably with the qualitative biological responses (+++, ++, etc.) reported previously in the literature.[8] Thus, DH-CSA (**2**) is about half as active as CSA while (MeThr[1])CSA (**4**), which has been reported to be inactive[8], is about one-thousandth as active in this assay. The new analogues (MeAbu[1])CSA (**5**) and (MeAbu[1],Sar[10])CSA (**6**) both show less activity than the (MeThr[1])CSA analogue **4**, although all three do inhibit Con A stimulation of murine thymocytes at concentrations of 10 μM. It is not yet known whether these responses are all caused by the same mechanism.[24]

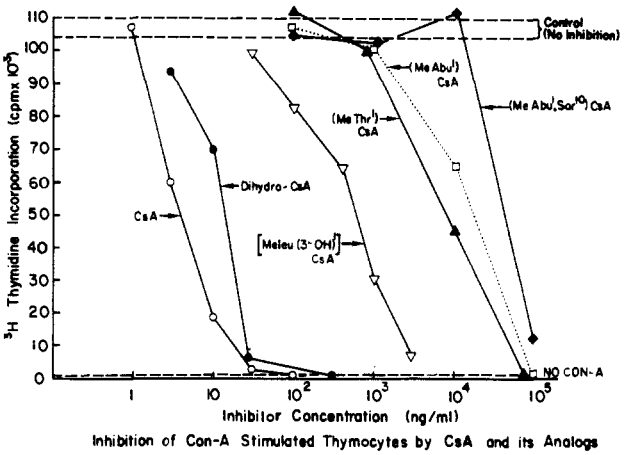


Figure 3. Inhibition of tritiated thymidine incorporation into murine thymocytes after Con A stimulation. Controls with and without added Con A are indicated by horizontal dashed lines. This experiment was repeated three times, with similar results. Cutlures: (○) CSA; (●) dihydro-CSA; (▽) [MeLeu(3-OH)[1]]CSA; (△) (MeThr[1])CSA; (□) (MeAbu[1])CSA; (■) (MeAbu[1],Sar[10])CSA.

The most interesting result is that the [MeLeu(3-OH)[1]]CSA analogue **7** has a biological activity about one-hundredth of that of cyclosporin. The analogue is clearly more active than either of the shorter analogues **4** and **5** but lacks full activity in spite of differing from DH-CSA (**2**) and CSA (**1**) by only three carbon atoms in the amino acid side chain in the 1-position (see Figure 1).[24] While it is possible that some racemization of one of the seven *N*-methyl amino acids might have occurred during synthesis, the NMR and CD data for the analogue compared with CSA itself are not consistent with this possibility.

The NMR data suggest that the modification of the MeBmt in position 1 of CSA to analogues with nonbranched side chain does not significantly alter the conformation of the cyclic ring system in chloroform. In Table III the *N*-methyl proton data show that unbranched analogues **4**, **5**, and **7** have only slightly different chemical shifts for the *N*-methyl groups than CSA. There are small differences between the amide resonances for the less active analgoues **4**, **5**, and **7** and CSA. The former are distinguished by the absence of an upfield resonance at 7.15 ppm for the D-Ala NH found in the spectrum for cyclosporin A in chloroform and the downfield shifted resonances for the amide protons in the less active analogues **4**, **5**, and **7**. Detailed conformational analysis of all analogues is beyond the scope of the present report, but these preliminary data suggest that the conformations of analogues **4**, **5**, and **7** are very close to that of cyclosporine in chloroform. Whether these conformation similarities persist in aqueous media is not known.

HPLC retention times of the various CSA analgoues in a standard solvent system were measured in order to determine whether the shortened side chain of the amino acid replacements in the 1-position altered the distribution

(18) Wang, B. S.; Heacock, E. A.; Collins, K. H.; Hutchinson, I. F.; Tilney, N. L.; Mannick, J. A. *J. Immunol.* **1981**, *127*, 89.
(19) Hess, A. D.; Tutschka, P. *J. Immunol.* **1980**, *124*, 2601.
(20) Larsson, E.-L. *J. Immunol.* **1980**, *124*, 2828.
(21) Burckhardt, J. J.; Guggenheim, B. *J. Immunol.* **1979**, *36*, 753.
(22) Dunlap, B. E.; Dunlap, S. A.; Rich, D. H. *Scand. J. Immunol.* **1984**, *20* 237.
(23) Zoaral, M. *Collect. Czech. Chem. Commun.* **1962**, *27*, 1273.
(24) Note Added in Proof: Using an interleukin-2 (IL-2) dependent cell line to measure IL-2 production in ConA stimulated thymocytes, we have found that **7** and **1** inhibit IL-2 production to the same extent they inhibit [^{3}H]TdR incorporation. Thus, cyclosporine and analogue **7** appear to inhibit T cells by the same mechanism.

properties of the analogues. In general, the retention time of the analogue increased with the length of the side chain in the 1-position as might be expected with the increasing hydrophobic properties of the molecule. DH-CSA (14 min) had a longer retention time than CSA (12 min). Thus, a simple relationship between hydrophobicity and biological activity is not apparent. Surprisingly, we noted that CSA elutes in a strikingly broad peak relative either to synthetic intermediates or to inactive diastereomeric cyclic analogues that eluted in sharp, well-resolved peaks. This observation suggests that the conformation of CSA may be slowly equilibrating during passage through the column, possibly among multiple conformations or *N,O*-acyl isomers. The closely related analogue (MeAbu[1],Sar[10])CSA (**6**), which shows multiple conformations in the NMR, elutes in a sharp peak under a variety of conditions so that the broad elution peaks are not a function of multiple conformations alone.

Experimental Section

The protected amino acids and the coupling reagents used in this study were obtained from commercial sources and used without further purification. *N*-methyl amino acids were obtained by alkylation of Boc-protected amino acids utilizing the procedure of McDermott and Benoiton.[9] The benzyl esters of *N*-methyl amino acids were synthesized by refluxing Boc amino acids in benzyl alcohol and *p*-toluenesulfonic acid in benzene. Tetrahydrofuran was distilled prior to use from sodium benzophenone ketyl. Dichloromethane and chloroform were distilled from P_2O_5 and stored over Linde 3-Å molecular sieves. 10% palladium on charcoal catalyst was obtained from Alfa Division.

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian Model EM-390, Bruker Model HX90E pulse Fourier transform NMR interfaced with a Nicolet 1080 computer disk unit, and Bruker WH 270 spectrometers.

Chemical shifts were reported as δ units relative to Me_4Si as an internal standard. Optical rotations were recorded on a Perkin-Elmer 241 automatic polarimeter. Elemental analyses were determined by Galbraith Laboratories, Inc., Knoxville, TN. TLC was performed on 0.25-mm-thickness silica gel glass plates (Merck, silica gel 60 F-254). For column chromatography, Brinkman silica gel 60, 70-270 mesh, was used. Spots were visualized by UV, ninhydrin, and chlorox-tolidine reagents. The following solvent systems were used: A, chloroform-methanol (9.4:0.6); B, chloroform–methanol–acetic acid (9:0.8:0.2); C, butanol–pyridine–acetic acid–water (15:10:3:12).

Procedure for N-Methylation of Boc-Thr and Boc-Ser. A stirred solution of amino acid (1.7 mmol) in dimethoxyethane (DME) (8 mL) was cooled at 0 °C in an ice bath, and methyl iodide (11.8 mmol) was added. Sodium hydride (50% dispersion, 3.9 mmol) was added in portions over 15 min. The reaction mixture was further stirred at 0–5 °C for 22 h and decomposed by water, and the organic layer was concentrated. The residue was taken up in water (15 mL) and washed with ether (1 × 10 mL). The aqueous layer was acidified with cold 1 N HCl to pH 3.0, and the oil that separated was extracted into ether (3 × 15 mL). The ethereal layer was washed with cold 1 N sodium thiosulfate solution (2 × 15 mL) and sodium chloride solution (2 × 15 mL), dried (Na_2SO_4), and concentrated. The crude product was dissolved in ether (5 mL) and cooled, and cyclohexylamine (1.8 mmol) was added. The salt that separated was filtered and dried over P_2O_5. Boc-MeThr(Bzl) cyclohexylamine (CHA) salt was isolated in 94% yield: mp 127–129 °C; $[\alpha]^{24}_D$ –22.2 (*c* 1, EtOH). Anal. ($C_{28}H_{38}N_2O_5$) C, H, N.

Boc-MeSer(Bzl) cyclohexylamine salt was isolated in 92% yield (93:7 ratio of Boc-MeSer(Bzl) to Boc-dehdyroalanine): mp 134–136 °C; $[\alpha]^{24}_D$ –8.6 (*c* 1, EtOH). Anal. ($C_{22}H_{34}N_2O_5$) C, H, N.

Boc-MeAbu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (14a). The hexapeptide **16a** (1.52 g, 1.92 mmol)[7] was dissolved in a mixture of CH_2Cl_2–TFA (1:2, 3 mL), and the solution was stirred at 0 °C for 3 h. The solvent was removed and the residue taken up in $CHCl_3$ (15 mL), cooled, and washed successively with cold saturated $NaHCO_3$ solution (3 × 10 mL) and cold NaCl solution (2 × 10 ml). The organic layer was dried (Na_2SO_4) and concentrated to an oily residue to give **16b**, yield 1.2 g (91%).

A solution of the above hexapeptide (1.19 g, 1.72 mmol) Boc-MeAbu-OH (**11a**) (0.43 g, 2 mmol) in CH_2Cl_2 (10 ml) was stirred at 0 °C, and *N*-methylmorpholine (0.19 mL, 1.7 mmol) and 1-hydroxybenzotriazole (HOBT) (0.52 g, 3.4 mmol) followed by DCC (0.45 g, 2.2 mmol) were added. The reaction mixture was stirred for 5 h at 0 °C and 20 h at room temperature, cooled, and filtered. The filtrate was concentrated and the residue dissolved in ethyl acetate (25 mL). The organic solution was washed with 1 N HCl (15 mL), saturated $NaHCO_3$ solution (2 × 20 mL), and NaCl solution (2 × 15 mL), dried (Na_2SO_4) and concentrated. The crude product was purified on a silica gel column using chloroform–methanol as eluant to give pure heptapeptide **14a**: yield 1.22 g (80%); ^{1}H NMR ($CDCl_3$) δ 0.85 (m, 24 H, Abu, MeAbu, MeLeu, Val-CH_3), 1.30 (d, J = 6 Hz, Ala-CH_3), 1.42 (s, 9 H, Boc), 1.56, 1.77 (2 m, 8 H, Abu, MeAbu, MeLeu, β-CH_2, MeLeu γ-CH), 2.04 (m, 1H, Val β-CH), 2.50, 2.64–3.07 (series of s, 12 H, *N*-CH_3), 4.0–4.77 (m, 7 H, α-CH), 5.11 (m, 3 H, Ph-CH_2 and Boc-NH), 7.34 (s, 5 H, Ar-H), 7.60–7.77 (m, 1 H, NH) 7.93 (d, 1 H, NH), 8.18 (d, 1 H, NH).

Boc-D-Ala-MeLeu-MeLeu-MeVal-MeAbu-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzxl (12a). The heptapeptide **14a** (0.48 g, 0.54 mmol) was stirred in a solution of TFA–CH_2Cl_2 (3:1) at 0 to –5 °C for 3 h and then worked up as described for deprotection of hexapeptide; yield 0.40 g (95%). To a stirred solution of above heptapeptide (0.4 g, 0.5 mmol) and tetrapeptide Boc-D-Ala-MeLeu-MeLeu-MeVal-OH (**13a**) (0.25 g, 0.46 mmol) in CH_2Cl_2 (8 mL) at 0 °C was added a solution of BOP[16] (0.24 g, 0.55 mmol) and *N*-methylmorpholine (0.06 mL, 0.55 mmol) in CH_2Cl_2 (1.5 mL). The reaction mixture was stirred for 3 h at 0 °C and 22 h at room temperature, diluted with $CHCl_3$ (40 mL), and worked up as for **14a**. The crude product was purified on a silica gel column, eluting with 80:20 and 70:30 *n*-hexane–acetone as eluant, to give the undecapeptide **12a**: yield 0.41 g (69%); ^{1}H NMR ($CDCl_3$) showed multiple conformations.

Boc-D-Ala-MeLeu-MeLeu-MeVal-MeAbu-Abu-Sar-MeLeu-Val-MeLeu-Ala-OH (17a). The undecapeptide **12a** (0.13 g, 0.098 mmol) was dissolved in 95% ethanol (20 mL), and 10% Pd–C (30 mg) was added. After the mixture was purged with nitrogen for 5 min, hydrogen gas was bubbled through the reaction mixture for 2.5 h at atmospheric pressure and ambient temperature. The reaction mixture was filtered through Celite and concentrated. The residue was triturated with ether to give 0.11 g (92%) of **17a**, R_f 0.45 (solvent B).

H-D-Ala-MeLeu-MeLeu-MeVal-MeAbu-Abu-Sar-MeLeu-Val-MeLeu-Ala-H (18a). To 0.11 g (0.09 mmol) of **17a** in CH_2Cl_2 (0.5 mL) at –20 °C was added a precooled solution of TFA (1 mL). The reaction mixture was stirred at –20 °C for 3 h, and then solvent was removed at –20 °C. The residue was dissolved in $CHCl_3$ (20 mL), washed with cold saturated $NaHCO_3$ solution (2 × 15 mL) and NaCl solution (2 × 10 mL), dried (Na_2SO_4), filtered, and evaporated to a white foamy solid: yield 0.09 g (90%); R_f 0.65 (solvent C).

***cyclo*-(MeAbu-Abu-Sar-MeLeu-Val-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal) or (MeAbu[1])CSA (5).** The free undecapeptide **18a** (0.1 g, 0.088 mmol) was dissolved in CH_2Cl_2 (350 mL), and (*N,N*-dimethylamino)pyridine (50 mg, 0.44 mmol) followed by BOP (0.15 g, 0.35 mmol) was added. The reaction mixture was stirred at room temperature for 42 h. The solvent was removed and the residue chromatographed on a silica gel column using 99:1 chloroform–methanol as eluant. The fractions containing pure cyclic peptide were pooled and concentrated to a white crystalline solid, yield 0.07 g (71%).

Ethyl *trans*-5-Isopropyl-2-oxazoline-4-carboxylate (8). Following a modification of a literature procedure for the synthesis of a homologue,[13] NaCN (0.42 g, 8.6 mmol, 15 mol %) in anhydrous ethanol (15 mL) maintained under N_2 atmosphere at 0 °C was reacted with a cooled mixture of ethyl isocyanoacetate (5.7 g, 50 mmol) and isobutyraldehyde (5.5 mL, 60 mmol) with stirring at 0 °C for 1 h and then worked up to give, after rapid chromatography on silica gel (60:40 hexane–acetone), 8 as a light oil: 78% yield; IR ($CHCl_3$) 1732, 1685, 1624 cm^{-1}; mass spectrum (M^+) m/e 186; ^{1}H NMR ($CDCl_3$) δ 0.96 (d, 3 H, J = 7 Hz, $CHCH_3CH_3$), 1.00 (d, 3 H, J = 7 Hz, $CHCH_3CH_3$) 1.20 (t, 3 H, $CO_2CH_2CH_3$), 1.87

(m, 1 H, J = 7 Hz, C*H*Me$_2$), 4.25 (q, 2 H, J = 7.2 Hz, CO_2CH_2), 4.35 (dd, 1 H, J = 7.6, 2.0 Hz, H-4), 4.46 (dd, 1 H, J = 7.6, 7.0 Hz, H-5), 6.95 (d, 1 H, J = 2.0 Hz, H-2); ^{13}C NMR ($CDCl_3$) δ 14.1, 17.2, 32.18, 61.6, 70.1, 86.46, 156.67, 171.08.

(±)-*threo*-*N*-Methyl-β-hydroxyleucine (10). To a stirred solution of oxazoline **8** (1.0 g, 4.6 mmol) in dry Et_2O (4 mL) at 0 °C was added methyl triflate (1.06 g, 6.5 mmol) via a syringe. The reaction mixture was stirred at room temperature for 1 h under N_2 atmosphere and then diluted with ether (15 mL), and H_2O (15 mL) was added. The aqueous layer was separated and diluted with concentrated HCl (3 mL). The mixture was refluxed for 15 h and then concentrated. The oily orange hydrochloride salt **10** was purified by ion-exchange chromatograhy over a basic ion-exchange resin (Dowex-1, hydroxide form) column, eluting with H_2O and 0.2 N acetic acid. Fractions containing the product were pooled and lyophilized to give **10** (0.48 g, 64%) as an off-white powder. Recrystallization from aqueous acetone gave white needles: mp 238–240 °C dec; TLC (solvent C) R_f 0.46; IR (KBr), 1616 cm^{-1}; ^{1}H NMR (D_2O) δ 0.96 (d, 3 H, J = 6.5 Hz, $CHCH_3CH_3$), 0.97 (d, 3 H, J = 6.9 Hz, $CHCH_3CH_3$), 1.83 (m, 1 H, C*H*Me$_2$), 2.74 (s, 3 H, N-CH_3), 3.57 (d, 1 H, J = 7.2 Hz, H-2), 3.69 (dd, 1 H, J = 7.2, 5.1 Hz, H-3); ^{13}C NMR (D_2O) δ 18.47, 21.67, 32.83, 35.32, 69.94, 77.63, 174.60. Anal. Calcd for $C_7H_{15}NO_3$: C, 52.15; H, 9.40; N, 8.69. Found: C, 52.0; H, 9.22; N, 8.62.

***N*,*O*-Isopropylidene-*N*-methyl-β-hydroxyleucine (11c).** A suspension of *threo*-*dl*-*N*-methyl-β-hydroxyleucine [(MeLeu(3-OH)] (0.08 g, 0.49 mmol) in dry acetone (50 mL) was refluxed for 24 h (in the presence of N_2 atmosphere) until a clear solution was obtained. The solvent was concentrated to 0.5 mL and was used as such in the next step.

***N*,*O*-Isopropylidene-MeLeu(3-OH)-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (14c).** The above residue of 11c was diluted with dry THF (5 mL), and N-methylmorpholine (0.55 mL, 0.5 mmol) was added. This solution was cooled to 0 °C, and a solution of hexapeptide **16b** (0.28 g, 0.4 mmol) in THF (3 mL) followed by 1-hydroxybenzotriazole (HOBT) (0.15 g, 1 mmol) and DCC (0.13 g, 0.6 mmol) was added. The reaction mixture was stirred at 0 °C for 4 h and 36 h at room temperature. The reaction mixture was cooled, filtered, evaporated to dryness, and worked up as for **16a**. The crude product was purified on a silica gel column using chloroform-methanol (99:1) as eluant, to give pure heptapeptide **14c** as oil: yield 0.28 g (73%); ^{1}H NMR ($CDCl_3$) multiple conformers δ 0.7–1.08 (m, 27 H, CH_3 of Abu, MeLeu, Val, MeLeu (3-OH), 1.2 (s, 6 H, CH_3 isopropylidene), 1.33 (d, 3 H, Ala-CH_3), 1.40–1.50 (m, 10 H, Abu, MeLeu, β-CH_2, MeLeu-γ-CH, Val β-CH), 2.18–3.23 (6 major s inside m, 13 H, N-Me and Sar α-CH), 3.34 (t, 1 H, α-CH), 3.5–5.27 (series of m, 8 H, α-CH and benzyl CH_2), 6.49 (d, 1 H, NH), 6.62 (d, 1 H, NH), 7.35 (s, 5 H, ArH), 7.8–8.1 (m, 1 H, NH).

***N*-MeLeu(3-OH)-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl.** The protected peptide **14c** (0.18 g, 0.2 mmol) was stirred at room temperature in a solution of MeOH–1 N HCl (2:1) for 15 h under a N_2 atmosphere. The reaction mixture was concentrated and water (4 mL) added. The aqueous layer was basified with $NaHCO_3$ solution, and the oily material separated out was extracted with $CHCl_3$ (2 × 10 mL). The organic layer separated and washed with NaCl solution, dried (Na_2SO_4), and concentrated to give N-deprotected heptapeptide in 85% yield. ^{1}H NMR showed the loss of the isopropylidene group.

Biological Methods. Female, 4–5-week-old BALB/c mice were obtained from Harlan Sprague-Dawley, Indianapolis, IN, and were maintained in our laboratories until used. Murine single-cell thymocyte suspensions were prepared as described previously.[22] Briefly, excised thymus tissue was minced, pressed through wire mesh with a syringe barrel, and centrifuged at 200g for 3 min, and the cells were washed 1× with media and distributed into 96 well microtiter plates (10^6 cells/well) in a final volume of 250 μL. Con A was added to the cell suspension at a final concentration of 1 μg/mL. Inhibitors were added in 5 μL of 25% EtOH (controls received 5 μL of 25% EtOH alone). In these experiments, Dulbecco's MEM-Hams F12 (1:1, v/v) media containing 5 ng/L of insulin, 5 ng/L transferrin, and 5 pg/L of selenium was used in place of EHAA media with mouse serum. The level of mitogenic activation was determined at 24 and 48 h by pulsing with [^{3}H]thymidine for 12 h, followed by harvesting and measurement of cellular [^{3}H]-TdR incorporation by scintillation counting as described previously.[22]

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Potent Vasopressin Antagonists Lacking the Proline Residue at Position 7

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As part of a program to design potent antidiuretic vasopressin antagonists and to define the minimum effective pharmacophore requirements for vasopressin (VP) antagonist activity, we studied the importance of the C-terminal tripeptide of a previously reported peptide antagonist of arginine-vasopressin (AVP, 1). The proline residue at position 7 in AVP is proposed to impart a conformational constraint to the peptide backbone that is essential for V_2-receptor agonist activity. Since the structure–activity relationships for VP agonists and antagonists are different, we investigated the effect of proline on antagonist activity, by synthesizing analogue **3** lacking this residue. This analogue was found to retain a high degree of antidiuretic antagonist activity. Since deletion of the Gly residue at position 9 of the antagonist did not adversely affect VP antagonist potency, several vasopressin antagonist analogues (**4–7** and **9**) that lacked both the Pro and Gly residues were also studied. These, too, were found to block vasopressin V_2-receptor activity. Our results indicate that neither the proline nor glycine residues are essential for antagonism of the V_2 receptor.

Some highly potent and selective antagonists of the antidiuretic and vascular effects of the nonapeptide arginine-vasopressin (AVP) have been described.[1-7] These antagonists were derived by modification of amino acid residues at various positions of AVP. In an effort to determine the miniumum structural requirements for anti-

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(1) Sawyer, W. H.; Pang, P. K. T.; Seto, J.; McEnroe, M.; Lammek, B.; Manning, M. *Science (Washington, D.C.)* **1981**, *212*, 49.
(2) Manning, M.; Lammek, B.; Kalodziejczyk, A. M.; Seto, J.; Sawyer, W. H. *J. Med. Chem.* **1981**, *24*, 701.

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