Synthesis, Conformation, and Immunosuppressive Activities of Three Analogues of Cyclosporin A Modified in the 1-Position[1]

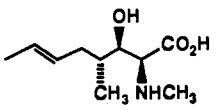
Johannes D. Aebi,† Donald T. Deyo, Chong Qing Sun, Dominique Guillaume,‡ Brian Dunlap, and Daniel H. Rich*

School of Pharmacy, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received August 16, 1989*

The syntheses of three new cyclosporin A (CsA) analogues that contain novel MeBmt derivatives in the 1-position are described. The MeBmt analogue that contains an additional methyl group on C4, (2*S*,3*R*,6*E*)-4,4-dimethyl-3-hydroxy-2-(*N*-methylamino)-6-octenoic acid ($MeBm_2t$), was synthesized in four steps beginning with the reaction of Pmz-Sar-O^{t}Bu with (4*E*)-2,2-dimethyl-4-hexenal. The C4 desmethyl analogue of MeBmt, (2*S*,3*R*,6*E*)-3-hydroxy-2-(*N*-methylamino)-6-octenoic acid (MeBth), was synthesized in nine steps by a route based on the Sharpless chiral epoxidation procedure. The alkynyl derivative of MeBmt, (2*S*,3*R*,4*R*)-4-methyl-3-hydroxy-2-(*N*-methylamino)-6-octynoic acid (MeByt), was synthesized by a modification of the procedure described by Tung et al. for the synthesis of MeBmt. Each MeBmt analogue was protected as the *N*,*O*-acetonide and coupled with the hexapeptide Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl. The resulting heptapeptide was deprotected and coupled with Fmoc-D-Ala-MeLeu-MeLeu-MeVal-OH. The resulting undecapeptides were deprotected and cyclized to give the corresponding CsA analogues. Conformational analysis by 1D and 2D NMR methods was carried out for each analogue in chloroform, and the results are compared with the corresponding solution conformations of CsA and dihydrocyclosporin. The immunosuppressive activities of each analogue, determined in concanavalin A stimulated thymocytes, are lower than obtained for CsA. The results establish the important effect the methyl group and the double bond in MeBmt have on the solution conformation of the 1-position residue in CsA and on immunosuppressive activity.

The selective and potent immunosuppressive activity of cyclosporin A (CsA, **1a**; Figure 1) has led to its becoming the leading therapeutic agent for preventing rejection of transplanted human organs.[2,3] CsA also has potential in the treatment of other diseases,[3] but its hepatic and renal toxicity limits its use in noncritical conditions.[4,5] Thus, there is a clear need for an improved understanding of the structural features of CsA that contribute to both its immunosuppressive and toxic properties.

The structure of CsA (**1a**) was established by chemical degradation of the natural peptide[6] and X-ray crystallography.[7] CsA is a lipophilic, cyclic undecapeptide that is distinguished by the presence of seven N-methylated amino acids, a D-alanine in position 8, and a novel amino acid, (4*R*)-4-[(*E*)-butenyl]-4,*N*-dimethyl-L-threonine (MeBmt, **2**), in position 1. CsA appears to adopt a single conformation (>95%) in hydrophobic solvents,[8a,b] which is somewhat more folded than the X-ray structure and which differs in the orientations of the MeLeu-10 and MeBmt side chains. The major difference concerns the MeBmt side chain, which extends into solvent in the solution conformation but is folded under the peptide ring system in the X-ray structure. Both conformations have a type II′ β-turn for residues 2 → 5 and a cis amide bond between MeLeu-9 and MeLeu-10.



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The elegant total synthesis of CsA reported by Wenger[9a–c] coupled with the biological data for numerous CsA analogues reported by the Sandoz[9d–f] group and others[10,11] has clarified many of the major features of these compounds needed for immunosuppressive activity. Conformationally restricted analogues that stabilize the type II′ β-turn for residues 2 → 5 have been used to probe the importance of this turn to activity,[10] and CsA analogues modified in the 1-position have demonstrated the crucial role MeBmt plays in the biological activity of CsA.[9d–f,11] For example, MeLeu(3-hydroxy)[1]-CsA has 0.1% of the immunosuppressive activity of CsA yet differs from CsA by only a three-carbon shorter side chain in position 1.[11a] Other parts of MeBmt contribute to biological activity since the removal or acetylation of the hydroxyl group in MeBmt produces analogues with very low immunosuppressive activity and reduction of the double bond forms dihydrocyclosporin (DH-CsA, **1b**), which retains about 50% of the activity of CsA.[9f]

† Present address: F. Hoffman-La Roche AG, PF/MD Grenzacherstrasse 124, CH-4002 Basel, Switzerland.

‡ Present address: Faculté des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire, 75006 Paris, France.

(1) IUPAC–IUB Joint Commission on Biochemical Nomenclature. Nomenclature and symbolism for amino acids and peptides, recommendations 1983. *Eur. J. Biochem.* **1984**, *138*, 9–37. Additional abbreviations: DMSO, dimethyl sulfoxide; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; THF, tetrahydrofuran; TsCl, *p*-toluenesulfonyl chloride; TFA, trifluoroacetic acid; DMF, *N*,*N*-dimethylformamide; DEAD, diethyl azodicarboxylate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; NMR, nuclear magnetic resonance; ROE, rotating-frame Overhauser effect; DMAP, 4-(dimethylamino)pyridine; $(PrPO_2)_3$, propylphosphonic anhydride.

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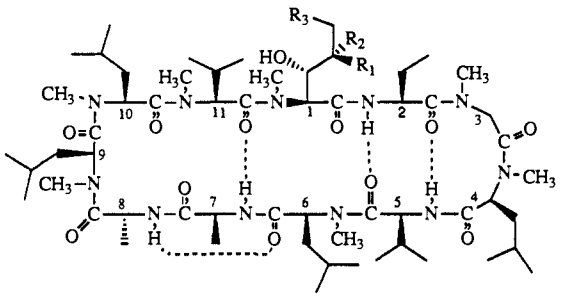


	R_1	R_2	R_3
1a	CH_3	H	$CH{=}CHCH_3$
1b	CH_3	H	$CH_2CH_2CH_3$
3	CH_3	CH_3	$CH{=}CHCH_3$
4	H	H	$CH{=}CHCH_3$
5	CH_3	H	$C{\equiv}CCH_3$
37	H	CH_3	$CH{=}CHCH_3$

Figure 1. Structures of cyclosporin A (CsA, 1a) and analogues modified in the 1-position.

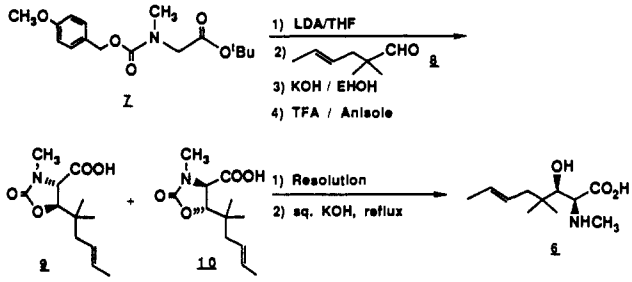
In order to determine more precisely which structural features found in the MeBmt (2) portion of CsA are needed for full immunosuppressive activity, we have synthesized three new analogues of CsA in which the MeBmt side chain has been altered. $MeBm_2t^1$-CsA (3), with (2*S*,3*R*,6*E*)-4,4-dimethyl-3-hydroxy-2-(*N*-methylamino)-6-octenoic acid in the 1-position, corresponds to an analogue that contains an additional methyl group at C4′, $MeBth^1$-CsA (4), with (2*S*,3*R*,6*E*)-3-hydroxy-2-(*N*-methylamino)-6-octenoic acid in the 1-position, corresponds to an analogue in which the methyl group at C4′ of MeBmt has been deleted, and $MeByt^1$-CsA (5), with (2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(*N*-methylamino)-6-octynoic acid in the 1-position, corresponds to an analogue in which a triple bond replaces the double bond of MeBmt. The conformations of all three analogues were analyzed by using 1D and 2D NMR techniques to evaluate the effects these changes produced on the peptide ring system conformation, and the immunosuppressive activity of each analogue was determined.

Synthesis

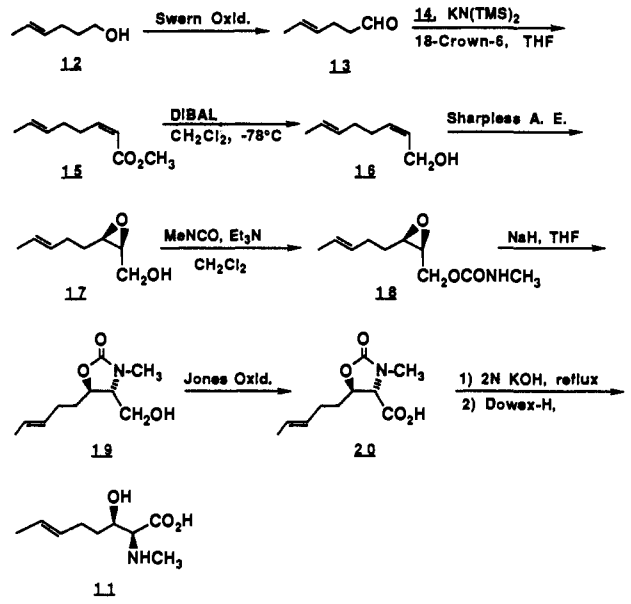
The synthesis of the cyclosporin analogues $MeBm_2t^1$-CsA (3), $MeBth^1$-CsA (4), and $MeByt^1$-CsA (5) utilized the general procedures introduced by Wenger[9a-c] and employed by us in our earlier work.[10,11] We regarded the preparation of analogues 3–5 as a two-step process, the first step being the synthesis of the MeBmt analogues and the second step the incorporation of these novel amino acids into the cyclosporin cyclic array. Several methods for synthesizing MeBmt have been reported,[12] and two of these were modified to prepare the three analogues of MeBmt reported here.

The MeBmt derivative that contains the added methyl group at C4, $MeBm_2t$ (6), was obtained in an efficient manner by utilizing the approach reported by Aebi et al.[12d] (Scheme I). The lithium enolate of *N*-[[(*p*-methoxybenzyl)oxy]carbonyl]sarcosine *tert*-butyl ester (Pmz-Sar-O^{t}Bu, 7) was reacted with (4*E*)-2,2-dimethyl-4-hexenal (8) to give a racemic mixture of the *trans*-oxazolidinonecarboxylates 9 and 10. The desired 9 was separated from the undesired isomer by resolving the mixture with (+)-ephedrine. Removal of the oxazolidinone protecting group by reaction of 9 with refluxing 2 N KOH followed by purification by ion-exchange chromatography on a Dowex H^+ column afforded 6.

Scheme I



Scheme II



The C4 desmethyl analogue of MeBmt, MeBth (11), first reported by Weber and Evans,[12b] was synthesized by the method of Sun and Rich,[13] which is based upon the Sharpless asymmetric epoxidation procedure[14a] (Scheme II). Oxidation of alcohol 12 gave the aldehyde 13 which was reacted with trimethyl phosphonoacetate (14) in the presence of potassium bis(trimethylsilyl) amide [KN(TMS)$_2$] and 18-crown-6 to give the cis ester 15 as the major product. Reduction of 15 followed by asymmetric epoxidation[14a] of the resulting alcohol 16 smoothly afforded epoxide 17. Reaction with methyl isocyanate gave the carbamate 18, which was subjected to sodium hydride catalyzed rearrangement to cleanly give the oxazolidinone 19. Oxidation to the acid 20 followed by hydrolysis gave MeBth (11) in 28% overall yield.

The alkynyl derivative of MeBmt, MeByt (21), was synthesized by using a modification of the procedure described by Tung et al.[12c] for the synthesis of MeBmt (Scheme III). The known hepta-2,5-diyn-1-ol (22) was reduced with lithium aluminum hydride to afford allylic alcohol 23, which was subjected to Sharpless' catalytic asymmetric epoxidation[14b] to afford the chiral epoxide 24a. The primary hydroxyl group was protected as the trityl

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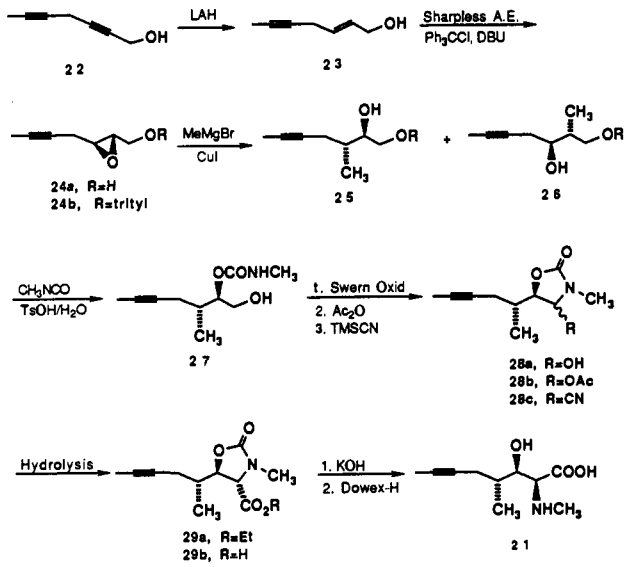
Scheme III



ether **24b**, and the epoxide then was reacted with excess MeMgBr/CuI to give **25** and **26** in a 1:2 ratio. Numerous attempts to improve the regioselectivity of this reaction to the level attained for MeBmt[12c] were unfruitful. Compound **25** was isolated by flash-column chromatography and treated with excess methyl isocyanate in a sealed-tube reaction. The trityl group was removed by reaction with $TsOH{\cdot}H_2O$ in methanolic ether to yield the methyl carbamate **27**. Swern oxidation of **27** gave the carbaminol **28a**, which results from the intramolecular attack by the nitrogen on the intermediate aldehyde. Acetylation of the hydroxyl group gave **28b**, which was reacted with trimethylsilyl cyanide in the presence of catalytic $BF_3{\cdot}Et_2O$ to afford nitrile **28c** as a mixture of epimers. Hydrolysis of nitrile **28c** with potassium carbonate in 95% ethanol and subsequent treatment with 2 N HCl/95% ethanol yielded the carboethoxyoxazolidinone **29a**. Hydrolysis of the ester by the reported procedures[12a] gave the acid **29b**, which was saponified to give amino acid **21**.

The synthesis of the corresponding CsA analogues **3–5** was carried out by using the strategy devised by Wenger[9a-c] as modified in our previous work (Scheme IV).[10,11] The appropriate MeBmt derivative was protected as the *N,O*-acetonide (refluxing acetone, 24 h to give **30a,b** and **31**) and then coupled with hexapeptide **32** by using DCC/HOBt to afford the heptapeptide **33a–c**. The acetonide protecting group of **33a–c** was removed (HCl/methanol) to afford **34a–c**, which was subsequently coupled with the tetrapeptide **35**.[20] Double deprotection of **36a–c** (dilute NaOH in ethanol)[15] followed by cyclization with propylphosphonic anhydride[16] afforded the cyclosporin analogues **3–5**.

NMR Results. The three analogues **3–5** are present in only one conformation (>95%) in chloroform. The carbon and proton NMR spectra for each analogue were obtained at 500 MHz (^1H frequency) in chloroform. Each spectrum was completely assigned by a combination of 1D and 2D homo- and heteronuclear techniques developed by Kessler et al.[8] as described previously.[10] The chemical shift data for each of the amide protons, α-protons, and NCH_3 protons are reported in Table I. Table II lists the assignment of the carbonyl carbons, α-carbons, and NCH_3 carbon resonances. We also report the chemical shifts of di-

Table I. Chemical Shifts of Cyclosporin and Cyclosporin Analogues

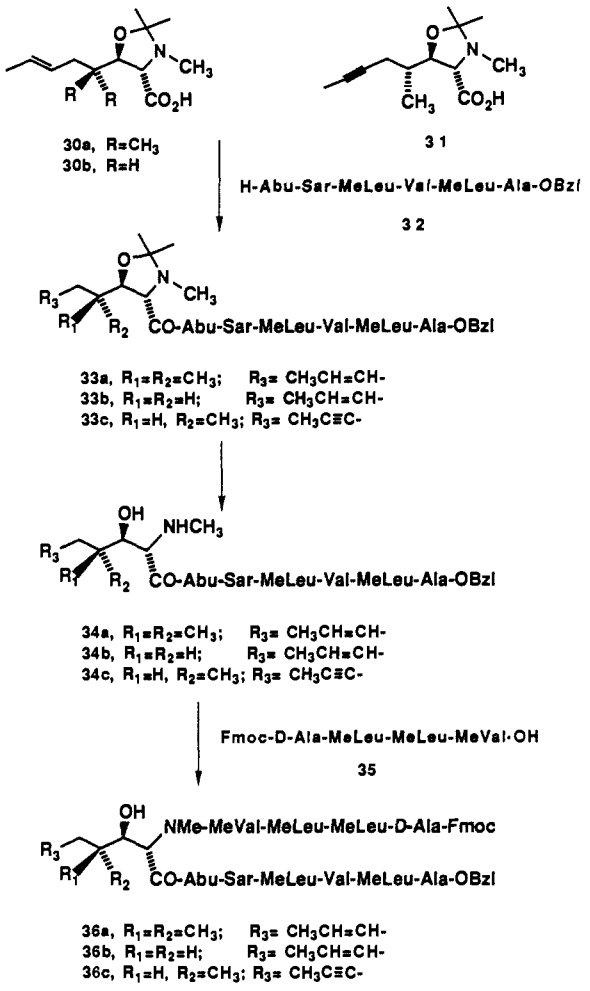
	NH			α-CH									NCH3						
	2	7	5	8	1	2	3_{re}	3_{si}	4	5	6	7	8	9	10	11	1	3	4	6	9	10	11
CsA (1a)	7.96	7.68	7.48	7.17	5.47	5.03	3.23	4.76	5.34	4.66	5.02	4.52	4.83	5.70	5.10	5.14	3.51	3.40	3.11	3.25	3.12	2.70	2.71
$MeBm_2t^1$-CsA (3)	8.20	7.90	7.65	7.25	5.38	4.98	3.15	4.67	5.30	4.72	5.19	4.47	4.75	5.66	5.06	4.98	3.50	3.40	3.05	3.21	3.16	2.65	2.68
$MeBth^1$-CsA (4)	8.21	7.80	7.50	7.10	5.62	5.00	3.20	4.72	5.28	4.64	4.95	4.58	4.87	5.67	5.10	5.10	3.41	3.38	3.10	3.25	3.13	2.67	2.63
$MeByt^1$-CsA (5)	8.17	7.7	7.45	7.25	5.33	4.98	3.16	4.70	5.29	4.70	5.11	4.48	4.83	5.69	5.11	5.18	3.45	3.37	3.18	3.27	3.16	2.69	2.67
DH-CsA (1b)	7.90	7.62	7.46	7.16	5.47	5.04	3.18	4.72	5.30	4.62	4.96	4.52	4.82	5.70	5.04	5.15	3.51	3.40	3.10	3.25	3.10	2.70	2.71

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

	carbonyls					Cα					N-CH3			
	DH-CsA (1b)	$MeBth^1$-CsA (4)	$MeByt^1$-CsA (5)	$MeBm_2t^1$-CsA (3)	CsA (1a)	DH-CsA (1b)	$MeBth^1$-CsA (4)	$MeByt^1$-CsA (5)	$MeBm_2t^1$-CsA (3)	CsA (1a)	DH-CsA (1b)	$MeBth^1$-CsA (4)	$MeByt^1$-CsA (5)	$MeBm_2t^1$-CsA (3)	CsA (1a)
1		169.9	170.1	70.6	169.65	59.0	60.95	59.6	56.3	58.75	34.1	33.6	33.5	33.3	34
2	173.9	173.8	173.5	173.2	173.0	48.7	48.7	48.65	48.4	48.8					
3	171.0	171.15	170.45	170.7	170.5	50.3	50.3	50.1	50.1	50.4		39.5	39.4	39.3	39.4
4		169.9	169.8	170.1	169.35	55.4	55.4	55.3	55.2	55.5	31.3	31.3	31.3	31.2	3.1.3
5	173.5	173.5	173.5	173.2	173.1	55.4	55.3	55.2	54.9	55.4					
6	171.5	171.6	171.5	171.5	170.9	55.3	54.9	55.0	54.5	55.3	31.5	31.35	31.5	31.2	31.5
7	171.2	171.2	171.0	171.2	170.4	48.6	48.4	48.5	48.3	48.7					
8	173.8	173.6	173.4	173.5	172.9	45.1	45.0	44.9	44.7	45.2					
9		170.2	170.0	170.4	169.75	48.2	48.3	48.0	48.0	48.3	29.5	29.7	29.6	29.6	29.65
10		169.7	170.45	170.3	169.45	57.6	57.7	57.3	57.3	57.5	29.8	29.65	29.8	29.8	29.8
11	173.4	173.1	173.4	173.5	172.85	57.8	58.15	57.7	58.3	57.9	29.8	29.8	29.8	30.0	29.8

(15) Shute, R. E.; Rich, D. H. *Tetrahedron Lett.* **1987**, *28*, 3419.
(16) Wissmann, H.; Kleiner, H. J. *Angew. Chem.* **1980**, *92*, 129.

Scheme IV



hydro-CsA (**1b**, Figure 1), in which the double bond of the MeBmt side chain has been reduced by catalytic hydrogenation.

Cyclic Peptide System. Even though the synthetic variations in the structure of the MeBmt side chains described here were not expected to modify the overall conformation of the peptide ring system, we carried out a detailed conformational analysis of the cyclic peptide ring system for each analogue. The amide proton temperature coefficients we observed and those reported previously for CsA and some analogues at low or high temperature[10] showed similar behavior except for NH-7 of MeBth^1-CsA (**4**). This indicates that at room temperature the amide proton hydrogen-bonding pattern of the CsA analogues is essentially the same as in CsA. In a similar fashion, the ROESY[17] experiment carried out on each analogue produced the same ROE pattern obtained for CsA or for CsA analogues having a similar cyclic peptide conformation.[10] Specifically, for each analogue we observed an ROE between the *si* proton of the Sar-3 residue and the NCH_3 protons of MeLeu-4, which indicates that the type II' β-turn is present in each analogue. The cis geometry of the amide bond between residues 9 (MeLeu) and 10 (MeLeu) found in CsA is also present in each of the three analogues, which was established by the ROE between the α-protons of these two residues. Finally, all three analogues and CsA give ROEs between the NCH_3 of MeVal-11 and the NH of Ala-7 and between the α-proton of residues 6 and 1. Taken together, these data indicate that the overall conformation of each analogue is very similar to the conformation of CsA with respect to the cyclic peptide system.

(17) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207.

Table III. MeBmt or MeBmt Analogue α-CH, β-CH Coupling Constant for Cyclosporin Derivatives in Chloroform

	compound				
	CsA (1a)	DH-CsA (1b)	MeBth^1-CsA (4)	MeByt^1-CsA (5)	MeBm$_2$t^1-CsA (3)
J, Hz	5.7	5.7	5.5	7.8	7.0

The second point of interest was to determine if the modifications of the MeBmt side chains that had been introduced in analogues **3–5** had changed the orientation of the 1-position side chain. It has been shown that the MeBmt side chain, in hydrophobic solvents, extends into the solvent (χ_1 = −45° to −60°) but is folded under the peptide ring system in the X-ray structure ($\chi_1 \simeq 191°$).[7,8] The chloroform conformation appears to be stabilized by a hydrogen bond between the hydroxyl group of MeBmt and the MeBmt carbonyl.[8]

At this point in our study of the conformation of analogues **3–5**, it was unrealistic to try to determine the exact position of the MeBmt side chain in chloroform from the NMR data because of the expected high mobility[8a] of this side chain. Therefore, we chose to compare the χ_1 and χ_2 angles for the 1-position for each of the three analogues with the corresponding torsion angles for CsA and DH-CsA to get a general idea of the position of the modified MeBmt side chain with respect to the cyclic peptide system. To do this, we examined closely the coupling constant data and ROE data for each 1-position analogue. The variation in the α-C*H*,β-C*H* coupling constant should give, by the use of a Karplus type curve, the corresponding value of χ_1 for the 1-position. A second parameter that we used is the effect of the 1-position structure on an ROE involving the α-CH or the β-CH of residue 1. For example, β-C*H* of the MeBmt side chain in CsA gives an ROE with other protons on the cyclic backbone. These ROEs are easy to observe since the β-C*H* resonance is distinct (no overlap with other shifts) and can be used to help determine the position of the MeBmt side chain. We have applied these two techniques to try to determine the orientation of the MeBmt side chain in CsA analogues **3–5** in chloroform. A third potentially useful parameter for orienting the 1-position side chain to the peptide ring system is the chemical shift of CH_3-4' of the MeBmt side chain, which resonates at 0.72 ppm in the case of CsA,[8c] and its proximity to the α-CH of MeLeu-6, which is detected by an ROE.[8a] A similar chemical shift for the corresponding methyl is present in the case of DH-CsA (**1b**), so that the upfield chemical shift of the C4' methyl group is not the result of an anisotropic effect of the double bond in MeBmt.

When these methods were applied to MeByt^1-CsA (**5**), we were surprised that no methyl resonated near 0.70 ppm, which led us to suspect that the side chain of MeByt in **5** is oriented differently than MeBmt in CsA (**1a**) with respect to the peptide ring system. This was supported by the larger coupling constant between α-C*H* and β-C*H* (7.8 Hz for **5** vs 5.7 Hz for CsA or DH-CsA) (Table III), which suggested χ_1 is increased. We tried to derive more information from the ROE spectrum, but no ROEs were detected between the β-CH and a proton on the cyclic peptide backbone in **5**. This is different from CsA where an ROE can be observed between the β-CH(1) and NH-Abu and the α-CH of MeBmt.[8a] Although it can be dangerous to overinterpret a missing ROE, the absence of this ROE in this case is consistent with the altered α-CH,β-CH

coupling constant and suggests that the orientation of the 1-position side chain in MeByt-CsA (5) is different from that in CsA and DH-CsA.

The removal of CH_3-4′ in the case of MeBth^1-CsA (4), which eliminated the possibility for ROEs to this group, suppressed one method for establishing the 1-position side-chain orientation. For this analogue, we tried to establish only χ_1 for the 1-position. The very close agreement in the chemical shift data for all the α-protons and NCH_3 groups indicated that the overall shape of the two molecules must be very similar. The major difference between 4 and CsA lies in the chemical shift of α-C*H* of MeBmt, and this effect can be explained by the removal of the steric constraint imposed by the 4′-CH_3 group on χ_1 in MeBmt. Furthermore, the α-C*H*,β-C*H* coupling constant for 4 is very similar to that found for CsA and DH-CsA. The two ROEs involving the β-C*H*(1) observed for CsA are also present in the ROE spectrum for MeBth^1-CsA. Taken together, these data indicate that χ_1 for the 1-position of MeBth^1-CsA is very similar to that found for this torsion angle in CsA.

In the case of MeBm$_2$t^1-CsA (3), no resonance for a methyl group corresponding to either of the methyl groups on C4′ was found near 0.72 ppm, which suggests that the magnetic environments of the C4′ methyl groups in 3 differ from that in CsA. Furthermore, the coupling constant between the 1-position α,β-protons in 3 is now 7 Hz, which indicates a modification of the χ_1 angle. Finally, different ROEs are observed for the MeBm$_2$t portion of 3 relative to the MeBmt portion of CsA. For example, although the α-CH–β-CH ROE is still present in 3, no ROE is observed between β-CH(1) and NH-Abu. On the other hand, an ROE between β-CH(1) and NCH_3-1 of the MeBm$_2$t portion of 3 is present. Together, these data indicate that χ_1 for the MeBm$_2$t side chain in 3 is now different from χ_1 calculated for MeBmt in the solution conformation of CsA.

Biological Results. The biological activities of CsA analogues 3–5 vs CsA (1a) were determined by using the inhibition of concanavalin A stimulated thymocytes as previously described.[10,11] The relative immunosuppressive activities are as follows: CsA, 100%; MeBth^1-CsA, 10–13%; MeByt^1-CsA, 10%; and MeBm$_2$t^1-CsA, 20–30%.

Discussion

The three cyclosporin analogues reported here all contain subtle modifications in the MeBmt portion of the peptide structure that significantly reduce immunosuppressive activity. Replacement of the double bond by a triple bond or removal of the methyl group at C4′ lowers immunosuppressive activity by about 90%, whereas addition of an extra methyl group at C4′ lowers immunosuppressive activity by 70–80%. These results further illustrate the remarkably stringent requirements for immunosuppressive activity exhibited by the cyclosporin system. To date, no analogues more potent than CsA have been reported.[9-11]

Our preliminary data indicated that the NMR spectra of the CsA analogues described here differed substantially from NMR data reported for CsA in spite of what appeared to be relatively minor structural changes in the molecule. In order to explore to what extent the overall conformation of the 33-membered ring system or the orientation of the side chain in solution had been altered by the synthetic modifications of the MeBmt residue, and thereby contributed to the NMR changes noted, we carried out a more detailed analysis of the conformation of each analogue by COSY and ROESY methods.[17]

Our results indicate that the conformation of the 33-membered ring system in each analogue is very similar to that of CsA with respect to the major structural features that distinguish the peptide ring system conformation of CsA. Thus, the amide bond hydrogen-bonding patterns, the type II′ β-turn, the cis amide bond between MeLeu-9 and MeLeu-10, and the various transannular interactions are all retained in each analogue.

In contrast, the orientations of two of the 1-position side chains (MeByt, MeBm$_2$t) differed significantly in chloroform from that of MeBmt in CsA in this solvent. The NMR data we have obtained do not permit a precise determination of these side-chain conformations, but they do show when χ_1 is altered in chloroform as a function of the synthetic modifications at position 1. The acetylenic analogue 5, which we anticipated might retain the CsA side-chain conformation, in fact was shown to adopt an altered conformation. The characteristic upfield shift for the C4′ methyl group in CsA and its proximity to the α-proton of MeLeu^6 were not observed. Furthermore, the coupling constant between the α- and β-protons of MeByt is larger (J = 7.8 Hz) than that for CsA (5.7 Hz), indicating that χ_1 for the 1-position residue has been altered. Due to the lack of distinguishing ROE signals for the MeByt residue, we were unable to define the side-chain orientation more precisely.

The removal or addition of methyl groups at C4′ was expected to alter the side-chain conformational distribution significantly, since the cyclosporin analogue obtained by altering the configuration at C4′ [(4*S*)-MeBmt^1-CsA (37)] retains the cyclic peptide ring system conformation in chloroform but adopts a new χ_1 for the 1-position side chain.[11b] Our solution NMR results show that χ_1 is altered significantly in one of the new C4′ analogues reported here. Whereas MeBth^1-CsA (4), which corresponds to the analogue that is formed by deleting the methyl at C4′, gave coupling constant data and ROE data for this residue that indicated that χ_1 for the 1-position in 1a and 4 are very similar, the corresponding NMR data for the MeBm$_2$t analogue 3 indicated that χ_1 has been altered by the incorporation of the addition of a methyl group at C4′. The coupling constant between the α- and β-protons on MeBm$_2$t is larger (7 Hz) than that for MeBmt in CsA, a new ROE between the β-proton and *N*-methyl group of MeBm$_2$t is apparent, and no ROE is detected between the MeBm$_2$t β-proton and the NH of Abu in the 2-position. Together, these data indicate that the side chain is oriented differently in 3 than in the parent 1a.

Our NMR data are not sufficiently complete to more precisely determine these conformations. In an attempt to clarify the conformational preferences of the 1-position residue as a function of the C4′ substitution, we have carried out force field calculations of the CsA system in which the 1-position residue has been replaced with MeBth, MeBm$_2$t, and (4*S*)-MeBmt.[18] These in vacuo calculations suggest the side chains of MeBth^1-CsA (4) and MeBm$_2$t^1-CsA (3) will be oriented differently than MeBmt in CsA in chloroform. A similar conclusion has been obtained for (4*S*)-MeBmt^1-CsA.[11b] The calculations predict that, in vacuo, in the lowest energy conformations, χ_1 for MeBm$_2$t will be about +190°, which is consistent with the observed coupling constant for this residue (Table III). In

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this case, the NMR data agree with the results of the in vacuo molecular mechanics calculations.

However, the NMR data for MeBth[1]-CsA (4) in chloroform are consistent with the 1-position side chain retaining the MeBmt conformation (χ_1) found in CsA. Specifically, the α,β-proton coupling constant (5 Hz) for analogue 4 restricts χ_1 to near −45°. The ROE data, which are essentially identical with those obtained for CsA, are consistent with the MeBmt and MeBth side chains adopting equivalent conformations in their respective analogues. This contrasts with the results of the energy calculations[18] which predict folded conformations for the 1-position side chain with χ_1 somewhere between 180° and 210° rather than the observed $\chi_1 = -45°$. Thus, the energy calculations do not correctly predict the chloroform solution conformation with respect to χ_1 for this analogue. These results suggest that it may be necessary to include solvent when calculating the relative stabilities of individual conformations of MeBth[1]-CsA (4).

In addition to establishing the importance of the 4′-methyl group and the trans olefin group in CsA for immunosuppressive activity, our data also indicate that these structural features are important contributors to the chloroform solution conformation. Placing an (*S*)-methyl group on C4′, as in both (4*S*)-MeBmt[1]-CsA and $MeBm_2t^1$-CsA, alters the conformation of the 1-position side chain with respect to the peptide ring system. For the (4*S*)-MeBmt[1]-CsA (37) and $MeBm_2t^1$-CsA (3) analogues, the NMR data restrict χ_1 to about 190°, rather than the −45° for this side chain in CsA. The chloroform solution conformations for analogues 3 and 37 are consistent with low-energy conformers identified by systematic search and in vacuo force field calculations, which predict a series of stable conformations in which χ_1 is near 190° ± 10°.

However, the NMR data indicate that the chloroform solution conformation of the MeBth[1]-CsA analogue 4 is essentially the same as that of CsA, with respect to both the overall conformation of the 33-membered ring system and the orientation of the 1-position side chain. This result differs from the predicted low-energy conformations for MeBth[1]-CsA (4), suggesting that the solvent plays a more important role in determining the solution conformation of this CsA analogue.

Although the NMR data provide interesting insight into the effects that small changes in the MeBmt residue have upon the solution conformations of this CsA side chain, these conformations are probably not related to the conformations of these analogues at the CsA receptor. Systematic search and subsequent energy minimizations of the 1-position conformers that are accessible from the solution and crystal conformations of CsA have suggested that the bioactive conformations of CsA and analogues 3, 4, and 37 are closely related to the X-ray crystal structure of CsA.[18] The average side-chain orientation of the 1-position residue of four CsA derivatives is $\chi_1 = 191°$, $\chi_2 = 64°$, $\chi_3 = 177°$, and $\chi_4 = 225°$. This bioactive conformation is compatible with the results of Quesniaux et al., who concluded on the basis of antibody binding studies that CsA adopts a conformation in aqueous media in which the MeBmt side chain is folded across the cyclic undecapeptide ring system.[23]

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter (1.000-dm cell) at room temperature. Infrared (IR) spectra were recorded on a Perkin-Elmer 599B spectrometer (data in cm^{-1}). ^1H NMR spectra were recorded on a Bruker WP-200, WP-270, or AM-500 instrument. Coupling constants are reported in hertz (Hz); chemical shifts, in ppm (δ units) downfield from tetramethylsilane. Silica gel chromatography was carried out under low pressure (−15 psi) using Merck grade 60 silica, 230–400 mesh. TLCs were run on Merck Kieselgel 60-F_{254} with fluorescent indicator visualized by ultraviolet (UV) or 7% phosphomolybdic acid (PMA) in ethanol. Elemental analyses were determined by Galbraith Laboratories, Inc., Knoxville, TN.

Tetrahydrofuran (THF) was distilled from sodium metal/benzophenone ketyl. All nonaqueous reactions were carried out under a dry nitrogen atmosphere in oven-dried (140°, 12 h) glassware unless otherwise indicated (argon). Ethereal solutions of methyllithium and methylmagnesium bromide were obtained from Aldrich, as were all other reagents, and were used as received.

(4*E*)-2,2-Dimethyl-4-hexenal (8). A solution of (±)-3-buten-2-ol (7.79 g, 108.1 mmol), isobutyraldehyde (7.48 g, 103.7 mmol), benzene (3 mL), and *p*-toluenesulfonic acid monohydrate (0.08 g, dehydrated with 2 × 20 mL of benzene) was heated (bath temperature 155 °C) with a Vigreux column (6 cm) and a Dean–Stark trap for 24 h. Distillation with a Vigreux column (6 cm) gave, after a small forerun, 5.1 g (39%, ca. 8% of the cis isomer) of 8 as a colorless oil: bp 145–148 °C (760 Torr); ^1H NMR ($CDCl_3$, 200 MHz) δ 1.04 (s, 6 H, 2 CH_3-C_2), 1.65 (dd, J = 0.5, 7.0 Hz, CH_3-C_5), 2.14 (dd, J = 0.5, 7.0 Hz, 2 H-C_3), 5.20–5.58 (m, 2 H, H-C_4, H-C_5), 9.46 (s, 1 H, –CHO). Anal. ($C_8H_{14}O$) C, H.

(4*S*,5*R*)-3-Methyl-5-[(3′*E*)-1′,1′-dimethyl-3′-pentenyl]-2-oxazolidinone-4-carboxylic Acid (9). A cooled solution (−78 °C) of LDA (13.90 mmol, 1.1 equiv) in THF/hexane (90 mL, 8:1) was treated dropwise with Pmz-Sar-O^{t}Bu (7) (3.91 g, 12.64 mmol, 1.1 equiv) in 10 mL of THF.[12d] After 40 min at −78 °C, (4*E*)-2,2-dimethyl-4-hexenal (8) (1.80 g, 14.54 mmol, 1.15 equiv, 92% trans isomer) was added to the colorless enolate solution. Stirring was continued for 30 min at −78 °C and 30 min at 0 °C, and then 1 N KOH in anhydrous ethanol (12 mL) was added. The solution was then heated at reflux (bath temperature 120 °C) for 25 min, cooled, concentrated in vacuo (40 °C/16 Torr), and treated with ether (40 mL)/water (40 mL). The aqueous phase was extracted a second time with ether (40 mL) and then cooled (0 °C) and acidified (6 N HCl, pH <2). After extraction of the aqueous phase with ether (3 × 50 mL), the organic phase was dried ($MgSO_4$) and concentrated in vacuo to give 3.36 g of crude acids. The oil was treated with anisole (3 mL) and at 0 °C with TFA (12 mL). After 1 h at 0 °C, the solvents were evaporated in vacuo (35 °C/16 Torr), and the residue was dissolved in saturated $NaHCO_3$/ether (40 mL/30 mL). The aqueous phase was washed a second time with ether (30 mL) and cooled (0 °C) and acidified (6 N HCl, pH <2). After extraction of the water phase with ether (3 × 40 mL), the organic phase was dried ($MgSO_4$) and evaporated in vacuo to give 2.88 g (94%, 94% ds, according to the ^1H NMR probably as trans isomer) of oxazolidinones 9 and 10 as a yellow oil. The racemic mixture of 9 and 10 (2.88 g, 11.94 mmol) was treated with (1*S*,2*R*)-(+)-ephedrine (2.72 g, 16.43 mmol) and crystallized from ether/EtOH (at room temperature, then 0 °C). The crystals were filtered and recrystallized twice from methylene chloride/ether at room temperature to give 1.08 g (21%, >95% ds, based on 200 MHz ^1H NMR) of diastereomerically pure oxazolidinone ephedrine salt. This material was treated in 60 mL of ether with 1 N HCl (40 mL) followed by two extractions with ether (40 mL). The organics were dried ($MgSO_4$) and concentrated to yield crystalline 2-oxazolidinone 9 (0.64 g, 21%, containing ca. 5% of the cis double bond isomer, according to 200 MHz ^1H NMR). An analytical sample of 9 was crystallized from ether/pentane: mp 101.1–102.0 °C; $[\alpha]^{rt}_D$ +58.7° (*c* 1.08, $CHCl_3$); IR ($CHCl_3$) 3500–2400, 1750, 1468, 1435, 1400, 1135, 1040, 970 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 0.92 [s, 6 H, $C(CH_3)_2$], 1.68 (d, J = 5.0 Hz, 3 H, 3 H-$C_{5'}$), 1.88–2.17 (m, 2 H, 2 H-$C_{2'}$), 2.95 (s, 3 H, CH_3-N), 4.06 (d, J = 4.5 Hz, 1 H; H-C_4), 4.12 (1 H, COOH), 4.25 (d, J = 4.5 Hz, 1 H, H-C_5), 5.29–5.60 (m, 2 H, olefin H); MS *m/e* 242 (M^+ + 1, 4), 241 (M^+, 11), 196 (4), 142 (19), 100 (18), 55 (51), 42 (51), 40 (100). Anal. ($C_{12}H_{19}NO_4$) C, H, N.

(2*S*,3*R*,6*E*)-4,4-Dimethyl-3-hydroxy-2-(methylamino)-6-octenoic Acid (6). The solution of 2-oxazolidinone 9 (0.159 g,

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0.657 mmol) in 3 mL of 2 N KOH was heated at reflux for 5 h, then cooled to room temperature, acidified with Dowex H^+ (50× 8–100) (pH <4), and heated for 5 min at 80 °C. The mixture of Dowex H^+/water was filtered through 10 mL of Dowex H^+ (1.7 × 5 cm column) and eluted with 1.5 M NH_4OH. Evaporation and freeze-drying of the PMA-positive fractions gave 0.11 g (78%) of the white crystalline acid **6**. The first fraction (25 mL) and 130 mL of the fractions after the PMA-positive fractions were evaporated (according to the ^1H NMR a mixture of the acids **9** and **6**), dissolved in 3 mL of 2 N KOH, and heated for 15 h at reflux. Dowex H^+ treatment gave 0.025 g of further pure acid. The final yield was 0.135 g (95%): mp 190–195 °C (after freeze-drying); $[\alpha]^{rt}_D$ −13.2° [c 0.49, H_2O at pH 7 (phosphate buffer tritrisol, pH 7.00, from Merck)]; ^1H NMR (D_2O, HDO at 4.63 ppm) δ 0.71 (s, 6 H, 2 CH_3-C_4), 1.45 (d, J = 4.0 Hz, 3 H, 3 H-C_8), 1.69–1.97 (m, 2 H, 2H-C_5), 2.49 (s, 3 H, N-CH_3), 3.31 (d, J = 6.0 Hz, 1 H, H-C_2), 3.49 (d, J = 6.0 Hz, 1 H, H-C_3), 5.28–5.40 (m, 2 H, olefin H). Anal. ($C_{11}H_{21}NO_3$) C, H, N.

4(*E*)-Hexenal (13). A solution of oxalyl chloride (7.10 g, 56 mmol, 1.4 equiv) in methylene chloride (150 mL) was cooled at −78 °C, and anhydrous DMSO (8.74 g, 112 mmol, 2.8 equiv) was added dropwise. After 5 min, a solution of 4(*E*)-hexen-1-ol (**12**) (used as received from Aldrich) in methylene chloride (15 mL) was transferred via a cannula into the reaction mixture. The resultant white suspension was stirred at −78 °C for 30 min, and then neat Et_3N (17.8 g, 176 mmol, 4.4 equiv) was added. The mixture was stirred at −30 °C for 1 h and diluted with pentane (500 mL), washed with 1 M aqueous $KHSO_3$ solution (2 × 400 mL) and water (2 × 400 mL), and dried (Na_2SO_4). The pentane and methylene chloride were removed by distillation at atmosphere pressure, and the residue was purified by microdistillation to afford 3.65 g (93%) of **13** as a colorless liquid (80 °C/100 mmHg), which was used immediately in the next reaction: R_f 0.35 (10% ethyl acetate/hexane); ^1H NMR (270 MHz, $CDCl_3$) δ 1.60 (dd, J = 0.9, 6.0 Hz, 3 H, 3 H-C_6), 2.30 (m, 2 H, 2 H-C_3), 2.45 (m, 2 H, 2 H-C_2), 5.30–5.55 (m, 2 H, olefin H), 9.73 (d, J = 1.5 Hz, 1 H, –CHO).

(2*Z*,6*E*)-Methyl Octadienoate (15). A solution of 18-crown-6 (4.10 g, 155 mmol, 4.2 equiv) in anhydrous THF (600 mL) was cooled to −78 °C, and trimethyl phosphonoacetate (**14**) (6.7 g, 36.7 mmol, 1.0 equiv) was added. To the resultant white suspension was added a 0.5 M solution of potassium bis(trimethylsilyl) amide in toluene (73.4 mL, 36.7 mmol, 1.0 equiv). The mixture was stirred at −78 °C for 30 min, and then the aldehyde **13** (3.6 g, 36.7 mmol, 1.0 equiv) in THF (10 mL) was added dropwise. The mixture was stirred at −78 °C for another 1 h and quenched with saturated NH_4Cl solution. Most of the THF was removed by rotary evaporation, and the residue was extracted with methylene chloride (3 × 50 mL). The combined organic solution was washed with saturated $NaHCO_3$ (80 mL) and brine (2 × 50 mL) and then dried (Na_2SO_4) and concentrated to give an oily residue. The crude product contained a 7:1 ratio of 2*Z*:2*E* isomers (71%), and the 2*Z* isomer was separated by careful chromatography with 2% ethyl acetate/hexane.

2*Z* isomer of 15: R_f 0.48 (10% ethyl acetate/hexane); IR ($CHCl_3$) 3050, 2940, 2910, 1715, 1640, 1440, 1410, 1190, 1170, 965 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.63 (dd, J = 0.9, 6.0 Hz, 3 H, 3 H-C_8), 2.09 (m, 2 H, 2H-C_5), 2.67 (m, 2 H, 2 H-C_4), 3.68 (s, 3 H, –$COOCH_3$), 5.42 (m, 2 H, H-C_6, H-C_7), 5.75 (dt, J = 1.7, 11.5 Hz, 1 H, H-C_2), 6.20 (dt, J = 7.3, 11.5 Hz, 1 H, H-C_3). MS: Exact mass calcd for $C_9H_{14}O_2$, 154.0994; found (EI), 154.0992 (M^+).

2*E* isomer of 15: R_f 0.39 (10% ethyl acetate/hexane); ^1H NMR (200 MHz, $CDCl_3$) δ 1.66 (dd, J = 0.9, 6.0 Hz, 3 H, 3 H-C_8), 2.15 (m, 2 H, 2 H-C_5), 2.25 (m, 2 H, 2 H-C_4), 3.73 (s, 3 H, –$COOCH_3$), 5.43 (m, 2 H, H-C_6, H-C_7), 5.83 (dt, J = 1.5, 15.6 Hz, 1 H, H-C_2), 6.96 (dt, J = 6.6, 15.6 Hz, 1 H, H-C_3).

(2*Z*,6*E*)-Octadien-1-ol (16). To a solution of **15** (0.87 g, 5.65 mmol, 1.0 equiv) in methylene chloride (5 mL) cooled at −78 °C was added dropwise 1 M of DIBAL in methylene chloride (17 mL, 17 mmol, 3.0 equiv). The mixture was stirred at −78 °C for 2 h and then quenched carefully with CH_3OH (2 mL). The resultant mixture was diluted with methylene chloride (150 mL) and shaken with 50% saturated Rochelle salt solution (150 mL). The layers were separated, and the aqueous phase was extracted with methylene chloride (3 × 30 mL). The combined organic solution was washed with 50% saturated Rochelle salt solution (100 mL) and brine (100 mL) and then dried (Na_2SO_4) and evaporated in vacuo. The residue was purified by flash chromatography (20% ethyl acetate/hexane) to give 0.71 g (100%) of the title compound **16** as a colorless oil: R_f 0.36 (33% ethyl acetate/hexane); IR ($CHCl_3$) 3700–3300 (br), 3600, 3000, 2940, 2850, 1650, 1450, 1430, 1375, 1230, 1010, 970 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.61 (dd, J = 1.0, 4.6 Hz, 3 H, 3 H-C_8), 1.90–2.20 (m, 4 H, 2 H-C_4, 2 H-C_5), 4.16 (d, J = 5.9 Hz, 2 H, 2 H-C_1), 5.40 (m, 2 H, H-C_6, H-C_7), 5.57 (m, 2 H, H-C_2, H-C_3). MS: Exact mass calcd for $C_8H_{14}O$, 126.1045; found (EI), 126.1049 (M^+).

(2*S*,3*R*,6*E*)-2,3-Epoxy-6-octen-1-ol (17). To a solution of titanium(IV) isopropoxide (1.65 mL, 5.55 mmol, 1.0 equiv) in methylene chloride (60 mL) cooled at −25 °C was added diethyl L-(+)-tartrate (1.26 g, 6.11 mmol, 1.1 equiv). The mixture was stirred at −25 °C for 10 min, and then the alcohol **16** (0.70 g, 5.55 mmol, 1.0 equiv) in methylene chloride (2 mL) was added. After 5 min, a solution of 3 M *tert*-butyl hydroperoxide in toluene (3.67 mL, 11.0 mmol, 2.0 equiv) was added, and the reaction mixture was stored at −20 °C for 40 h. The workup followed the procedure described by Sharpless.[14a] The residue was purified by flash chromatography (25% ethyl acetate/hexane) to afford 0.60 g (76%) of **17** as a colorless oil, which solidified upon cooling at 0 °C: R_f 0.24 (60% ethyl acetate/hexane); $[\alpha]_D$ −10.24° (c 2.0, $CHCl_3$); IR ($CHCl_3$) 3560–3300 (br), 2940, 2860, 1455, 1370, 1200, 1060, 970 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.62–1.69 (m, 2 H, 2 H-C_4), 1.67 (dd, J = 1.2, 4.8 Hz, 3 H, 3 H-C_8), 2.17 (m, 2 H, 2 H-C_5), 3.04 (m, 1 H, H-C_3), 3.16 (m, 1 H, H-C_2), 3.67 (dd, J = 6.7, 12.1 Hz, 1 H, H-C_1), 3.85 (dd, J = 4.0, 12.1 Hz, 1 H, H-C_1), 5.45 (m, 2 H, olefin H). MS: Exact mass calcd for $C_8H_{14}O_2$, 142.0994; found (EI), 142.0999 (M^+).

Analysis of the acetyl derivative of **17** by the shift reagent $Eu^{III}(hfc)_3$ (270 MHz, C_6D_6) indicated an optical purity of 92%.

(2*S*,3*R*,6*E*)-2,3-Epoxy-1-[(*N*-methylcarbamoyl)oxy]-6-octene (18). To a solution of epoxy alcohol **17** (0.27 g, 1.90 mmol, 1.0 equiv) in methylene chloride (20 mL) were added sequentially triethylamine (0.685 mL, 4.92 mmol, 2.5 equiv) and methyl isocyanate (0.22 mL, 3.7 mmol, 2.0 equiv). The mixture was stirred at room temperature under nitrogen for 20 h and then quenched with saturated NH_4Cl solution (20 mL). The layers were separated, and the aqueous phase was extracted with methylene chloride (4 × 10 mL). The combined methylene chloride solution was dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography (30% ethyl acetate/hexane) to give 0.35 g (92.5%) of **18** as a colorless oil: $[\alpha]_D$ −20.4° (c 0.8, $CHCl_3$); IR ($CHCl_3$) 3460, 3000, 2960, 1745, 1520, 1450, 1210, 1140, 1000, 960 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.55 (m, 2 H, 2 H-C_4), 1.59 (d, J = 5.0 Hz, 3 H, 3 H-C_8), 2.13 (m, 2 H, 2 H-C_5), 2.78 (d, J = 5.3 Hz, 3 H, CH_3-N), 2.99 (m, 1 H, H-C_3), 3.15 (m, 1 H, H-C_2), 3.94 (dd, J = 7.0, 12.0 Hz, 1 H, H-C_1), 4.37 (dd, J = 4.0, 12.0 Hz, 1 H, H-C_1), 4.70 (br, 1 H, NH), 5.45 (m, 2 H, olefin H). MS: Exact mass calcd for $C_{10}H_{17}NO_3$, 199.1208; found (EI), 199.1207 (M^+).

(3′*E*,4*R*,5*R*)-4-(Hydroxymethyl)-3-methyl-5-(3′-pentenyl)-2-oxazolidinone (19). To a suspension of NaH (0.14 g, 6.0 mmol, 4.0 equiv) in THF (30 mL) was added a solution of **18** (0.3 g, 1.5 mmol, 1.0 equiv) in THF (2 mL). The mixture was stirred at room temperature under nitrogen for 20 h and then quenched by careful addition of saturated NH_4Cl solution (10 mL). The layers were separated, and the aqueous phase was extracted with methylene chloride (3 × 15 mL). The combined organic solution was dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography (40% ethyl acetate/hexane) to give 0.28 g (94%) of **19** as a colorless oil: $[\alpha]_D$ +75.2° (c 4.0, $CHCl_3$); IR ($CHCl_3$) 3500–3300 (br), 3000, 2940, 1740, 1435, 1410, 1250, 1210, 1150, 1030, 965 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.59 (d, J = 5.0 Hz, 3 H, 3 H-$C_{5'}$), 1.68 (m, 2 H, 2 H-$C_{1'}$), 2.09 (m, 2 H, 2 H-$C_{2'}$), 2.82 (s, 3 H, CH_3-N), 3.28 (m, 1 H, H-C_4), 3.50–3.80 (m, 3 H, C_4-CH_2OH), 4.30 (q, J = 5.6 Hz, 1 H, H-C_5), 5.37 (m, 2 H, olefin H). MS: Exact mass calcd for $C_{10}H_{17}NO_3$, 199.1208; found (EI), 199.1209 (M^+).

(3′*E*,4*S*,5*R*)-3-Methyl-5-(3′-pentenyl)-2-oxooxazolidine-4-carboxylic Acid (20). To a vigorously stirred solution of **19** (80 mg, 0.4 mmol) in acetone (5 mL) was added Jones reagent dropwise at such a rate to maintain the orange color. After stirring for 1 h, the reaction mixture was quenched with 10% aqueous $NaHSO_3$ solution and diluted with water (10 mL). The mixture was saturated with NaCl and extracted with ethyl acetate (4 ×

10 mL). The combined ethyl acetate extracts were concentrated to about 10 mL and extracted with 1 N $NaHCO_3$ solution (3 × 5 mL). The combined solution was acidified to pH 2 with 1 N HCl. The mixture was saturated with NaCl and extracted with ethyl acetate (4 × 10 mL). The combined ethyl acetate extracts were washed with brine (20 mL), dried ($MgSO_4$), and concentrated in vacuo to give 57 mg (67%) of crystalline acid **20**. An analytical sample was obtained by recrystallization from ether and pentane: mp 90 °C; $[\alpha]_D$ +37.5° (*c* 0.9, $CHCl_3$); IR ($CHCl_3$) 3300–2700 (br), 1750, 1435, 1400, 1215, 1040, 965 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.65 (d, J = 5.0 Hz, 3 H, 3 H-$C_{5'}$), 1.85 (m, 2 H, 2 H-$C_{1'}$), 2.20 (m, 2 H, 2 H-$C_{2'}$), 2.95 (s, 3 H, CH_3-N), 3.93 (d, J = 5.2 Hz, 1 H, H-C_4), 4.50 (m, 1 H, H-C_5), 5.45 (m, 2 H, olefin H), 8.70 (br, 1 H, -COOH); MS *m/e* 213 (M^+). Anal. ($C_{10}H_{15}NO_4$) C, H, N.

(2*S*,3*R*,6*E*)-3-Hydroxy-2-(methylamino)-6-octenoic Acid (11). A solution of **20** (0.31 g, 1.41 mmol) in 2 N KOH aqueous solution (6 mL) was heated to reflux for 5 h. The reaction mixture was cooled to room temperature and treated with Dowex H^+ (50× 8–100) to pH 4. The mixture was filtered through a Dowex H^+ column (1 × 20 cm) and eluted with 1.5 M aqueous NH_3 solution (350 mL). The aqueous solution was evaporated in vacuo to dryness, and the residue was crystallized from ethanol and water (drops) to give 0.26 g (95%) of crystalline amino acid 11: mp 248 °C; $[\alpha]_D$ +8.95° [*c* 0.8, H_2O at pH 7 (phosphate buffer tritrisol, pH 7, from Merck)], +17.0° (*c* 0.9, 0.4 M HCl);[12b] IR (KBr) 3300–2500 (br), 3240, 3000, 2960, 2850, 1645 (shoulder), 1590, 1425, 1355, 1305, 960 cm^{-1}; ^1H NMR (270 MHz, D_2O) δ 1.48 (d, J = 5.0 Hz, 3 H, 3 H-C_6), 1.35–1.60 (m, 2 H, 2 H-C_4), 1.85–2.10 (m, 2 H, 2 H-C_5), 2.58 (s, 3 H, CH_3-N), 3.25 (d, J = 7.3 Hz, 1 H, H-C_2), 3.73 (m, 1 H, H-C_3), 5.25–5.50 (m, 2 H, olefin H); MS *m/e* 188 (M^+ + 1). Anal. ($C_9H_{17}NO_3$) C, H, N.

***(E)*-Hept-2-en-5-yn-1-ol (23).** The title compound was prepared by a modification of the reported procedure.[21] To a stirred mixture of ether (40 mL) and $LiAlH_4$ (3.40 g, 87 mmol) was added, over 30 min, a solution of alcohol **22** (9.50 g, 88 mmol) in 10 mL of ether, during which time the mixture came to a gentle reflux. Following addition, the mixture was heated at gentle reflux for 7 h. The resulting thick slurry was diluted with additional ether (50 mL) and cautiously treated with water (5 mL), 2 N sodium hydroxide (5 mL), and again water (5 mL). The mixture was stirred at ambient temperature for 15 min and filtered. The filters were concentrated and short path distilled to a pale yellow oil: bp 67–72 °C (1.5 Torr); IR ($CHCl_3$) 3605, 3000, 2915, 1205, 1070 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.83 (t, J = 2.0 Hz, 3 H), 2.90 (m, 2 H), 4.15 (m, 2 H), 5.62–5.77 (m, 1 H), 5.85–6.00 (m, 1 H). Anal. ($C_7H_{10}O$) C, H.

(2*S*,3*S*)-2,3-Epoxyhept-5-yn-1-ol (24a). The general procedure described by Sharpless et al. was used.[14b] A mechanically stirred suspension of 2 g of powdered, activated 4-Å sieves (Aldrich) in dry methylene chloride (120 mL) was cooled in a carbon tetrachloride/dry ice bath to −10 °C. To this were added diethyl L-tartrate (1.3 mL, 7.6 mmol) and titanium tetraisopropoxide (1.6 mL, 5.4 mmol). The mixture was chilled to −25 °C and treated with *tert*-butyl hydroperoxide (55 mL of a 3 N solution in isooctane). To this mixture was added alcohol **23** (7.90 g, 72 mmol) in 20 mL of methylene chloride. The mixture was stirred for 4 h, during which it warmed to 0 °C. The mixture was placed in a −20 °C freezer overnight. With good stirring at ambient temperature, the mixture was treated with 20 mL of water, followed 30 min later by 10 mL of 4 N sodium hydroxide. After 30 min, the cloudy white solution was diluted with 300 mL of water and extracted two times with methylene chloride (total volume 500 mL). The organic layer was dried over magnesium sulfate and concentrated to afford 6.71 g (74%) as a white crystalline solid. Analytically pure material was obtained by recrystallization from 20% hexane/cyclohexane: R_f 0.33 (25% acetone/hexane); mp 55–56 °C; $[\alpha]_D$ −20.3° (*c* 0.60, $CHCl_3$); IR ($CHCl_3$) 3060, 3000, 1490, 1445, 1205, 695 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.80 (t, J = 2.0 Hz, 3 H), 2.40–2.68 (m, 2 H), 3.12 (m, 2 H), 3.68 (m, 1 H), 3.95 (br d, J = 13.0 Hz, 1 H). Anal. ($C_7H_{10}O_2$) C, H.

(2*S*,3*S*)-2,3-Epoxyhept-5-ynyl Triphenylmethyl Ether (24b). A solution of triphenylmethyl chloride (16.4 g, 59 mmol) in methylene chloride (150 mL) was cooled in an ice/H_2O bath and treated with DBU (10.0 mL, 22.1 mmol), followed, after 1 min, by epoxy alcohol **24a** (6.65 g, 53 mmol) in 15 mL of methylene chloride which was added over 20 min. The reaction mixture was removed from the ice bath and stirred at ambient temperature for 36 h. The reaction mixture was poured into 300 mL of ether/150 mL of water, and the organic layer was washed successively with 5% $KHSO_4$, 1 N $NaHCO_3$, and brine. Drying ($MgSO_4$) and concentration afforded 18.2 g (93%) of the protected epoxide as a yellow oil. Crystallization from ether afforded white crystals: mp 111–112 °C; IR ($CHCl_3$) 3620, 3020, 2940, 1195 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.80 (t, J = 2.0 Hz, 3 H), 2.50 (m, 2 H), 3.00–3.22 (m, 3 H), 3.31 (q, J = 7.0 Hz, 1 H), 7.15–7.50 (m, 15 H). Anal. ($C_{28}H_{24}O_2$) C, H.

(2*R*,3*R*)-3-Methyl-2-hydroxyhept-5-ynyl Triphenylmethyl Ether (25). To a mechanically stirred solution of copper(I) iodide (1.30 g, 6.8 mmol) in 100 mL of dry THF cooled to −30 °C under argon was added MeMgBr (5.0 mL of a 3 M solution in ether, 15 mmol). After 15 min, BF_3-Et_2O (1.0 mL, 5.2 mmol) was added, followed directly by protected epoxide **24b** (1.10 g, 3.0 mmol) in 10 mL of THF. After 1 h, methyllithium (5.0 mL of a 1.4 M solution in ether, 7.0 mmol) was added and the reaction mixture was warmed to 0 °C. After 1 h, the reaction mixture was quenched with 10 mL of 20% ammonium chloride, removed from the cold bath, and stirred at ambient temperature for 30 min. The mixture was treated with Celite and filtered through a 1-in. pad of Celite, rinsing well with ether. The combined organics were washed once with water and once with brine, dried ($MgSO_4$), and concentrated. Purification on column chromatography (10% *tert*-butyl methyl ether/hexane) afforded 0.19 g (27%) of **25** and 0.26 g (37%) of **26**. A total of 0.51 g of the epoxide **24b** was also recovered.

25: R_f 0.30 (12.5% methyl *tert*-butyl ether/hexane); IR ($CHCl_3$) 3515, 3030, 2945, 1605, 1505, 1460, 1065 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.80 (d, J = 7.0 Hz, 3 H), 1.88 (m, 3 H), 2.23 (m, 2 H), 2.45 (m, 1 H), 3.10 (dd, J = 7.0, 8.0 Hz, 1 H), 3.30 (dd, J = 4, 11.0 Hz, 1 H), 3.60 (m, 1 H), 7.15–7.50 (m, 15 H). Anal. ($C_{27}H_{28}O_4$) C, H.

26: ^1H NMR ($CDCl_3$) δ 0.91 (d, J = 7.0 Hz, 3 H), 1.79 (m, 3 H), 2.00 (m, 1 H), 2.28 (m, 2 H), 3.06 (dd, J = 7.0, 8.0 Hz, 1 H), 3.25 (d, J = 4.0 Hz, 1 H), 3.63 (m, 1 H), 7.15–7.50 (m, 15 H).

(2*R*,3*R*)-3-Methyl-2-[(*N*-methylcarbamoyl)oxy]hept-5-yn-1-ol (27). A solution of **25** (0.70 g, 1.8 mmol), methyl isocyanate (1.0 mL, 18.4 mmol), and 20 mL of toluene was heated in a sealed tube at 110 °C for 7 h. The reaction mixture was concentrated in vacuo, diluted with 50 mL of hexane, and reconcentrated. The resulting yellow oil was dissolved in 40 mL of 50% methanol/ether and treated with $TsOH$-H_2O (50 mg, 0.26 mmol). After stirring at room temperature for 38 h, the reaction mixture was treated with 5 mL of 2 N $NaHCO_3$ and concentrated. The resulting residue was partitioned between 100 mL of methylene chloride and 50 mL of brine. The aqueous layer was extracted twice with methylene chloride, and the combined organics were dried ($MgSO_4$), filtered, and concentrated. Purification on a silica gel column (30% ethyl acetate/hexane) afforded **27** (0.25 g, 72%) as a yellow oil: R_f 0.28 (50% ethyl acetate/hexane); IR ($CHCl_3$) 3465, 1710, 1515, 1195 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.05 (d, J = 7.0 Hz, 3 H), 1.80 (m, 3 H), 1.90–2.30 (m, 3 H), 2.61 (m, 1 H), 2.78 (d, J = 5.0 Hz, 3 H), 3.60–3.92 (m, 2 H), 4.63–4.85 (m, 2 H). Anal. ($C_{10}H_{17}NO_3$) C, H, N.

(4*R*,5*R*)-4-Hydroxy-3-methyl-5-[(1′*R*)-1′-methyl-3′-pentynyl]-2-oxazolidinone (28a). To a stirred solution of oxalyl chloride (0.14 mL, 1.6 mmol) in 10 mL of methylene chloride, cooled to −40 °C, was added anhydrous DMSO (0.17 mL, 20 mmol) dropwise over 2 min. After 5 min, **27** (0.27 g, 1.36 mmol) in 4 mL of methylene chloride was added dropwise over 5 min. After 15 min, 1 mL of triethylamine was added to the mixture and the cold bath removed. With good stirring the reaction was allowed to warm to ambient temperature over 1 h. The reaction mixture was poured into 100 mL of ether/50 mL of water, the layers were separated, and the aqueous layer was reextracted with ether. The organic layers were dried ($MgSO_4$) and concentrated. Purification on a silica gel column (50% ethyl acetate/hexane) afforded **28a** (0.18 g, 67%) as a yellow oil: R_f 0.20 (50% ethyl acetate/hexane); IR ($CHCl_3$) 3460–3240 (br), 2915, 1745, 1445, 1030 cm^{-1}; ^1H NMR δ 1.08 (d, J = 7.0 Hz, 3 H), 1.81 (t, J = 2.0 Hz, 3 H), 1.88 (m, 1 H), 2.20–2.30 (m, 2 H), 2.92 (s, 3 H), 4.12 (dd, J = 3.0, 8.0 Hz, 1 H), 4.37 (d, J = 10.0 Hz, 1 H), 4.94 (dd, J = 3.0, 10 Hz, 1 H). MS: Exact mass calcd for $C_{10}H_{15}NO_3$, 197.10518; found (HR-FAB), 197.10519. Anal. ($C_{10}H_{15}NO_3$) C, H, N.

Ethyl (4*S*,5*R*)-3-Methyl-5-[(1′*R*)-1′-methyl-3′-pentynyl]-2-oxazolidinone-4-carboxylate (29a). To a well-stirred

mixture of **28a** (0.15 g, 0.76 mmol), 4-(dimethylamino)pyridine (0.01 g, 0.76 mmol), triethylamine (0.3 mL), and 15 mL of methylene chloride was added acetic anhydride (0.09 mL, 0.91 mmol) dropwise over 1 min. After stirring for 2 h, the reaction mixture was poured into 100 mL of ether/50 mL of water, the layers were separated, and the aqueous layer was extracted once with ether. The combined organics were successively washed with 10% $KHSO_4$ and 1 N $NaHCO_3$, dried over magnesium sulfate, and concentrated. The unstable crude acetate **28b** was used at once in the next reaction.

28b: IR ($CHCl_3$) 2970, 2920, 2250, 1765, 1440, 1220, 1120 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.08 (d, J = 7.0 Hz, 3 H), 1.80 (t, J = 2.0 Hz, 3 H), 1.96 (m, 1 H), 2.12 (s, 3 H), 2.20–2.30 (m, 2 H), 2.88 (s, 3 H), 2.22 (dd, J = 1.0, 8.0 Hz, 1 H), 6.12 (d, J = 1.0 Hz, 1 H).

The acetate **28b** (148 mg, 0.62 mmol) in 10 mL of nitromethane was treated with trimethylsilyl cyanide (0.5 mL, 3.7 mmol) and 3 mol % BF_3·Et_2O. After stirring for 1 h at room temperature, the reaction mixture was concentrated in vacuo to yield 116 mg (91% of the epimeric nitrile **28c** as a 1.3:1.0 mixture of cis to trans). The diastereomeric compounds could be separated on silica gel column (35% ethyl acetate/hexane).

Cis isomer: R_f 0.63 (50% ethyl acetate/hexane); IR ($CHCl_3$) 1780, 1405, 1055 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.12 (d, J = 7.0 Hz, 3 H), 1.82 (t, J = 2.0 Hz, 3 H), 2.05 (m, 1 H), 2.30–2.40 (m, 2 H), 3.03 (s, 3 H), 4.38 (d, J = 6.0 Hz, 1 H), 4.63 (dd, J = 6.0, 7.0 Hz, 1 H); MS m/e 207 (M^+ + 1, 19), 180 (M^+ – CN, 19), 147 (27), 126 (44), 82 (55), 68 (70), 53 (100).

Trans isomer: R_f 0.55 (50% ethyl acetate/hexane); IR ($CHCl_3$) 1780, 1400, 1200 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.12 (d, J = 7.0 Hz, 3 H), 1.80 (t, J = 2.0 Hz, 3 H), 2.28 (m, 1 H), 2.40–2.50 (m, 2 H), 3.05 (s, 3 H), 4.46 (dd, J = 6.0, 15.0 Hz, 1 H), 4.49 (d, J = 6.0 Hz, 1 H); MS m/e 207 (M^+ + 1, 10), 180 (M – CN, 15), 147 (20), 126 (38), 82 (40), 68 (152), 53 (82).

Nitrile **28c** (116 mg, 0.56 mmol) was dissolved in 10 mL of 95% ethanol and treated with 2 equiv of K_2CO_3 (0.16 g, well powdered). After stirring vigorously for 9 h, the reaction mixture was partitioned between methylene chloride (100 mL) and water (50 mL). The layers were separated, and the aqueous layer was extracted with 2 × 20 mL of methylene chloride. The combined organics were dried ($MgSO_4$) and concentrated in vacuo to a yellow oil. This residue was immediately dissolved in 17 mL of 95% ethanol, treated with 1 mL of 2 N HCl, and stirred at room temperature for 6 h. The mixture was neutralized with 1 N $NaHCO_3$ and partitioned between 100 mL of methylene chloride/50 mL of water. The aqueous was extracted with 1 × 30 mL of methylene chloride, and the combined organics were dried ($MgSO_4$) and concentrated in vacuo to a yellow oil. Purification on silica gel column (30% ethyl acetate/hexane) afforded 97 mg (68%) of **29a**: R_f 0.44 (50% ethyl acetate/hexane); $[\alpha]_D$ +25.2° (c 0.91, $CHCl_3$); IR ($CHCl_3$) 3005, 2985, 1760, 1755, 1400, 1045 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.10 (d, J = 7.0 Hz, 3 H), 1.34 (t, J = 7.0 Hz, 3 H), 1.80 (t, J = 2.0 Hz, 3 H), 2.00 (m, 1 H), 2.29 (m, 2 H), 2.95 (s, 3 H), 4.05 (d, J = 5.0 Hz, 1 H), 4.23–4.40 (m, 3 H). Anal. ($C_{13}H_{19}NO_4$) C, H, N.

(2*S*,3*R*,4*R*)-3-Hydroxy-4-methyl-2-(methylamino)-6-octynoic Acid (21). To a well-stirred solution of **29a** (102 mg, 0.40 mmol) in 2 mL of methanol cooled to 0 °C was added 2 mL of 2 N NaOH. The mixture was allowed to warm to room temperature over 2 h. The methanol was removed in vacuo and the mixture acidified (0 °C, 6 N HCl) to pH 2 and extracted three times with ether. Drying ($MgSO_4$) and concentration afforded 83 mg (90%) of the carboxylic acid **29b**: ^1H NMR ($CDCl_3$) δ 1.10 (d, J = 7.0 Hz, 3 H), 1.82 (br s, 3 H), 2.00 (m, 1 H), 2.24–2.34 (m, 2 H), 2.98 (s, 3 H), 4.12 (d, J = 5.0 Hz, 1 H), 4.43 (dd, J = 5.0, 8.0 Hz, 1 H), 6.4 (bs, 1 H). Acid **29b** was suspended in 2 N KOH (2 mL) and heated at reflux for 5 h. After being cooled to room temperature, the mixture was treated with Dowex (50× 8–100) resin (H^+ form) until the pH was <5. The mixture was heated at 75–80 °C for several minutes with good stirring, cooled to room temperature, and applied to column with about 20 mL more Dowex resin. The column was eluted with 200 mL of 1.5 M NH_4OH. Fractions that contained products (seen by developing TLC plates with phosphomolybdic acid) were concentrated in vacuo to afford 75 mg of **21** as a white solid: mp 233–235 °C; $[\alpha]_D$ –13.6° (c 0.45, pH 7 phosphate buffer); ^1H NMR (D_2O) δ 0.93 (d, J = 7.0 Hz, 3 H), 1.60 (br s, 3 H), 1.76 (m, 2 H), 2.00–2.30 (m, 2 H), 2.61 (s, 3 H), 3.52 (d, J = 5.0 Hz, 1 H), 3.74 (m, 1 H). Anal. ($C_{10}H_{17}NO_3$) C, H, N.

General Procedures for the Synthesis of 1-Position Analogues of CsA. [[(4*S*,5*R*,3′*E*)-5-(1′,1′-Dimethyl-3′-pentenyl)-2,2,3-trimethyl-4-oxazolidinyl]carbonyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (33a). A suspension of (2*S*,3*R*,6*E*)-4,4-dimethyl-3-hydroxy-2-(methylamino)-6-octenoic acid (**6**) (0.17 g, 0.25 mmol) (dried by azeotropic distillation of H_2O with two 50-mL portions of benzene) in 100 mL of anhydrous acetone was heated at reflux for 24 h until a clear solution was obtained. The acetone solution was concentrated to 2.5 mL under vacuum, and the remaining solution was used directly for synthetic purposes.

The solution of freshly prepared acetonide **30a** was diluted immediately with 1.2 mL of THF and *N*-methylmorpholine (0.03 mL, 0.27 mmol). Then *N*-hydroxybenzotriazole ($HOBt·H_2O$; 0.08 g, 0.50 mmol) was dehydrated (by azeotropic distillation of H_2O with 2 × 15 mL of toluene) and added to the solution together with hexapeptide **32** (0.17 g, 0.25 mmol)[9b,11] diluted in 1.2 mL of THF. The resulting solution was cooled (0 °C), and DCC (0.052 g, 0.246 mmol) was added. After $^1/_2$ h (0 °C) the solution was allowed to warm to room temperature and stirred for 26 h at room temperature. The mixture was diluted with 40 mL of methylene chloride and washed with saturated $NaHCO_3$ (30 mL). The aqueous phase was extracted with methylene chloride. The combined organics were dried ($MgSO_4$) and concentrated. After flash chromatography (10–20% acetone in hexane) the heptapeptide **33a** was obtained in 86% yield (0.20 g): R_f 0.32 (40% acetone/hexane); $[\alpha]_D$ –128.4° (c 1.16, $CHCl_3$); IR ($CHCl_3$) 3360, 2960, 1740, 1655, 1309, 1460 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz; at room temperature there are at least four conformers; if distinguishable, only the main conformer is described) δ 0.74–1.05 (m, 27 H, CH_3), 1.15–1.50 [m, 9 H, 2 CH_3-C(2^1),CH_3-C(2^7)], 1.66 [m, CH_3-C($4'^1$)], 1.55–2.40 (m, 11 H, CH_2, CH), 2.29 (s, CH_3-N^1), 3.94, 3.02, and 3.18 (s, 9 H, CH_3-N), 3.78 [m, 1 H, H-C(5^1)], 4.38–5.50 (m, 12 H, benzyl, olefin α-C-H), 6.62 (d, J = 9.0 Hz, H-N), 6.71 (d, J = 9.5 Hz, H-N), 7.34 (s, 5 H, aromatic H), 7.84 (d, J = 10 Hz, H-N). Anal. ($C_{50}H_{83}N_7O_9$) C, H, N.

[(2*S*,3*R*,6*E*)-4,4-Dimethyl-3-hydroxy-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (34a). A solution of protected hexapeptide **33a** (0.26 g, 0.28 mmol) in 1.5 mL of MeOH was stirred for 48 h at room temperature in the presence of 1.2 mL of 1 N HCl (the HCl solution was added in two portions; 2 h after the addition of 0.9 mL of 1 N HCl, 0.3 mL of 1 N HCl was added). The acid in the reaction medium was neutralized with $NaHCO_3$ (0.63 g, 7.50 mmol) and the solvent evaporated in vacuo (16 Torr), taking care the temperature did not rise above 30 °C. The residue was taken up in 2% MeOH/methylene chloride and flash chromatographed on 20 g of silica gel with 2–3% MeOH/methylene chloride as eluent to give 0.11 g (44%) of **34a** (including about 15% of the methyl ester). Starting material (0.08 g, 31%) was recovered. The methyl ester heptapeptide was not separated. Using the same conditions, but 1 equiv of 1 N HCl, gave pure benzyl ester, only in 31% yield. The recovered starting material was hydrolyzed in dioxane/aqueous HCl to give after flash chromatography an additional 20% of N-deprotected heptapeptide **34a**: ^1H NMR ($CDCl_3$, 200 MHz, at room temperature two main conformers were visible, 3:2) δ 0.76–1.09 (m, 27 H, CH_3), 1.34 and 1.35 [d, J = 7.5 Hz, 3 H, CH_3-C(2^7)], 1.65 [m, 3 H, CH_3-C(7^1)], 1.40–2.21 (m, 11 H, CH_2, CH); 2.40 and 2.41 (s, 3 H, CH_3-N^1), 3.58 (br s, 3 H, 2 H-N, HO), 2.80, 2.99, 3.05, 3.08, 3.21, and 3.30 (s, 9 H, CH_3-N), 3.58 [m, 1 H, H-C(5^1)], 4.01 and 4.22 [AB system, J = 15 Hz, 2 H-C(2^3)], 4.32–5.51 (m, 12 H, benzyl, olefin α-C-H), 6.72 (d, J = 8.0 Hz, H-N), 6.93 (d, J = 9.5 Hz, H-N), 7.34 (s, 5 H, aromatic H), 7.65 (m, 1 H, H-N), 7.99 (m, 1 H, H-N). Anal. ($C_{47}H_{79}N_7O_9$) C, H, N.

[(2*S*,3*R*,6*E*)-3-Hydroxy-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (34b). The title compound was prepared in 54% yield from (2*S*,3*R*,6*E*)-3-hydroxy-2-(methylamino)octenoic acid (**11**) according to the procedure used to prepare **33a** and **34a**. R_f 0.13 (6% MeOH/methylene chloride); $[\alpha]^{rt}_D$ –110.7° (c 0.55, $CHCl_3$); ^1H NMR

($CDCl_3$) δ 0.65–1.10 (m, 21 H, CH_3), 1.20–2.25 [m, 19 H, CH, CH_2, CH_3-C(7^1), CH_3-C(2^7)], 2.36, 2.40 (s, 3 H, CH_3-N^1), 2.76 (br s, 2 H, OH, NH), 2.99, 3.05, 3.10, 3.12, 3.22, 3.33 (s, 9 H, CH_3-N), 3.50–5.48 [m, 13 H, H-C(3^1), α-H, benzyl, olefin], 6.70 (d, J = 8.0 Hz, H-N), 6.95 (d, J = 9.0 Hz, N-H), 7.33 (s, 5 H, aromatic H), 7.55 (d, J = 8.0 Hz, N-H), 7.75 (d, J = 9.0 Hz, N-H), 7.95 (d, J = 7.0 Hz, N-H). MS: Exact mass calcd for $C_{45}H_{76}N_7O_9$ (M^+ + 1), 858.5704; found (HR-FAB), 858.5708.

[(2*S*,3*R*,4*R*,6*E*)-3-Hydroxy-4-methyl-2-(methylamino)-6-octynoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (34c). The title compound was prepared in 43% yield from [2*S*,3*R*,4*R*,6*E*]-3-hydroxy-4-methyl-2-(aminomethyl)-6-octynoic acid (**21**) according to the procedure for **33a** and **34a**: R_f 0.26 (10% MeOH/methylene chloride); $[\alpha]^{rt}_D$ −122.2° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.78–1.18 (m, 24 H, CH_3), 1.25–2.52 [m, 18 H, CH_1, CH_2, CH_3-C(2^7), CH_3-C(7^1)], 2.75–3.38 (m, 14 H, CH_3-N, H-O, H-N, major conformer CH_3N at 2.70, 3.00, 3.13, 3.28), 3.58–5.25 [m, 11 H, α-CH, benzyl, H-C(3^1)], 6.65 (m, N-H), 7.00 (d, J = 8.0 Hz, N-H), 7.33 (s, 5 H, aromatic H), 7.62 (d, J = 7.0 Hz, N-H), 7.95 (d, J = 9.0 Hz, N-H), 8.06 (d, J = 7.0 Hz, N-H), 8.30 (d, J = 8.0 Hz, N-H). MS: Exact mass calcd for $C_{46}H_{75}N_7O_9$, 870.570453; found (HR-FAB), 870.56892.

[(9-Fluorenylmethyl)oxy]-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl[(2*S*,3*R*,6*E*)-4,4-dimethyl-3-hydroxy-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (36a). To a solution of heptapeptide **34a** (0.10 g, 0.113 mmol) and tetrapeptide **35** (0.093 g, 0.124 mmol, 1.1 equiv)[20] in 1.5 mL of methylene chloride were added *N*-methylmorpholine (0.027 g, 0.242 mmol, 2.15 equiv) and BOP reagent[22] (0.082 g, 0.186 mmol, 1.65 equiv). The mixture was stirred for $3^1/_2$ days at room temperature. The solution was diluted with methylene chloride (30 mL) and washed with saturated $NaHCO_3$ (30 mL). The aqueous phase was extracted with methylene chloride (2 × 30 mL). The organics were dried (Na_2SO_4) and concentrated. The residue was flash chromatographed on 20 g of silica gel with 10–20% acetone in hexane as eluent, and 0.08 g (46%) of pure undecapeptide **36a** was isolated. Another diastereomeric undecapeptide, with a larger R_f (0.28, 40% acetone in hexane), was isolated (0.035 g, 20%). R_f 0.22 (40% acetone/hexane); $[\alpha]^{rt}_D$ −171.6° (*c* 0.57, $CHCl_3$); IR ($CHCl_3$) 3410, 3300, 2955, 1715, 1636, 1500, 1465, 1410 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz; several conformers were observed at room temperature, and only the main conformer is described) δ 0.68–1.05 (m, 45 H, CH_3), 1.20–2.10 [m, 26 H, CH_3-C(2^1), CH_3-C(7^5), CH_3-C(2^{11}), CH_2, CH], 2.31 [m, 2 H, H-C(5^5), H-C(3^9)], 2.62, 2.81, 2.96, 3.06, 3.21, and 3.30 (s, 21 H, CH_3-N), 3.86 [m, 1 H, H-C(3^5)], 4.01–5.82 [m, 18 H, H-N^1, $-OCH_2Ph$, OCH_2CH (Fmoc), olefin H, α-CH], 6.82 (d, J = 9.0 Hz, 1 H, H-N), 6.90 (d, J = 6.0 Hz, 1 H, H-N), 7.28–7.80 (m, 10 H, H-N, 9 aromatic H). Anal. ($C_{85}H_{131}N_{11}O_{15}$) C, H, N.

[(9-Fluorenylmethyl)oxy]-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl[(2*S*,3*R*,6*E*)-3-hydroxy-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (36b). The title compound was prepared in 59% yield from heptapeptide **34b** according to the procedure for **36a**: R_f 0.20 (35% acetone/hexane); $[\alpha]^{rt}_D$ −151.2° (*c* 0.75, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 0.73–1.08 (m, 31 H, CH_3), 1.20–2.33 [m, 29 H, CH_3-C(2^1), CH_3-C(7^5), CH_3-C(2^{11}), C-H, CH_2], 2.97, 3.03, 3.04, 3.10, 3.17, 3.31, and 3.39 (s, 21 H, CH_3-N), 3.95–5.51 [m, 20 H, OCH_2Ph, OCH_2CH (Fmoc), olefin H, α-CH, H-C(3^5)], 5.78 (d, J = 9.0 Hz, H-N^1), 7.10 (d, J = 9.0 Hz, H-N), 7.27–7.76 (m, 13 H, aromatic H), 7.80 (d, J = 8.0 Hz, H-N), 7.90 (d, J = 9.0 Hz, H-N). Anal. ($C_{83}H_{127}N_{11}O_{15}$) C, H, N.

[(9-Fluorenylmethyl)oxy]-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-octynoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanine Benzyl Ester (36c). The title compound was prepared in 47% yield from heptapeptide **34c** according to the procedure for **36a**: R_f 0.37 (40% acetone/hexane); $[\alpha]^{rt}_D$ −154.3° (*c* 0.70, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.70–1.08 (m, 42 H, CH_3), 1.25–2.45 [m, 28 H, CH_3-C(2^1), CH_3-C(7^5), CH_3-C(2^{11}), CH_2, CH], 2.83, 3.00, 3.04, 3.11, 3.28, and 3.35 (s, 21 H, CH_3-N), 3.70–5.50 [m, 18 H, OCH_2CH (Fmoc), $-OCH_2PH$, α-CH, H-C(3^5)], 6.78 (d, J = 7.0 Hz, N^1-H), 7.28–7.80 (m, 13 H, aromatic H), 7.72 (d, J = 7.0 Hz, H-N), 7.88 (d, J = 8.0 Hz, H-N), 8.08 (d, J = 8.0 Hz, H-N). Anal. ($C_{84}H_{127}N_{11}O_{15}$) C, H, N.

***cyclo*-[[(2*S*,3*R*,6*E*)-4,4-Dimethyl-3-hydroxy-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl] (MeBm$_2$t^1-CsA, 3).** A solution of fully protected linear undecapeptide **36a** (0.08 g, 0.0517 mmol) in 2 mL of EtOH (0 °C) was treated with 0.52 mL of 0.2 N NaOH and stirred in the cold for 1 h.[15] At that time an additional 0.26 mL of 0.2 N NaOH was added and stirring was continued at 0 °C. After $5^1/_2$ h no starting material was left (TLC; 40% acetone in hexane). The mixture was acidified with 0.78 mL of 0.2 N HCl (pH 5–6) and was treated with 10 mL of saturated NaCl/30 mL of methylene chloride. The layers were separated and the aqueous portion was extracted with methylene chloride (4 × 20 mL). The combined organics were dried (Na_2SO_4) and concentrated to an oil (0.10 g) which was carefully dried. With vigorous stirring, the residue was dissolved in 300 mL of methylene chloride and treated with DMAP (0.038 g, 0.31 mmol, 6 equiv) and $(PrPO_2)_3$ [0.042 mL of a 50% w/w solution in methylene chloride (Fluka), 0.259 mmol, 5 equiv].[16] This solution was stirred at room temperature for 36 h and then concentrated to 1 mL and directly applied to 25 g of silica gel. Flash chromatography with 10–40% acetone in hexane as eluent gave 0.039 g (62%) of pure MeBm$_2$t^1-CsA (**3**). For analytical purposes a sample of **3** was precipitated from ether with pentane: R_f 0.26 (40% acetone/hexane); mp 134.0–137.0 °C; $[\alpha]^{rt}_D$ −266.7° (*c* 0.78, $CHCl_3$); IR ($CHCl_3$) 3300, 2955, 1665, (shoulder), 1520, 1465, 1410 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz), δ 0.72–1.09 [m, 45 H, 2 CH_3-C(4^1), CH_3-C(3^2), 2 CH_3-C(4^4), 2 CH_3-C(3^5), 2 CH_3-C(4^6), 2 CH_3-C(4^9), 2 CH_3-C(4^{10}), 2 CH_3-C(3^{11})], 1.16–2.19 [m, 14 H, H-C(5^1), 2 H-C(2^2), 2 H-C(3^4), H-C(4^4), 2 H-C(3^6), H-C(4^6), 2 H-C(3^9), H-C(4^9), 2 H-C(3^{10}), H-C(4^{10})], 1.26 [d, J = 7.0 Hz, 3 H, CH_3-C(2^8)], 1.31 [d, J = 7.0 Hz, 3 H, CH_3-C(2^7)], 1.62 [d, J = 6.0 Hz, 3 H, CH_3-C(7^1)], 2.26–2.45 [m, 2 H, H-C(3^{11}), H-C(3^5)], 2.65 (s, 3 H, CH_3-N^{10}), 2.66 (s, 3 H, CH_3-N^{11}), 3.05 (s, 3 H, CH_3-N^4), 3.16 (s, 3 H, CH_3-N^9), 3.17 [d, J = 13.0 Hz, 1 H, H-C(2^3)], 3.22 (s, 3 H, CH_3-N^6), 3.41 (s, 3 H, CH_3-N^3), 3.48 (s, 3 H, CH_3-N^1), 3.86 [d, J = 6.5 Hz, 1 H, H-C(3^1)], 4.45 [m, 1 H, H-C(2^7)], 4.67 [d, J = 13.0 Hz, 1 H, H-C(2^3)], 4.72 [t, J = 9.0 Hz, 1 H, H-C(2^5)], 4.83 [m, 1 H, H-C(2^8)], 4.97 [m, 2 H, H-C(2^2), H-C(2^{11})], 5.06 [t, J = 7.0 Hz, 1 H, H-C(2^{10})], 5.18 [dd, J = 5.0, 11.0 Hz, 1 H, H-C(2^6)], 5.25–5.41 [m, 4 H, H-C(2^1), H-C(6^1), H-C(7^1), H-C(2^4)], 5.66 [dd, J = 4.0, 11.0 Hz, 1 H, H-C(2^9)], 7.24 (d, J = 5.2 Hz, 1 H, H-N^8), 7.61 (d, J = 8.8 Hz, 1 H, H-N^5), 7.86 (d, J = 6.9 Hz, 1 H, H-N^7), 8.18 (d, J = 7.7 Hz, 1 H, H-N^2). MS: Exact mass calcd for $C_{63}H_{114}N_{11}O_{12}$ (M^+ + 1), 1216.8648; found (HR-FAB), 1216.8650.

***cyclo*-[[(2*S*,3*R*,6*E*)-3-Hydroxy-2-(methylamino)-6-octenyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl] (MeBth^1-CsA, 4).** The title compound was prepared in 62% yield from undecapeptide **36b** according to the procedure for **3**: R_f 0.22 (35% acetone/hexane); $[\alpha]^{rt}_D$ −225.3° (*c* 0.60, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 0.75–1.15 [m, 39 H, CH_3-C(3^2), 2 CH_3-C(4^4), 2 CH_3-C(3^5), 2 CH_3-C(4^6), 2 CH_3-C(4^9), 2 CH_3-C(4^{10}), 2 CH_3-C(3^{11})], 1.2–2.2 [m, 17 H, 2 H-C(4^1), 2 H-C(3^2), 2 H-C(3^4), H-C(4^4), 2 H-C(3^6), H-C(4^6), 2 H-C(3^9), H-C(4^9), 2 H-C(3^{10}), H-C(4^{10}), H-C(3^{11})], 1.22 [d, J = 7.0 Hz, 3 H, CH_3-C(2^8)], 1.31 [d, J = 7.0 Hz, 3 H, CH_3-C(2^7)], 1.58 [m, 3 H, CH_3-C(7^1)], 2.23 [m, 2 H, 2 H-C(5^1)], 2.39 [m, 1 H, H-C(3^5)], 2.64 (s, 3 H, CH_3-N^{10}), 2.70 (s, 3 H, CH_3-N^{11}), 3.1 (s, 3 H, CH_3-N^4), 3.15 (s, 3 H, CH_3-N^9), 3.2 [d, J = 11.0 Hz, 1 H, H-C(2^3)], 3.29 (s, 3 H, CH_3-N^6), 3.4 (s, 3 H, CH_3-N^3), 3.45 (s, 3 H, CH_3-N^1), 3.95 [m, 2 H, H-C(3^1), HOC(3^1)], 4.58 [m, 1 H, H-C(2^7)], 4.61 [t, J = 7.5 Hz, 1 H, H-C(2^5)], 4.71 [d, J = 11.0 Hz, 1 H, H-C(2^3)], 4.82 [m, 1 H, H-C(2^8)], 4.95 [d, J = 6.5 Hz, 1 H, H-C(2^6)], 6.98 [m, 1 H, H-C(2^2)], 5.08 [m, 2 H, H-C(2^{10}), H-C(2^{11})], 5.28 [dd, J = 4.0, 10.4 Hz, 1 H, H-C(2^4)], 5.37 [m, 2 H, H-C(6^1), H-C(7^1)], 5.6 [d, J = 6.5 Hz, 1 H, H-C(2^1)], 5.65 [dd, J = 4.0, 10 Hz, 1 H, H-C(2^9)], 7.08 (d, J = 8.0 Hz, 1 H, H-N^8), 7.65 (d, J = 8.5 Hz, 1 H, H-N^5), 7.78 (d, J = 8.0 Hz, 1 H, H-N^7), 8.2 (d, J = 8.0 Hz, 1 H, H-N^2). MS: Exact mass calcd for $C_{61}H_{110}N_{11}O_{12}$ (M^+H), 1188.8335; found (HR-FAB), 1188.8338.

***cyclo*-[[(2*S*,3*R*,4*R*,6*E*)-4-Methyl-3-hydroxy-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-sarcosyl-*N*-methyl-L-**

leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl] (MeByt[1]-CsA, 5). The title compound was prepared in 39% yield from undecapeptide **36c** according to the procedure for **3**: R_f 0.42 (40% acetone/hexane); $[\alpha]^{rt}_D$ −229.1° (*c* 0.50, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 0.78–1.11 [m, 42 H, CH_3-C(4^1), CH_3-C(3^2), 2 CH_3-C(4^4), 2 CH_3-C(3^5), 2 CH_3-C(4^6), 2 CH_3-C(4^9), 2 CH_3-C(4^{10}), 2 CH_3-C(3^{11})], 1.18–2.62 [m, 19 H, HC(4^1), 2 H-C(5^1), 2 H-C(3^2), 2 H-C(3^4), H-C(4^4), H-C(3^5), 2 H-C(3^6), H-C(4^6), 2 H-C(3^9), H-C(4^9), 2 H-C(3^{10}), H-C(4^{10}), H-C(3^{11})], 1.26 [d, *J* = 7.0 Hz, 3 H, CH_3-C(2^8)], 1.36 [d, *J* = 7.0 Hz, 3 H, CH_3-C(2^7)], 1.76 [m, 3 H, CH_3-C(7^1)], 2.70 (s, 6 H, CH_3-N^{11}, CH_3-N^{10}), 3.06 (s, 3 H, CH_3-N^4), 3.16 (s, 3 H, CH_3-N^9), 3.18 [d, *J* = 11.5 Hz, 1 H, H-C(2^3)], 3.26 (s, 3 H, CH_3-N^6), 3.38 (s, 3 H, CH_3-N^3), 3.49 (s, 3 H, CH_3-N^1), 3.85 [m, 1 H, H-C(3^1)], 4.48 [m, 1 H, H-C(2^7)], 4.68 [m, 2 H, H-C(2^3), H-C(2^5)], 4.82 [m, 1 H, H-C(2^8)], 4.98 [m, 1 H, H-C(2^2)], 5.10 [m, 2 H, H-C(2^6), H-C(2^{10})], 5.20 [d, *J* = 10.0 Hz, 1 H, H-C(2^{11})], 5.30 [dd, *J* = 4.0, 10 Hz, 1 H, H-C(2^4)], 5.36 [d, *J* = 8.0 Hz, 1 H, H-C(2^1)], 5.70 [dd, *J* = 3.5, 10 Hz, 1 H, H-C(2^9)], 7.26 (d, *J* = 8.0 Hz, 1 H, H-N^8), 7.43 (d, *J* = 8.0 Hz, 1 H, H-N^5), 7.76 (d, *J* = 7.0 Hz, 1 H, H-N^7), 8.18 (d, *J* = 7.5 Hz, 1 H, H-N^2). MS: Exact mass calcd for $C_{62}H_{110}N_{11}O_{12}$ (M^+ + 1), 1200.8308; found (HR-FAB), 1200.8335.

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Registry No. **1a**, 59865-13-3; **1b**, 59865-15-5; **3**, 114865-22-4; **4**, 114891-20-2; **5**, 124562-06-7; **6**, 124562-07-8; **7**, 108536-03-4; **8**, 920-08-1; **9**, 124648-21-1; (±)-**9**, 124562-32-9; **9** [(+)-ephedrine salt], 124562-33-0; 11, 104324-14-3; **12**, 928-92-7; **13**, 25166-87-4; 14, 5927-18-4; **15**, 25184-12-7; **15** (2*E*-isomer), 25172-05-8; **16**, 124562-08-9; **17**, 121741-83-1; **18**, 124562-09-0; **19**, 121741-84-2; **20**, 108536-06-7; **21**, 124562-10-3; **22**, 26817-34-5; **23**, 124562-11-4; **24a**, 124562-12-5; **24b**, 124562-22-7; **25**, 124562-13-6; **26**, 124562-14-7; **27**, 124562-15-8; **28a**, 124562-16-9; **28b**, 124562-23-8; *cis*-**28c**, 124562-24-9; *trans*-**28c**, 124562-25-0; **29a**, 124581-07-3; **29b**, 124562-26-1; **30a**, 124562-17-0; **30b**, 124581-08-4; **31**, 124562-18-1; **32**, 81135-30-0; **33a**, 124562-19-2; **33b**, 124562-27-2; **33c**, 124562-28-3; **34a**, 124562-20-5; **34b**, 124562-29-4; **34c**, 124562-30-7; **35**, 115141-96-3; **36a**, 124562-21-6; **36b**, 124562-31-8; **36c**, 124581-09-5; (±)-$CH_3CH(OH)CH{=}CH_2$, 6118-14-5; Me_2CHCHO, 78-84-2; MeNCO, 624-83-9; Ph_3CCl, 76-83-5; Me_2CO, 67-64-1; (+)-ephedrine, 321-98-2.

NMR Studies of the Conjugation of Mechlorethamine with Glutathione

Michael P. Gamcsik,*[†] Terence G. Hamill,[‡] and Michael Colvin[‡]

Division of NMR Research, Department of Radiology, The Johns Hopkins University School of Medicine, and The Oncology Center, The Johns Hopkins Hospital, Baltimore, Maryland 21205. *Received June 8, 1989*

Many cancer cells are resistant to chemotherapeutic treatment with mechlorethamine and other alkylating agents. These drug-resistant cells often show an increase in the intracellular concentration of glutathione and an increase in the activity of glutathione-*S*-transferase when compared to the sensitive cells. Both of these components are thought to be involved with inactivation of the drug either through conjugation with glutathione or by hydrolysis. NMR spectroscopy was used to monitor the nonenzymatic conjugation of mechlorethamine with glutathione. Several intermediates along the pathway to the doubly glutathione substituted mustard, including both mustard–aziridinium adducts, can be observed. The assignment of the ^1H NMR spectrum of these adducts are presented. At 30 °C, pH 7.0, no hydrolyzed mustard was detectable. With the use of ^{13}C-labeled mustard, the conjugation reaction can be shown to proceed through an aziridinium intermediate rather than by direct nucleophilic substitution.

The nitrogen mustards are a class of bifunctional alkylating agents commonly used in cancer chemotherapy. These agents are believed to exert their cytotoxic effect through the alkylation of cellular components.[1] Alkylation is thought to occur via the formation of a reactive mustard aziridinium intermediate. Cancer cells displaying resistance to these alkylating agents often have an increased intracellular concentration of glutathione (GSH) and a higher activity of the enzyme(s) glutathione-*S*-transferase (GST).[2] The role these species play in conferring the drug resistance of these cells is not clear. However, it has been proposed that the drug may be inactivated by hydrolysis or by conjugation with GSH.[3,4] It is not known whether inactivation must be catalyzed by GST or will occur spontaneously under physiological conditions.

In this paper, we report the NMR characterization of the inactive derivative of the nitrogen mustard mechlorethamine which is formed by the conjugation with GSH. The formation of this bisthioether can be monitored dynamically by ^1H NMR spectroscopy. Several intermediates in the reaction of GSH with mechlorethamine can be observed, including the stepwise formation of the two aziridinium derivatives and the mono-GSH-substituted adduct.

Mechlorethamine specifically labeled with the ^{13}C nucleus was used to prove that the formation of the conjugated adduct proceeds through the aziridinium intermediate and not through direct nucleophilic substitution of the chlorine atom with the thiolate of GSH.

This report presents the NMR characterization of the inactive analogues of this alkylating agent and forms a basis for the study of the enzyme-catalyzed reactions in vitro and in vivo.

[†] Department of Radiology.
[‡] The Oncology Center.

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