ANALOGS OF CYCLOSPORIN A MODIFIED AT THE D-ALA[8] POSITION

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The conversion of [2-deutero-3-fluoro-D-Ala[8]]cyclosporin A (1) to a dehydroalanine analog [Δ-Ala[8]]cyclosporin A (2) was achieved with lithium diisopropylamide in THF at low temperature. This dehydro compound is a useful intermediate for the preparation of position 8 analogs of cyclosporin A formed from it by the conjugate addition of thiol compounds. NMR conformational studies have provided evidence for the restoration of D-stereochemistry in the modified Ala[8] residues. The preparation of several of these cyclosporin analogs and their bioactivities are described.

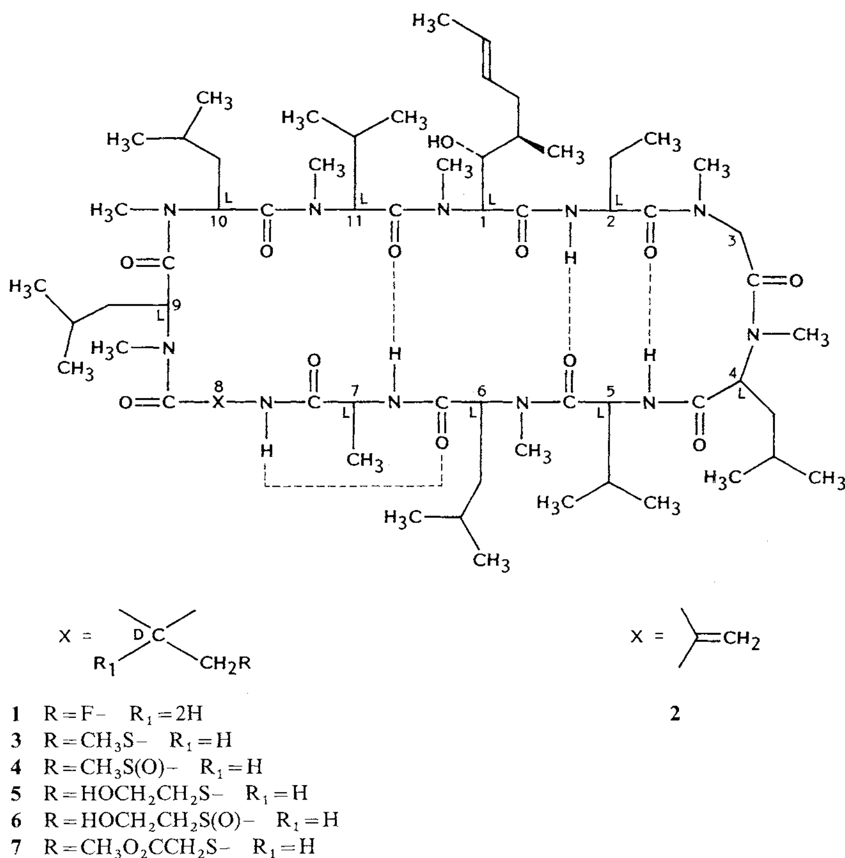
Cyclosporin A (CyA) is a potent immunosuppressant which has received widespread clinical application in therapy accompanying organ transplantation and promising reports of its efficacy have appeared in the treatment of rheumatoid arthritis, psoriasis, uveitis and myasthenia gravis[1]. CyA's usefulness is limited by renal and hepatic toxicities[2] and numerous analogs have been investigated in an effort to find one with improved potency and therapeutic index[3~6]. Toward the latter goal we decided to introduce substituent groups into CyA which might be expected to alter its *in vivo* metabolism. We chose the [D-Ala[8]] residue as a possible site for these modifications since this amino acid position is accessible to biosynthetic modification[6,7]. Importantly, cyclosporin analogs retaining good immunosuppressive activity have been described in which D-Ala[8] has been replaced by D-Ser[6] and *O*-acetyl-D-Ser[8]. Other analogs in which this position has been substituted with β-chloro-D-Ala[7], D-Abu[7], or D-Lys[9] are also active immunosuppressants. More recently, it has been reported that the D-Ala[8] residue is not involved in cyclophilin binding[10,11] and thus modified D-Ala[8] residues could be positioned on or near a putative effector surface of the cyclophilin-CyA complex[12].

The availability of [2-deutero-3-fluoro-D-Ala[8]]CyA (1)[13] provided a potentially useful intermediate for the preparation of residue 8 modified cyclosporins. We were intrigued by the report that a stable polyanion of CyA could be generated at low temperature and alkylated to produce [Sar[3]]CyA analogs without appreciable epimerizations or other destructive alterations of the cyclosporin molecule[14,15]. We were also aware that fluorine is readily β-eliminated from fluoro-D-alanine in the α anionic species which is formed when it inactivates alanine racemase[16]. In fact, when **1** was treated with a large excess of lithium diisopropylamide (LDA) under polyanion forming conditions a dehydroalanine analog [Δ-Ala[8]]CyA (**2**) was formed in moderate yield. It was possible to add thiol-containing compounds to the dehydroalanine residue at position 8 and the characterization of these new cyclosporin analogs is reported below.

Chemistry

The preparation of residue-3 substituted cyclosporins has been achieved by the reaction of a variety of electrophiles with a polyanionic cyclosporin formed in anhydrous THF at −78°C[14,15]. A large excess of LDA (11 to 16 equivalents) was used to ionize the various active hydrogen atoms in this molecule and to form a highly reactive carbanion on the alpha carbon atom of Sar[3]. To eliminate HF from **1** we

Fig. 1. Structures of cyclosporins modified at the D-Ala[8] position.



employed 21 equivalents of LDA at $-78°C$ in THF for 30 minutes and allowed the temperature to rise slowly to $-30°C$ over 4 hours whereupon the reaction mixture was again cooled to $-78°C$ and quenched with aqueous HOAc. Conversion to **2** went remarkably well under these conditions. The isolated yield was reproducibly about 35%, and allowing for recovered starting material, the conversion was about 55%.

[Δ-Ala^8]CyA (**2**) is a substituted acrylamide to which we were able to add thiol compounds in a Michael reaction in modest yields to afford the products shown in Fig. 1. From this reaction we isolated in each case only one diastereomeric product. Inspection of CPK models of an enolate anion intermediate built using the NMR derived solution conformation[17,18] of CyA suggested that the least hindered orthogonal protonation of this anion would generate D-stereochemistry. This possibility had to be rigorously established and for this purpose, we turned to NMR studies of the methanethiol adduct **3**.

Comparison of the ^{1}H and ^{13}C NMR assignments of CyA and **3**, showed remarkable similarity except those expected for the resonances of the modified residue at position 8 and minor changes of some of the neighboring L-Ala^7 and MeLeu^9 residues (see Table 1). The ^{13}C resonances of all other carbons were consistent within 0.2 ppm and the data strongly suggested similar conformations. All four N-H protons have similar chemical shifts in the two compounds respectively, implicating their hydrogen bonds NH---C=O to have remained intact, in particular the bifurcated hydrogen bond between the N-H of the Ala^8 residue and the MeLeu^6 and MeLeu^9 C=O's, postulated by KESSLER *et al.*[17,18] in CyA. Dreiding model building

Table 1. ^{1}H and ^{13}C NMR assignments of backbone resonances of CyA and **3** in C_6D_6[a].

Assignment[b]		CyA		**3**	
		^{1}H	^{13}C	^{1}H	^{13}C
NHCO	Abu^{2}	8.27 d (9.8)	173.7 s	8.42 d (9.6)	173.6 s
	Ala^{7}	7.95 d (7.1)	171.2 s	8.07 d (7.4)	171.6 s
	Ala^{8}	7.61 d (7.8)	174.2 s	7.64 d (8.1)	172.6 s
	Val^{5}	7.44 d (8.7)	174.2 s	7.46 d (8.8)	174.1 s
α-CH	ML^{9}	5.87 dd (4.2, ~10.6)	48.4 d	5.92 dd (5.1, 10.1)	48.9 d
	MB^{1}	5.75 d (7.6)	59.4 d	5.76 d (7.9)	59.3 d
	ML^{4}	5.59 dd (4.1, 11.6)	55.7 d	5.60 dd (4.0, 11.6)	55.6 d
	ML^{6}	5.38 dd (5.5, 10.4)	55.5 d	5.43 dd (5.5, 10.4)	55.3 d
	ML^{10}	5.33 dd (5.5, 8.5)	57.8 d	5.33 dd (5.2, 8.6)	57.7 d
	MV^{11}	5.25 d (11.1)	58.3 d	5.22 d (10.5)	58.5 d
	Abu^{2}	5.12 dt (9.6, 7.4)	49.0 d	5.10 dt (9.7, 7.2)	49.1 d
	Val^{5}	4.88 dd (8.7, 10.0)	55.5 d	4.90 dd (8.8, 10.0)	55.5 d
	Ala^{8}	4.83 dq (7.9, 6.8)	48.3 d	5.13 br dt (~6.9, ~7.2)	49.0 d
	Ala^{7}	4.82 dq (~7.2)	49.0 d	4.81 dq (~7.2)	49.0 d
	Sar^{3}	3.99 d (13.8), 2.17 d (13.8)	49.5 t	4.00 d (13.6), 2.20 d (13.8)	49.5 t
β-CH	MB^{1}	4.19 dt (7.4, 5.6)	74.4 d	4.18 m	74.3 d
	Ala^{7}	1.68 d (7.2)	16.1 q	1.64 d (7.3)	16.3 q
	Ala^{8}	1.06 d (6.6)	17.9 q	2.74 dd (7.4, 13.5), 2.57 dd (6.7, 13.5)	37.2 t
	SMe	—	—	1.74 s	16.5 q
CONMe	MB^{1}	3.72 s	33.8 q	3.68 s	33.5 q
	ML^{6}	3.22 s	31.6 q	3.22 s	31.6 q
	Sar^{3}	3.06 s	39.0 q	3.06 s	39.0 q
	MV^{11}	2.98 s	30.4 q	2.99 s	30.5 q
	ML^{9}	2.92 s	29.4 q	3.17 s	29.5 q
	ML^{10}	2.85 s	30.0 q	2.85 s	30.0 q
	ML^{4}	2.58 s	30.8 q	2.59 s	30.8 q

[a] ^{1}H and ^{13}C chemical shifts are given in ppm downfield of TMS at 400 and 100 MHz, respectively; coupling constants in parentheses are given in Hz.

[b] The residues are numbered (superscript) as in ref 5 and are abbreviated as follows: MB^{1} = [(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine (MeBmt); Abu^{2} = α-aminobutyric acid; Sar^{3} = sarcosine; ML^{4,6,9,10} = *N*-methylleucine (MeLeu); Val^{5} = valine; Ala^{7,8} = alanine; MV^{11} = *N*-methylvaline (MeVal).

moreover suggested that the *N*-methyl groups are sensitive indicators of diamagnetic anisotropy of the peptide carbonyl bonds. That their widely dispersed chemical shifts (δ 2.55~3.75) are almost identical in both compounds as well as the similar $^{3}J_{\alpha CHNH}$ values, are indicative of a rigid backbone and similar conformations. The slight upfield shift of the MeLeu^{9} *N*Me resonance can in large part be attributed to the substituent change at the Ala^{8} position.

The similar conformations of CyA and **3** were more critically demonstrated by comparison of pure absorptive mode 2D-NOE data in C_6D_6, recorded with identical experimental parameters, using mixing times of 0.35 and 0.5 second. The experiments were carried out in C_6D_6 as better dispersion, compared to $CDCl_3$, of signals in the methyl (δ 0.5~2.00) and α-CH regions (δ 4.4~5.7) was observed.

Materials and Methods

T Cell Proliferation Assay

The immunosuppressive activity of CyA analogs was evaluated as previously described[19,20]. T cells

were isolated by nylon wool column separation from spleen of C57Bl/6 mice. The cells were suspended at 10^6 cells/ml in RPMI-1640 culture medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum. The cell suspension was distributed into 96 well flat-bottom microculture plates (Costar, Cambridge, MA) at 200 μl/well. Various concentrations of the CyA analogs or of CyA, used as a reference, were added in triplicates to the wells. Control wells received medium only. The cultures were then stimulated with ionomycin (250 ng/ml) + PMA (10 ng/ml) and incubated at 37°C in a humidified atmosphere of 5% CO_2 - 95% air for 44 hours. The proliferation of T lymphocytes was assessed by measurement of tritiated thymidine incorporation. After 44 hours of culturing, the cells were pulse-labeled with 2 μCi/well of tritiated thymidine (NEN, Cambridge, MA). After another 4 hours of incubation, cultures were harvested on glass fiber filters using a multiple sample harvester. Radioactivity of filter discs corresponding to individual wells was measured by standard liquid scintillation counting methods. Mean counts per minute of replicate wells were calculated and the results expressed as percent inhibition of tritiated thymidine uptake (proliferation) as follows:

$$\%\ \text{Inhibition} = 100 - \frac{\text{cpm cells treated with compound}}{\text{cpm cells in media only}} \times 100$$

The IC_{50} for the test compound was determined and expressed as percent of the IC_{50} of the control CyA.

In Vivo T Cell Activation

Female C57BL/6NTacfBR mice were purchased from Taconic Farms (Germantown, NY) and housed in a sterile, pathogen-free environment. *In vivo* T cell activation was achieved with an intravenous injection of Con A (400 μg/animal; ICN Biologicals, Lisle, IL). One hour after the Con A injection, spleens were removed and single cell suspensions prepared. Red blood cells were lysed with ACK lysing solution (Gibco, Grand Island, NY) and dead cells removed with high phosphate buffered saline. Spleen cells were washed in L-15, 5% fetal calf serum, 10 mM HEPES, and α-methyl-D-mannopyranoside (2 mg/ml) and cultured in RPMI-1640, 10% fetal calf serum, 10 mM HEPES, nonessential amino acids, sodium pyruvate, L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. Cells were cultured in flat-bottom microtiter plates at 5×10^5 per well ($n = 12$ wells per condition) for 24 hours at 37°C in 7% CO_2 in the presence of 2 μCi of [^{3}H]thymidine. Cell activation was assessed by the incorporation of [^{3}H]thymidine into new DNA and measured by liquid scintillation counting.

CyA and CyA analogs were administered intravenously 30 minutes prior to the injection of Con A. Compounds were solubilized in ethanol, diluted in Cremophor, and further diluted 50-fold in sterile phosphate buffered saline. Inhibition of T cell proliferation was plotted with four-parameter curve fitting and, where applicable, ED_{50} values calculated. Under these conditions, CyA produces ED_{50} values from 5 to 10 mg/kg.

General Chemical Methods

FAB-MS data were obtained using a MAT731 mass spectrometer at 8 Kv in the FAB mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian XL-300 and XL-400 spectrometers (TMS standards). ^{1}H and ^{13}C NMR assignments in C_6D_6 and/or $CDCl_3$ were made by comparison with those reported for cyclosporin A by KESSLER *et al.*[17,18] and on the basis of ^{1}H-^{1}H COSY and RELAY experiments. Pure-absorptive mode 2D-NOESY spectra were accumulated in C_6D_6 using the standard pulse sequence with phase-sensitive detection[21]. Mixing times were 0.35 and 0.5 second and the delay times between scans were 2.65 and 2.5 seconds, respectively. The NOE's are designated strong (s), medium (m) or weak (w). Preparative and analytical HPLC separations were performed using DuPont Zorbax ODS columns maintained at 60°C with an LDC Spectromonitor III detector operated at 210 nm and with a Spectra-Physics SP 4100 computing integrator. Preparative TLC separations were achieved using 20 × 20 cm Analtech silica gel GF plates (0.5 mm thick).

[DehydroAla^8]CyA (2)

LDA in cyclohexane (0.6 ml, 0.9 mmol) was added to 2 ml THF stirred at −78°C under N_2. To this solution was added [2-deutero-3-fluoro-D-Ala^8]CyA (1) (50 mg, 0.042 mmol) in 0.1 ml THF. The mixture

was stirred at $-78°C$ for 30 minutes and the temperature was slowly raised to $-30°C$ over 4 hours. The mixture was then cooled to $-78°C$, quenched by adding HOAc (0.15 ml) in H_2O (0.9 ml) and added to saturated aq NaCl (20 ml) containing $NaHSO_4$ (0.2 g). The reaction products were extracted into EtOAc (3×20 ml). The latter extract was washed with saturated aq NaCl (2×20 ml), dried over Na_2SO_4 and taken to dryness under vacuum. The residue (47 mg) was purified by preparative TLC ($CHCl_3$ - EtOH, 96:4) using two developments to afford two major bands. From the more polar band was obtained **2** (17 mg, 34%) as a colorless solid; Rt 14 minutes on analytical HPLC (CH_3CN - H_2O, 80:20); FAB-MS *m/z* 1,200 $(M+H)^+$ consistent with molecular formula $C_{62}H_{109}N_{11}O_{12}$; ^1H NMR ($CDCl_3$) δ 0.69 (3H, d, J=6.5 Hz), 0.80 (3H, d, J=6.6 Hz), 0.92 (3H, d, J=6.7 Hz), 0.96 (3H, d, J= ~6.5 Hz), 0.98 (3H, d, J= ~6.5 Hz), 1.00 (3H, d, J=6.5 Hz), 1.01 (3H, d, J=6.3 Hz), 1.02 (3H, d, J= ~6.5 Hz), 1.34 (3H, d, J=7.3 Hz), 2.69 (s, NMe), 2.76 (s, NMe), 3.07 (s, NMe), 3.20 (s, NMe), 3.21 (s, NMe), 3.36 (s, NMe), 3.47 (s, NMe), 3.76 (t, J=6.5 Hz, $MB^1\beta H$), 4.42 (dq, J=7.0 Hz, $Ala^7\alpha H$), 4.65 (dd, J=8.5 and 9.5 Hz, $Val^5\alpha H$), 4.71 (d, J=14.2 Hz, $Sar^3\alpha H$), 4.97 (br s, $Ala^8\beta H$), 5.16 (d, J=10.6 Hz, $MV^{11}\alpha H$), 5.43 (d, J=6.0 Hz, $MB^1\alpha H$), 5.61 (dd, J=4.3 and 11.0 Hz, $ML^9\alpha H$), 5.73 (br s, $Ala^8\beta H$), 7.42 (d, J=8.6 Hz, Val^5NH), 7.64 (d, J=6.6 Hz, Ala^7NH), 8.03 (d, J=9.5 Hz, Abu^2NH), 8.28 (s, Ala^8NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 9.9 q, 15.8 q, 16.6 q, 17.9 q, 18.6 q, 19.5 q, 20.2 q, 21.2 q, 21.8 q, 22.0 q, 23.1 q, 23.4 q, 23.7 q ($2\times$), 23.9 q, 24.6 d, 24.7 d, 24.9 d, 25.0 d, 25.2 t, 29.0 q, 30.1 q, 30.4 q, 31.1 q, 31.2 q, 31.3 d, 32.5 q, 33.9 q, 35.6 t, 35.9 d, 36.0 t, 37.1 t, 39.2 q, 39.3 t, 40.8 t, 48.9 d, 49.2 d, 49.3 d, 50.2 t, 54.9 d, 55.2 d, 55.5 d, 57.5 d, 57.8 d, 58.3 d, 74.7 d, 108.3 t ($Ala^8\beta C$), 126.3 d, 129.5 d, 134.8 s ($Ala^8\alpha C$), 167.6 s (Ala^8CON), 170.0 s, 170.1 s, 170.3 s, 170.5 s, 170.8 s, 171.1 s, 171.9 s, 173.4 s, 173.50 s and 173.53 s. From the less polar band there was obtained 19 mg of recovered starting material **1**. The conversion yield to **2** was 55%.

[3-Methylthio-D-Ala⁸]CyA (3)

To a stirred solution of [Δ-Ala⁸]CyA (**2**) (45 mg, 0.037 mmol) in methanol (1.0 ml) was added sodium methylsulfide (60 mg) in methanol (1.5 ml). The mixture was kept 18 hours at 20°C. It was then added to 20 ml of saturated aq NaCl containing $NaHSO_4$ (0.3 g) and the mixture was extracted with EtOAc (4×15 ml). The organic extract was washed with saturated aq NaCl (2×15 ml), dried over Na_2SO_4 and concentrated to dryness under vacuum. The residue (38 mg) was purified by HPLC (CH_3CN - H_2O, 70:30, Rt 20.5 minutes) to afford **3** (12 mg, 26%); FAB-MS *m/z* 1,248 $(M+H)^+$ consistent with molecular formula $C_{63}H_{113}N_{11}O_{12}S$; ^1H NMR (C_6D_6) see Table 1; ^{13}C NMR (100 MHz, C_6D_6) δ 10.1 q ($Abu^2\gamma C$), 16.0 q ($Ala^7\beta C$), 16.3 q (Ala^8SMe), 17.9 q ($MB^1\delta C$), 18.1 q ($MB^1\eta C$), 18.6 q ($Val^5\gamma C$), 18.8 q ($MV^{11}\delta C$), 20.0 q ($Val^5\gamma C$), 20.0 q ($MV^{11}\gamma C$), 21.4 q ($ML^6\delta C$), 22.0 q ($ML^{10}\delta C$), 22.5 q ($ML^4\delta C$), 23.6 q ($ML^6\delta C$), 23.6 q ($ML^4\delta C$), 23.8 q ($ML^{10}\delta C$), 24.2 q ($ML^9\delta C$), 24.5 q ($ML^9\delta C$), 24.7 d ($ML^9\gamma C$), 25.2 d ($ML^{10}\gamma C$), 25.3 d ($ML^4\gamma C$), 25.6 t ($Abu^2\beta C$), 25.7 d ($ML^6\gamma C$), 29.5 q (ML^9NMe), 29.8 q ($MV^{11}NMe$), 30.0 q ($ML^{10}NMe$), 30.5 q ($MV^{11}NMe$), 30.8 q (ML^4NMe), 31.5 d ($Val^5\beta C$), 31.6 q (ML^6NMe), 33.5 q (MB^1NMe), 35.3 t ($MB^1\delta C$), 35.6 d ($MB^1\gamma C$), 36.5 t ($ML^4\beta C$), 37.2 t ($Ala^8\beta C$), 37.8 t ($ML^6\beta C$), 39.0 q (Sar^3NMe), 40.2 t (ML^9NMe), 41.4 t ($ML^{10}\beta C$), 48.9 d ($ML^9\alpha C$), 49.0 d ($Ala^8\alpha C$), 49.0 d ($Ala^7\alpha C$), 49.1 d ($Abu^2\alpha C$), 49.5 t ($Sar^3\alpha C$), 55.3 d ($ML^6\alpha C$), 55.5 d ($Val^5\alpha C$), 55.6 d ($ML^4\alpha C$), 57.7 d ($ML^{10}\alpha C$), 58.5 d ($MV^{11}\alpha C$), 59.3 d ($MB^1\alpha C$), 74.3 d ($MB^1\beta C$), 126.2 d ($MB^1\zeta C$), 130.8 d ($MB^1\eta C$), 169.6 s (ML^4CON), 170.0 s (MB^1CON), 170.5 s (ML^9CON), 170.5 s ($ML^{10}CON$), 171.0 s (Sar^3CON), 171.6 s (Ala^7CON), 171.8 s (ML^6CON), 172.6 s (Ala^8CON), 173.6 s (Abu^2CON), 174.0 s ($MV^{11}CON$), 174.1 s (Val^5CON).

[3-(Methoxycarbonylmethylthio)-D-Ala⁸]CyA (7)

A solution of methyl mercaptoacetate (25 mg, 0.24 mmol) in methanol (0.5 ml) was added to NaOMe (13 mg, 0.24 mmol) and the mixture was added to a solution of **2** (11 mg, 0.01 mmol) in methanol (0.5 ml). After 18 hours at 20°C work up as for compound **3** gave 15 mg of crude **7**. It was purified by HPLC (CH_3OH - H_2O, 85:15, Rt 16.9 minutes); FAB-MS *m/z* 1,306 $(M+H)^+$ consistent with molecular formula $C_{65}H_{115}N_{11}O_{14}S$.

[3-(2-Hydroxyethylthio)-D-Ala⁸]CyA (5)

A solution of 2-mercaptoethanol (21 mg, 0.27 mmol) in THF (0.5 ml) was added to NaOMe (10 mg, 0.18 mmol). To the stirred mixture was added **2** (18 mg, 0.015 mmol) in THF (0.8 ml). After 18 hours at 20°C work up as for compound **3** gave 26 mg of crude **5** which was purified by HPLC (CH_3CN - H_2O,

75:25, Rt 11.5 minutes); FAB-MS *m/z* 1,278 $(M+H)^+$ consistent with moleculor formula $C_{64}H_{115}N_{11}O_{13}S$; ^{1}H NMR ($C_6D_6$) δ 0.64 (3H, d, J=6.4 Hz), 0.85 (3H, t, J=7.2 Hz), 0.95 (6H, d, J=6.4 Hz), 0.99 (3H, d, J=6.5 Hz), 1.07 (3H, d, J=6.5 Hz), 1.66 (3H, d, J=7.2 Hz), 2.15 (d, J=13.9 Hz, Sar$^3\alpha$H), 2.56 (s, ML^{4}NMe), 2.84 (s, ML^{10}NMe), 2.98 (s, MV^{11}NMe), 3.04 (s, Sar^{3}NMe), 3.07 (s, ML^{9}NMe), 3.20 (s, ML^{6}NMe), 3.71 (s, MB^{1}NMe), 3.97 (d, J=13.8 Hz, Sar$^3\alpha$H), 4.15 (m, MB$^1\beta$H), 4.85 (dq, J= ~7.3 Hz, Ala$^7\alpha$H), 4.85 (dd, J=8.7 and 10.0 Hz, Val$^5\alpha$H), 5.10 (dt, J=9.7 and 7.4 Hz, Abu$^2\alpha$H), 5.15 (dt, J=5.8 and ~7.7 Hz, Ala$^8\alpha$H), 5.32 (dd, J=5.2 and 8.6 Hz, ML$^{10}\alpha$H), 5.37 (dd, J=5.5 and 10.3 Hz, ML$^6\alpha$H), 5.56 (dd, J=7.3 and ~12.1 Hz, ML$^4\alpha$H), 5.76 (d, J=7.5 Hz, MB$^1\alpha$H), 5.89 (dd, J=4.6 and 10.1 Hz, ML$^9\alpha$H), 7.46 (d, J=8.8 Hz, Val^{5}NH), 7.68 (d, J=8.0 Hz, Ala^{8}NH), 8.17 (d, J=7.3 Hz, Ala^{7}NH), 8.31 (d, J=9.6 Hz, Abu^{2}NH). ^{13}C NMR (75 MHz, C_6D_6) δ 10.1 q, 16.0 q, 17.8 q, 18.1 q, 18.5 q, 20.0 q, 20.1 q, 21.4 q, 21.9 q, 22.3 q, 23.6 q (2×), 23.8 q, 24.2 q, 24.5 q, 24.8 d, 25.2 d, 25.3 d, 25.5 t, 25.8 d, 29.5 q, 29.7 d, 30.0 q, 30.4 q, 30.8 q, 31.6 d, 31.6 q, 33.8 q, 34.9 t (CH_2S), 35.6 t (Ala$^8\beta$C), 35.9 d, 36.5 t (2×), 37.8 t, 39.0 q, 39.9 t, 41.5 t, 48.9 d, 49.0 d, 49.2 d, 49.5 t, 50.0 d (Ala$^8\alpha$C), 55.5 d, 55.6 d, 55.7 d, 57.8 d, 58.3 d, 59.4 d, 62.1 t (CH_2OH), 74.5 d, 126.3 d, 130.7 d, 169.6 s, 170.1 s, 170.3 s, 170.4 s, 171.2 s, 171.6 s, 172.2 s, 172.3 s, 173.8 s, 174.1 s, 174.3 s.

[3-(Methylsulfinyl)-D-Ala^8]CyA (**4**)

To a stirred solution of **3** (12.8 mg) in MeOH (3 ml) was added a solution of sodium periodate (15 mg) in water (1.5 ml). The reaction mixture was stirred at room temperature for 3 hours and evaporated *in vacuo* to remove methanol. EtOAc was added and the organic phase was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The residue was purified by preparative TLC eluting with 5% methanol in CH_2Cl_2 to afford the sulfoxide (12.0 mg, 93%). FAB-MS *m/z* 1,264 $(M+H)^+$ consistent with molecular formula $C_{63}H_{113}N_{11}O_{13}S$. Duplicate signals in the ^{1}H and ^{13}C NMR spectra are observed due to (R) and (S) sulfinyl stereoisomeric forms; ^{1}H NMR (C_6D_6) δ 0.66/0.68 (d, J= ~6.9 and ~6.9 Hz, MV^{11}Me), 1.07/1.08 (d, J=6.5 and 6.4 Hz, ML^{6}Me), 1.51/1.63 (d, J=7.2 and 7.3 Hz, Ala^{7}Me), 1.73/1.78 (s, SMe), 2.22/2.23 (d, J=13.8 and ~14.0, Sar$^3\alpha$H), 2.60/2.61 (s, ML^{4}NMe), 2.83/2.86 (s, ML^{10}NMe), 2.98/2.985 (s, MV^{11}NMe), 3.07/3.09 (s, Sar^{3}NMe), 3.20/3.22 (s, ML^{6}NMe), 3.29/3.48 (s, ML^{9}NMe), 3.62/3.66 (s, MB^{1}NMe), ~4.01 (2H, d, J=13.8 Hz, Sar$^3\alpha$H), 4.59/4.66 (dq, J= ~7 and ~7 Hz, Ala$^7\alpha$H), ~4.89 (2H, dd, J=9.0 and 10.0 Hz, Val$^5\alpha$H), 5.18/5.19 (d, J=11.0 and 11.0 Hz, MV$^{11}\alpha$H), 5.69/5.74 (d, J=8.9 and 8.3, MB$^1\alpha$H), 5.91/5.92 (dd, J= ~5.4, ~8.3 and ~5.4, ~8.3 Hz, ML$^9\alpha$H), 7.48/7.49 (d, J=8.9 and 8.8 Hz, Val^{5}NH), 7.74/8.01 (d, J=8.0 and 6.8 Hz, Ala^{8}NH), 8.14/8.17 (d, J=7.1 and 8.3 Hz, Ala^{7}NH), 8.43/8.46 (d, J=10.0 and 9.8 Hz, Abu^{2}NH). ^{13}C NMR (100 MHz, C_6D_6) δ 10.1 q (2×), 15.7/15.9 q, 17.77/17.87 q, 18.1 q (2×), 18.5 q (2×), 18.82/18.88 q, 19.9/20.1 q, 19.98 q (2×), 21.4 q (2×), 21.96/22.04 q, 22.5/22.8 q, 23.57/23.62 q, 23.73/23.77 q, 24.3 q (2×), 24.5/24.6 q, 24.7/25.2 d, 25.0 d (2×), 25.3 d (2×), 25.3/25.7 d, 25.7/25.9 t, 29.4/29.6 q, 29.9/30.1 q, 30.1/30.2 q, 30.6/30.7 q, 30.8 q (2×), 31.4/31.5 d, 31.51/31.56 q, 33.0/33.3 q, 35.1/35.2 t, 35.4/35.7 d, 36.42/36.45 t, 37.4/37.6 t, 38.6/39.0 q (CH_3SO), 38.86/38.94 q, 40.1/40.4 t, 41.3/41.4 t, 44.9/45.8 d (Ala$^8\alpha$C), 48.9/49.5 d, 49.0 d (2×), 49.0 t (2×), 49.4 d (2×), 55.1 d (2×), 55.4 d (2×), 55.6 d (2×), 57.1/57.4 t (Ala$^8\beta$C), 57.6 d (2×), 58.6/58.7 d, 59.2/59.3 d, 73.6/74.0 d, 126.18/126.22 d, 130.9 d (2×), 169.7/169.8 s, 169.83 s, (2×), 170.3/170.35 s, 170.3/170.6 s, 170.9/171.0 s, 171.3/171.9 s, 171.69/171.72 s, 172.03/172.07 s, 173.46/173.51 s, 173.5/174.0 s, 173.9/174.1 s.

[3-(2-Hydroxyethylsulfinyl)-D-Ala^8]CyA (**6**)

This compound was prepared from **5** according to the procedure used to synthesize **4**. The residue (25 mg) was purified by HPLC on a DuPont Zorbax ODS (0.94×25 cm) column at 60°C, using a solvent system of 75:25 CH_3CN-H_2O at a flow rate of 2.65 ml/minute. The effluent was monitored at 210 nm collecting fifteen fractions. Fraction seven, Rt 10.9 minutes, yielded 5.3 mg of **6**. FAB-MS *m/z* 1,294 $(M+H)^+$ consistent with molecular formula $C_{64}H_{115}N_{11}O_{14}S$. Duplicate signals in the ^{1}H and ^{13}C NMR spectra are observed due to (R) and (S) sulfinyl stereoisomeric forms. ^{1}H NMR (C_6D_6) δ 0.65/0.66 (d, J=6.5 and 6.5 Hz, MV^{11}Me), 1.06/1.08 (d, J= ~6.2 and ~6.2 Hz, ML^{6}Me), 1.55/1.65 (d, J=7.2 and 7.3 Hz, Ala^{7}Me), 2.17/2.19 (d, J=13.8 and 13.8 Hz, Sar$^3\alpha$H), 2.57/2.59 (s, ML^{4}NMe), 2.83/2.84 (s, ML^{10}NMe), 2.96/2.98 (s, MV^{11}NMe), 3.04/3.07 (s, Sar^{3}NMe), 3.176/3.181 (s, ML^{6}NMe), 3.23/3.32 (s, ML^{9}NMe), 3.64/3.68 (s, MB^{1}NMe), 3.97/3.98 (d, J=13.8 and 13.8 Hz, Sar$^3\alpha$H), 4.62/4.67 (dq, J= ~7.1 and ~7.0 Hz, Ala$^7\alpha$H), ~5.10 (2H, dt, J=9.6 and ~7.1 Hz, Abu$^2\alpha$H), 5.19/5.23 (d, J=11.0 and 11.0 Hz,

MV[11]αH), 5.30/5.33 (dd, J=6.0, 8.5 and 4.7, 8.3 Hz, ML[10]αH), 5.42/5.46 (dd, J=5.3, 10.2 and 5.8, 10.7 Hz, ML[6]αH), 5.70/5.74 (d, J=8.4 and 7.7 Hz, MB[1]αH), 7.480/7.485 (d, J=8.7 and 8.8 Hz, Val[5]NH), 7.69/7.98 (d, J=7.7 and 8.3 Hz, Ala[8]αH), 8.12/8.15 (d, J=7.1 and 7.0 Hz, Ala[7]NH), 8.26/8.40 (d, 9.9 and 8.4 Hz, Abu[2]NH).

Results and Discussion

KESSLER *et al.*[17,18] have studied the solution structure of CyA by ^{1}H NMR at 300 MHz in $CDCl_3$ and C_6D_6 which they recently confirmed by more extensive 2D-NOE work at 600 MHz and with molecular dynamics calculations[22]. With the exception of some flexibility in the Abu[2] and MeBmt[1] side chains, their results indicate a fairly rigid backbone and fixed conformations of most side chains. The backbone conformation deviates only slightly from that in the crystal structure. Comparison of the ^{1}H and ^{13}C chemical shift and ^{1}H-^{1}H coupling constant data of CyA and the methanethiol adduct **3** (see Table 1), strongly suggested a rigid backbone and similar conformations for the two compounds. Moreover, the pattern of positive NOE cross peaks of the contour plots and corresponding intensities from the pure absorptive mode 2D-NOE experiments were almost identical for the two compounds. The only *cis*-peptide bond between MeLeu[9] and MeLeu[10] is preserved in **3** as evidenced by the strong NOE between the respective α-protons, as well as the *trans*-conformation of all other amide bonds as indicated by the NOE's involving the *N*-methyl (strong) or N-H protons (weak to medium) and the α-proton of the preceding residue. In particular, the strong NOE from the α hydrogen of D-Ala[8] to the *N*-methyl of MeLeu[9] provided corroboration for the D-configuration of Ala[8] in CyA, which was established on the basis of total synthesis[23] and X-ray crystallography[18]. The comparable strong NOE in **3**, other NOE's involving Ala[8] protons of both CyA and **3** [Ala[8]αH - Ala[8]βH (s), Ala[8]αH - Ala[8]SMe (w), Ala[8]NH - Ala[7]αH (w), Ala[8]βH (w)], as well as the almost identical $^{3}J_{\alpha H,NH}$ values (Table 1), therefore strongly supports the D-configuration of the MeSAla[8] residue in **3**. By extension we have assigned a D-configuration to the other biologically active alkylthiol adducts described in this paper.

The ability of our new 8-position modified cyclosporins to inhibit lymphocyte proliferation is summarized in Table 2. The dehydro analog **2** retained modest activity as did the ester **7**, however, the sulfoxides **4** and **6** have little or no activity at the dose level tested. In contrast, analogs substituted by fluorine (**1**), methanethiol (**3**) and β-hydroxyethanethiol groups (**5**) were nearly as active as CyA itself.

Compounds **3** and **4** were tested in the *in vivo* T cell activation assay described in the Materials and Methods section. CyA typically produces ED_{50} values ranging from 5 to 10 mg/kg. Compound **3** had an ED_{50} of 9.6 mg/kg, which is similar to CyA itself. Compound **4**, however, demonstrated very little *in vivo* activity (a maximum of 28% inhibition at 28 mg/kg).

In summary several novel biologically active cyclosporin analogs have been prepared from a

Table 2. Inhibition of T cell proliferation *in vitro* by CyA analogs.

Compound	Suppressive activity[a,b] (% of CyA)
1	80.7
2	30
3	85.4
4	5.6
5	80.8
6	N.A.[c]
7	32.0

[a] Assay procedure described in Materials and Methods.
[b] CyA control was run in parallel within the same experiment. The relative suppressive activity was calculated from the ratio of IC_{50} between CyA and the test compound. Each value is the mean from 2~4 independent experiments.
[c] No activity when tested up to 2 mg/ml.

position 8 dehydroalanine analog of CyA. The *in vivo* properties of the methylthio analog **3** are being investigated further.

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References

1) BOREL, J. F.: Pharmacology of cyclosporine (Sandimmune) IV. Pharmacological properties *in vivo*. Pharmacol. Rev. 41: 259~371, 1989
2) MASON, J.: Pharmacology of cyclosporine (Sandimmune) VII. Pathophysiology and toxicology of cyclosporine in humans and animals. Pharmacol. Rev. 41: 423~434, 1989
3) WENGER, R. M.: Pharmacology of cyclosporine (Sandimmune) II. Chemistry. Pharmacol. Rev. 41: 243~247, 1989
4) WENGER, R. M.: Synthesis of cyclosporin and analogues: Structural and conformational requirements for immunosuppressive activity. Prog. Allergy 38: 46~64, 1986
5) WENGER, R. M.; T. G. PAYNE & M. H. SCHREIER: Cyclosporine: Chemistry, structure-activity relationships and mode of action. *In* Progress in Clinical Biochemistry. Vol. 3, pp. 157~191, Springer-Verlag, 1986
6) TRABER, R.; H. HOFMANN & H. KOBEL: Cyclosporins—new analogues by precursor directed biosynthesis. J. Antibiotics 42: 591~597, 1989
7) LAWEN, A.; R. TRABER, D. GEYL, R. ZOCHER & H. KLEINKAUF: Cell-free biosynthesis of new cyclosporins. J. Antibiotics 42: 1283~1289, 1989
8) WENGER, R.; R. P. TRABER, H. KOBEL & H. HOFMANN (Sandoz): Novel cyclosporins. U.S. 4,639,434, Jan. 27, 1987
9) WENGER, R.: Cyclosporine and analogues: Structural requirements for immunosuppressive activity. Transplant. Proc. 28 (Suppl. 5): 213~218, 1986
10) QUESNIAUX, V. F. J.; M. H. SCHREIER, R. M. WENGER, P. C. HIESTAND, M. W. HARDING & M. H. V. VAN REGENMORTEL: Molecular characteristics of cyclophilin-cyclosporine interactions. Transplantation 46: 235~275, 1988
11) FESIK, S. W.; R. T. GAMPE, Jr., T. F. HOLZMAN, D. A. EGAN, R. EDALJI, J. R. LULY, R. HELFRICH, V. KISHORE & D. H. RICH: Isotope-edited NMR of cyclosporin A bound to cyclophilin: evidence for a trans 9,10 amide bond. Science 250: 1406~1409, 1990
12) SCHREIBER, S. L.: Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 251: 283~287, 1991
13) HENSENS, O. D.; R. F. WHITE, R. T. GOEGELMAN, E. S. INAMINE & A. A. PATCHETT: The preparation of [2-deutero-3-fluoro-D-Ala^8]cyclosporin A by directed biosynthesis. J. Antibiotics 45: 133~135, 1992
14) SEEBACH, D. (Sandoz): Novel cyclosporins. U.S. 4,703,033, Oct. 27, 1987
15) SEEBACH, D.; W. MURTIASHAW, R. NAEF, S. I. SHODA, M. KRIEGER, P. BOLLINGER, A. LEUTWILER & R. M. WENGER: Aktive cyclosporinderivate durch C-alkylierung unter ersatz von H-re der sarkosin-einheit. Erzeugung polylithiierter peptide. Presented at the Swiss Chem. Soc. Mtg., Berne, Oct. 18, 1985 [Cited by WENGER, R. M.: Pharmacology of cyclosporin (Sandimmune) II. Chemistry. Pharmacol. Rev. 41: 243~247, 1989]
16) WALSH, C.: Suicide substrates: mechanism-based enzyme inactivators. Tetrahedron 38: 871~909, 1982
17) KESSLER, H.; H.-R. LOOSLI & H. OSCHKINAT: Assignment of the ^{1}H-, ^{13}C-, and ^{15}N-NMR spectra of cyclosporin A in $CDCl_3$ and C_6D_6 by a combination of homo- and heteronuclear two-dimensional techniques. Helv. Chim. Acta 68: 661~681, 1985
18) LOOSLI, H. R.; H. KESSLER, H. OSCHKINAT, H.-P. WEBER, T. J. PETCHER & A. WIDMER: The conformation of 'cyclosporin A' in the crystal and in solution. Helv. Chim. Acta 68: 682~703, 1985
19) DURETTE, P. L.; J. BOGER, F. DUMONT, R. FIRESTONE, R. A. FRANKSHUN, S. L. KOPRAK, C. S. LIN, M. R. MELINO, A. A. PESSOLANO, J. PISANO, J. A. SCHMIDT, N. H. SIGAL, M. J. STARUCH & B. E. WITZEL: A study of the correlation between cyclophilin binding and *in vitro* immunosuppressive activity of cyclosporine A and analogues. Transplant. Proc. 20 (Suppl. 2): 51~57, 1988
20) DUMONT, F. J.; M. J. STARUCH, S. L. KOPRAK, M. R. MELINO & N. H. SIGAL: Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. J. Immunol. 144: 251~259, 1990
21) STATES, D. J.; R. A. HABERKORN & D. J. REUBEN: A two dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. J. Magn. Reson. 48: 286~293, 1982

22) KESSLER, H.; M. GERHKE, J. LAUTZ, M. KOCK, D. SEEBACH & A. THALER: Complexation and medium effects on the conformation of cyclosporin A studied by NMR spectroscopy and molecular dynamics calculations. *In* NMR Methods for Elucidating Macromolecule-Ligand Interactions: An Approach to Drug Design. *Eds.*, R. E. SCHUMACHER & I. M. ARMITAGE, pp. 169 ~ 173, Pergamon Press, 1990

23) WENGER, R. M.: Total synthesis of cyclosporin A and cyclosporin H, two fungal metabolites isolated from the species *Tolypocladium inflatum* Gams. Helv. Chim. Acta 67: 502 ~ 525, 1984