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FR235222, a Fungal Metabolite, is a Novel Immunosuppressant that Inhibits Mammalian Histone Deacetylase

III. Structure Determination

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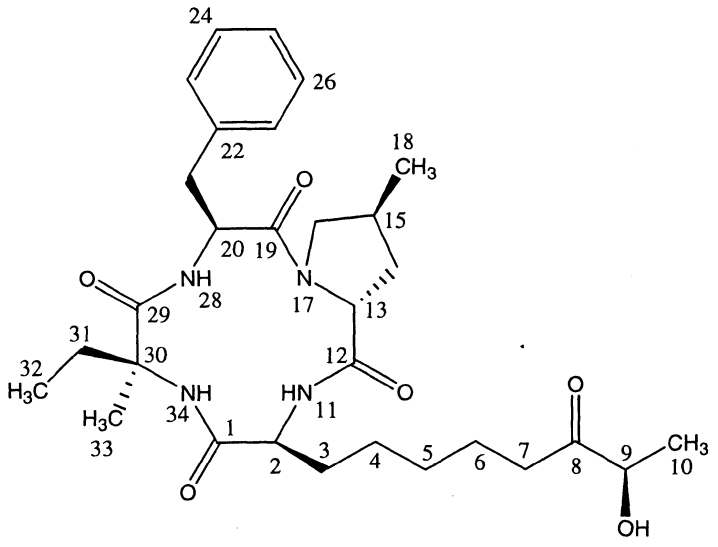
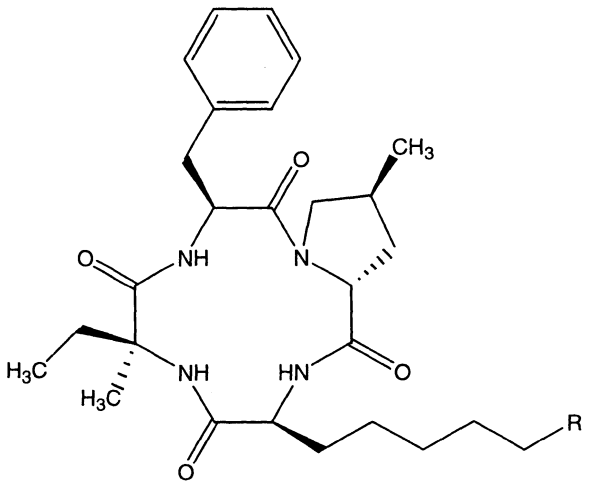
Cyclosporin A and tacrolimus dramatically changed the field of clinical transplantations, and they are also prescribed or currently developed for various autoimmune diseases. They suppress acute allograft rejection effectively, but chronic allograft rejection is a matter of issue on transplantations even when treated with these drugs[1], and their dosages are restricted because of their toxicities at high doses. Thus, different effects or safer properties are desirable for the next generation of immunosuppressant drugs. Taking the fact that cyclosporin A and tacrolimus share its mechanism as calcineurin inhibitors into consideration, we screened inhibitors of T-cell activation from microbial products to seek for new immunosuppressants with different mechanism of action and found a potent HDAC (histone deacetylase) inhibitor, FR235222 (**1**, Fig. 1). The isolation and biological properties of **1** are reported in the preceding papers[2,3]. Herein, structure determination of FR235222 including its absolute stereochemistry will be presented.

FR235222 (**1**) was isolated as white wax exhibiting melting point of 52~57°C and specific rotation of −129° (*c* 0.50, $CHCl_3$, 22°C). ESI-MS of **1** detected a protonated molecular ion at *m/z* 557. Analysis of HRESI-MS, ^{13}C NMR and HSQC established its molecular formula to be $C_{30}H_{44}N_4O_6$ (calcd. 557.3339 for M+H, found 557.3326). IR spectrum (neat) indicated the presence of hydroxyl, ketone and amide groups from absorptions at 3300, 1715, 1680 and 1660 cm^{-1}. In the ^1H NMR spectrum (Fig. 2) were observed all 44 protons including four exchangeable ones. The ^{13}C NMR and HSQC spectra showed characteristic signals for a ketone, four amide carbonyl, a mono-substituted benzene, and nineteen aliphatic carbons. Carbons at 63.0(s), 58.0(d), 54.4(d) and 53.3(d) ppm were assigned to α-carbons of a proline analogue, a

Fig. 1. Structures of FR235222 (**1**) and its derivatives.



1 (FR235222)



2 (R=-COOH)
3 (R=-COCH(CH_3)O-(*S*)-MTPA)
4 (R=-COCH(CH_3)O-(*R*)-MTPA)

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Fig. 2. ^{1}H NMR spectrum of **2** (500 MHz, in $CDCl_3$).

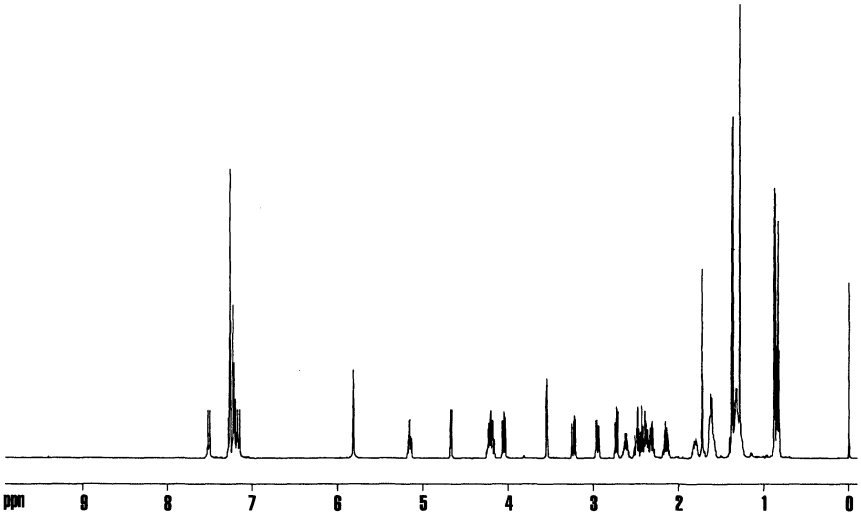
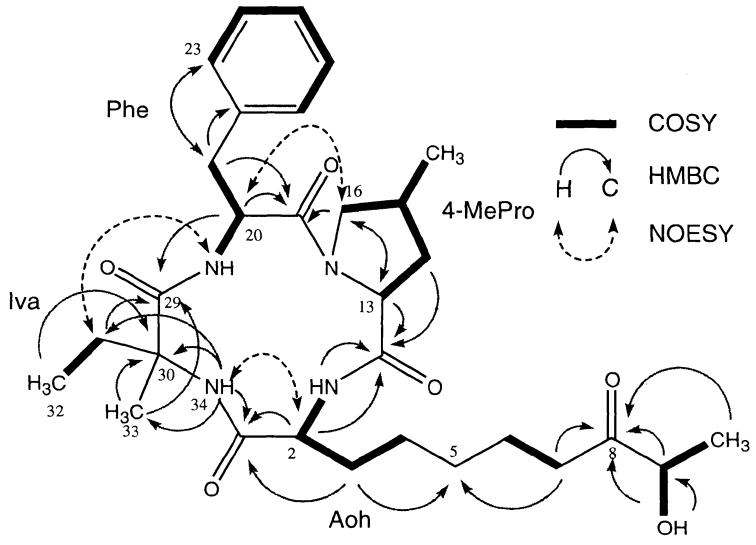


Fig. 3. NMR analysis of FR235222.



phenylalanine and two acyclic amino acids on the basis of NMR analysis (*vide infra*). The existence of a benzene, five other sp^2 carbons and the proline explain ten unsaturation degrees. Thus, FR235222 should be a macrocyclic compound to account for eleven unsaturation degrees required from its molecular formula. In the ^{1}H-^{15}N HSQC spectrum, three nitrogens observed at 119.6, 132.0 and 129.1 ppm respectively correlated with amide protons resonating at 7.52, 5.81 and 7.17 ppm.

COSY analysis clarified proton spin networks shown as bold lines in Fig. 3. Chemical shifts of C-16 (δ_C 53.8) and 16-H_2 (δ_H 4.05 and 2.73) indicate C-16 is adjacent to an amide nitrogen. HMBC correlations of 16-H_2/C-13 and 13-H/C-16 connected C-13 and C-16 *via* the nitrogen, unveiling 4-methylproline (4-MePro) structure. Isovaline (iva) featured by a quaternary sp^3 carbon (C-30) was elucidated by using HMBC data shown below. HMBC of 32-CH_3/C-30 showed a bond of C-30 and C-31, while C-30

was also attached to C-29 and C-33 by HMBCs from 33-CH_3 to C-29 and C-30. Then, HMBCs from 34-NH to C-30, C-31 and C-33 clarified a linkage of 34-NH and C-30 to complete iva structure. 2-Amino-8-oxo-9-hydroxydecanoic acid residue (aoh) was revealed as follows. Though ^1H NMR signals of 5-H_2 overlapped with those of 4-H_2 and 6-H_2, HMBCs from 3-H_2 and 7-H_2 to C-5 linked C-5 to C-4 and C-6. HMBC data from 7-H_2, 9-H and 10-CH_3 to C-8 concluded C-8 attached to C-7 and C-9, which led completion of C–C linkages from the α-carbon (C-2, δ_C 54.4) to the terminal methyl (10-CH_3). The oxygen functionality at C-9 (δ_C 72.6) was assumed to be hydroxyl due to observation of a COSY cross peak from an exchangeable proton (δ_H 3.56) to the oxymethine proton (δ_H 4.22). All amide carbons were unambiguously assigned with HMBC data from their β-protons and used to

Table 1. NMR assignments of FR235222 (**1**) and Mosher analysis.

	position	FR235222(**1**)				δ_H(**3**)-δ_H(**4**)
		δ_H($CDCl_3$)		δ_C($CDCl_3$)	δ_N($CDCl_3$)	
Aoh	1			174.1		
	2	4.20	(m)	54.4		-0.01
	3	1.82	(m)	28.8		-0.02
		1.61	(m)			-0.03
	4	1.31	(m)	25.3		NA*1
	5	1.32	(m)	28.8		NA*1
	6	1.62	(m)	23.2		-0.06
	7	2.48	(m)	37.2		-0.12
		2.43	(m)			-0.13
	8			212.4		
	9	4.22	(m)	72.6		0.02
	10	1.38	(3H, d, 7)	19.8		0.06
	11	7.17	(d, 10)		129.1	
	9-OH	3.56	(d, 4.7)			
4-MePro	12			171.9		
	13	4.67	(dd, 8.1&2.2)	58.0		
	14	2.38	(m)	33.0		
		1.38	(m)			
	15	2.62	(m)	32.8		
	16	4.05	(dd, 9.9&7.5)	53.8		
		2.73	(dd, 9.9&7.8)			
	17					
	18	0.88	(3H, d, 6.7)	18.1		
Phe	19			173.1		
	20	5.16	(ddd, 10,10&6)	53.3		
	21	3.24	(dd, 13.5&9.8)	35.7		
		2.96	(dd, 13.5&6)			
	22			137.0		
	23/27	7.23	(2H, m)	129.0		
	24/26	7.27	(2H, m)	128.6		
	25	7.20	(m)	126.7		
	28	7.52	(d, 10)		119.6	
Iva	29			175.6		
	30			63.0		
	31	2.32	(dq, 13.8&7)	27.8		
		2.16	(dq, 13.8&7)			
	32	0.84	(3H, t, 7)	8.4		
	33	1.28	(3H, s)	22.4		
	34	5.81	(s)		132.0	

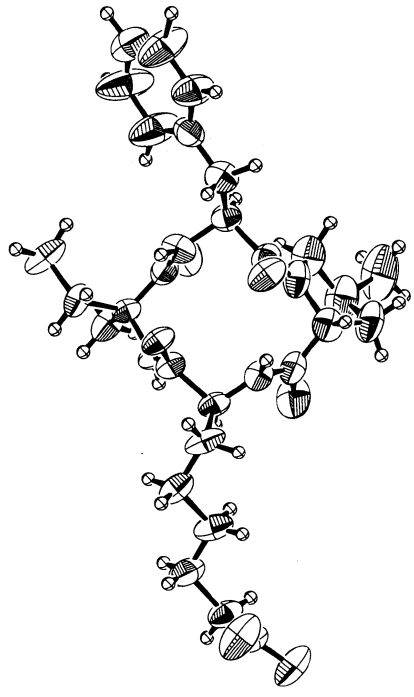
determine the amino acid sequence.

The sequence was elucidated with sequantial HMBCs and the amino acids sequence was further supported by NOEs as follows. ^{1}H-^{13}C HMBC correlations revealed the entire four sets of junctions, *i.e.* aoh-iva (34-NH/C-1), iva-Phe (20-H and 28-NH/C-29), Phe-4-MePro (16-H_2/C-19) and 4-MePro-aoh (2-H and 11-NH/C-12). NOEs (2-H/34-NH, 31b-H/28-NH, 20-H/16-H_2 and 13-H/2-H) agreed to this sequence to give the planar structure of **1** shown in Fig. 3. The ^{1}H, ^{13}C and ^{15}N NMR assignments of **1** are depicted in Table 1.

We then focused on stereochemical elucidation of **1**. The stereochemistry of the methyl group in 4-MePro proved to be difficult to solve by NMR analysis or chemical degradation, while all attempts to crystallize **1** suitable for X-ray crystallography were in vain. Fortunately, we were grateful to succeed in X-ray analysis of an oxidative derivative **2** (Fig. 1) obtained by treatment of **1** with $NaIO_4$. The crystals of **2** with approximate dimensions of $0.2\times0.1\times0.1\,mm^3$ were obtained from methanol solution. The structure was determined by a direct method using the program LODEM[4]. ORTEP drawing of **2** was shown in Fig. 4 and this concluded relative stereochemistry of the core peptide structure. The absolute configuration of Phe was determined by modified Marfey's method[5]. Hydrolysis of **1** with 6 N hydrochloric acid followed by treatment with FDVA (*N*-(3-fluoro-4,6-dinitrophenyl)-L-valinamide) and triethylamine to detect a FDVA derivative of Phe (Rt 17.3 minutes). LC-MS analysis allowed us to assign its L configuration by comparison with FDVA derivatives from L- and D-Phe (Rt 17.3 minutes and 20.2 minutes, respectively). The only remaining structural problem was the absolute stereochemistry at position 9, and it was solved with modified Mosher's method[6,7]. The differences of δ_H between (*S*)- and (*R*)-Mosher ester of **1** (**3** and **4**) are depicted in Table 1. Analysis of their contribution established the configuration of C-9 to be *R*.

In conclusion, the whole structure of FR235222 is as shown in Fig. 1, cyclo[-(2*S*,9*R*)-aoh-L-iva-L-Phe-(2*R*,4*S*)-4-MePro-]. FR235222 is composed of L-Phe and three unique amino acids, *i.e.* a 4-methylproline (4-MePro), an isovaline (iva) and a 2-amino-8-oxo-9-hydroxydecanoic acid (aoh). To the best of our knowledge, the 4-methylproline has been encountered for the first time in tetracyclic peptide chemistry[8~16]. The isovaline is also a rare amino acid and has been reported only in phoenistatin[14] as composites of tetracyclic peptides. The amino acid with α-hydroxyketone[15,16] is characteristic because α-epoxyketone function in stead of α-hydroxyketone has been assumed to be mandatory for HDAC inhibition *via* covalent binding since Yoshida's pioneer work on trapoxin[17]. We are now under extensive examination of FR235222 derivatives for better pharmacological properties, and the results will be reported in due course.

Fig. 4. ORTEP drawing of **2**.



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