Synthesis and Characterization of an Epimer of Tacrolimus, an Immunosuppressive Drug

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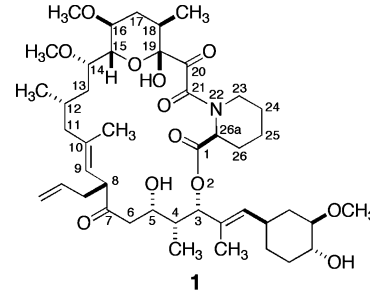
8-Epitacrolimus (**2**), a new L-pipecolic acid macrolide lactone, was obtained by base-catalyzed epimerization of tacrolimus (FK-506, **1**), an important immunosuppressive drug, and its structure determined by a single-crystal X-ray diffraction method. The compound was fully characterized by spectroscopic techniques. The epimer is of importance due to its potential biological effects as well as because of its possible formation during formulation, handling, and use of tacrolimus products.

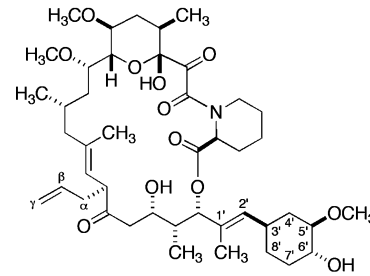
Tacrolimus (**1**),[1] a macrolide lactone also known as FK-506, fugimycin, or tsukubaenolide, is a marketed drug (Prograf, Advagraf, Protopic) used as an immunosuppressive agent to prevent rejection of transplanted organs[2] and also topically in a number of conditions.[3] Tacrolimus is produced by the bacterium *Streptomyces tsukubaensis*,[4] and its name is an acronym reportedly derived from "*t*sukuba m*acrol*ide *im*mu*no*suppressant". Tacrolimus belongs to the class of L-pipecolic acid-derived macrolides produced by *Streptomyces* species, also comprising rapamycin (sirolimus),[5] ascomycin,[6] meridamycin,[7] and other compounds, many of which possess very valuable pharmacological properties.[8] Tacrolimus exerts its action by binding to the immunophilin FKBP12 to give a complex that inhibits calcineurin, a calcium-dependent phosphatase participating in signal transduction that leads to lymphokine gene transcription.[9,10]

We found that in contrast to the treatment of tacrolimus with strong bases, which leads to degradation of the molecule,[1] treatment with 1,5-diazabicyclo[4.3.0]nonene (DBN) in CH_2Cl_2 at ambient temperature gives an apparent equilibrium mixture consisting of **1** and a new, slightly less polar compound in a ratio of 1:2 (reversed-phase HPLC, 210 nm). Preparative-scale isolation gave the reaction product as a crystalline material with mass spectrometric (electrospray LC-MS mode, *m*/*z* 804, [MH^+]) and ^1H and ^{13}C NMR data resembling those of **1** and indicative of formation of an isomer. Epimerization of tacrolimus at C-8 via reversible enolization of the carbonyl group is an immediately expected possibility, as suggested by the CD spectrum showing opposite Cotton effect for the n → π^* carbonyl group transition in comparison to **1** (Figure 1), although epimerization of the *N*-acylated pipecolic acid moiety (C-26a) cannot be ruled out.[11]

The new isomer was confirmed as being the 8*S* epimer of tacrolimus (**2**) by single-crystal X-ray diffractometry (Figure 2).[12] Treatment of **2** with DBN in CH_2Cl_2 gave a mixture of **1** and **2** in the same ratio as that obtained when starting from **1**. The solid-state conformation of **2** is very different from that of **1** (Figure 3). The ^1H and ^{13}C NMR spectra of **2**, similar to those of tacrolimus (**1**), showed the presence of amide bond rotamers of the major hemiketal tautomer, in addition to very weak signals of minor tautomers. The solvent-dependent ratio between the major and the minor rotamer of **2** was 5:1 and 3:1 in chloroform-*d* and benzene-d_6, respectively, as compared to the 2:1 ratio observed for **1** in chloroform-*d*. Extensive 2D NMR investigations (COSY, NOESY, HSQC, HMBC) allowed assignment of all ^{13}C NMR and most ^1H NMR signals of both rotamers (Table 1). That the major isomers present in the solution are amide bond rotamers and not, for example, C-19 epimers, was shown, inter alia, by the differences in chemical shifts of C-23 and C-26a and of the corresponding hydrogen atoms (Table 1).



1



2

Interestingly, neither 8-epitacrolimus (**2**) nor epimers of related macrolides have been reported prior to this work in spite of their easy formation and potential pharmacological and pharmaceutical significance. Thus, the implications of characterization of this epimer are twofold. First, the formation of **2** takes place under relatively mild basic conditions (and is also expected under conditions of acid and free-radical catalysis), and therefore **2** may be formed during manipulations of tacrolimus, e.g., during its isolation from fermentation broths, purification, or manufacturing of pharmaceutical formulations, and possibly also in vivo as a metabolite of tacrolimus (**1**). Second, even very minor structural modifications of **1** are known to result in significant alterations of pharmacological profiles, as in the case of ascomycin,[14] an analogue of **1** where the (8*R*)-propenyl group is replaced by an (8*R*)-ethyl group, and assessment of biological activity of **2** is therefore of considerable interest. Published X-ray structures of complexes of human and bovine calcineurin with FKBP12 bound to tacrolimus (**1**) show that the 2-propenyl group is embedded in a cleft lined by lipophilic amino acid residues and that C-8 is significantly displaced upon binding of the FKBP12 complex to calcineurin.[10] Consequently, epimerization at C-8 is expected to lead to alteration of FKBP12 and calcineurin binding. Therefore, the isolation of the

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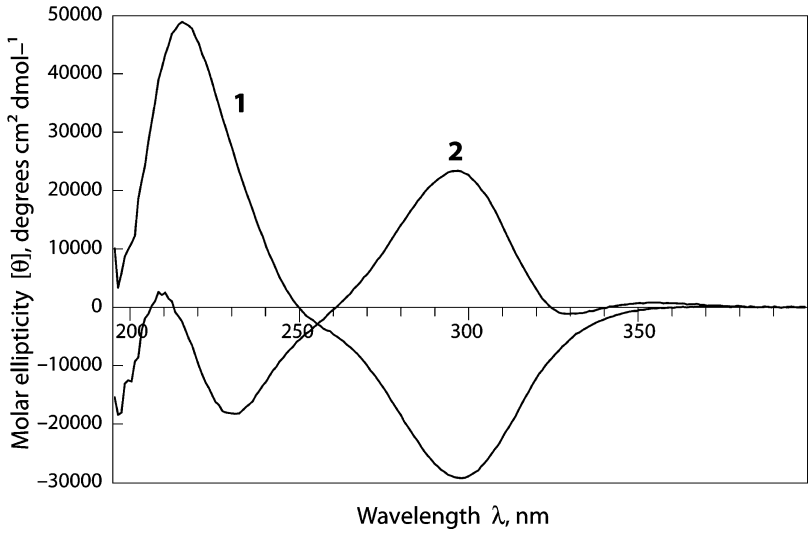

Figure 1. CD spectra of tacrolimus (**1**) and its epimer (**2**) in acetonitrile.

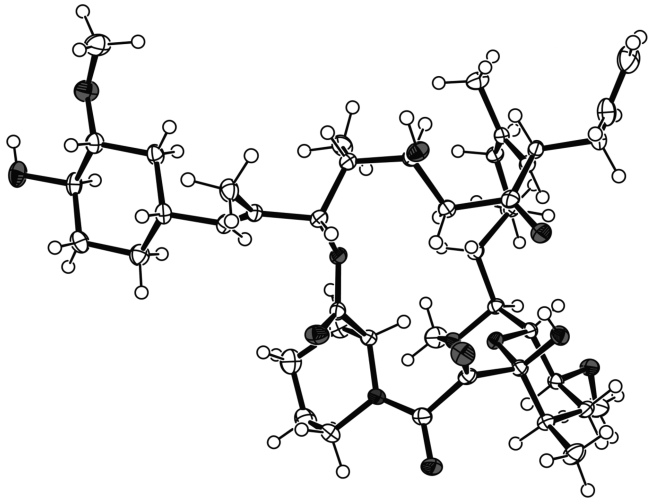

Figure 2. ORTEP[13] drawing of the crystal structure of compound **2**. Displacement ellipsoids are drawn at the 50% probability level. Hydrogen atoms are drawn as spheres of arbitrary size.

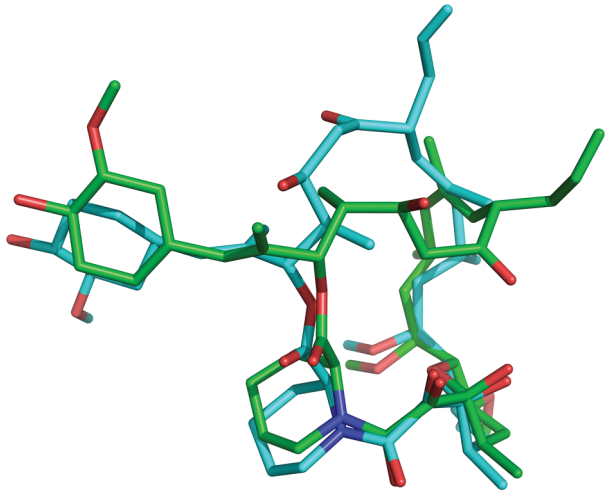

Figure 3. Superimposition of compound **2** (green) on the crystal structure[1] of **1** (cyan) showing differences in crystal-state conformation.

novel immunosuppresant analogue **2** reported in this work opens the possibility of exploration of its binding to various members of the family of FK506-binding proteins[15] and their calcineurin complexes, as well as studies of its action on other macromolecular targets.

Experimental Section

General Experimental Procedures. The optical rotation was measured with a Perkin-Elmer 241 polarimeter. UV spectra were recorded with a Perkin-Elmer Lambda 2 spectrophotometer, and IR spectra with a Perkin-Elmer FT model 1600 spectrophotometer. CD spectra were recorded on an Olis DSM 10 instrument. NMR spectra were recorded at 25 °C on a Bruker Avance 600 MHz spectrometer using chloroform-*d* or benzene-d_6 as solvent and TMS as internal reference. Phase-sensitive COSY spectra were recorded with 4k × 512 data points. Phase-sensitive NOESY spectra were recorded with the same data matrix size and mixing time of 600 ms. Gradient-enhanced HSQC and HMBC spectra (mixing time 65 ms) consisted of 2k × 380 data points. Analytical HPLC analyses were performed on a Shimadzu LC-10A system consisting of a controller, a solvent supply unit, a diode array detector, an autoinjector, and a column oven, using a Phenomenex Luna C_{18} column (4.6 mm × 150 mm, 3 μm), operated at 40 °C. LC-MS data were obtained using an Agilent 1100 chromatograph equipped with an autoinjector, a diode array detector, and a column oven, connected to a Bruker Esquire LC mass spectrometer equipped with electrospray ionization ion source. HRMS was measured on a JEOL HX-110/110 mass spectrometer operating in the EI mode. Preparative HPLC separations were performed on a Waters system consisting of a Waters model 590 pump and a Waters Lambda-Max model 481 detector, using a Supelco Discovery C_{18} column (21.2 mm × 250 mm, 5 μm). Tacrolimus (**1**) was obtained from LifeCycle Pharma.

Synthesis of 8-Epitacrolimus (2). Tacrolimus (**1**; 1 g, 1.2 mmol) was dissolved in CH_2Cl_2 (100 mL), the solution was cooled to 0 °C, and a solution of 1,5-diazabicyclo[4.3.0]nonene (302 mg, 2.4 mmol) in CH_2Cl_2 (40 mL) was added. The resulting solution was allowed to warm slowly to room temperature and was stirred overnight. The reaction mixture was poured into water (120 mL) containing AcOH (2.4 mmol). The organic layer was separated, washed with water (3 × 40 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo. The product was purified by preparative reversed-phase HPLC [CH_3CN-H_2O (60:40)] to yield 8-epitacrolimus (**2**) as an amorphous, white solid (147 mg, 14.7%). The product was crystallized for X-ray analysis from CH_3CN-H_2O (70:30).

8-Epitacrolimus, (3*S*,4*R*,5*S*,8*S*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-5,19-dihydroxy-14,16-dimethoxy-15,19-epoxy-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-3-[(1*E*)-2-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylethenyl]- 8-(2-propen-1-yl)-4,10,12,18-tetramethyl-3*H*-pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(4*H*,23*H*)-tetrone (2): Colorless prisms; mp 179−182 °C [CH_3CN-H_2O (70:30)]; $[\alpha]^{25}_D$ −1.2 (*c* 0.725, $CHCl_3$); UV (CH_3CN) λ_{max} (ε) 202 nm (9500); CD (CH_3CN) λ_{max} ([θ]) 231 (−18 300), 297 (+23 400); IR (KBr) λ_{max} 3580, 3431, 2934, 1753, 1724, 1704, 1633, 1452, 1193,

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) Spectroscopic Data[a] for 8-Epitacrolimus (**2**) (δ values, proton multiplicities, and coupling constants) in Benzene-d_6 and Chloroform-*d*

	^{1}H NMR data				^{13}C NMR data			
	$CDCl_3$		C_6D_6		$CDCl_3$		C_6D_6	
no.	major rotamer	minor rotamer	major rotamer	minor rotamer	major rotamer	minor rotamer	major rotamer	minor rotamer
1					168.4	168.9	168.2	168.9
3	5.35 (br s)	5.38 (br s)	5.69 (br s)	5.67 (br s)	75.3	76.0	75.7	76.3
4	2.05	n.a.	2.01	2.05	38.9	39.0	39.6	40.2
5	4.03 (dd, 10.6, 4.8)	3.95 (dd, 10.4, 4.5)	4.18 (dd, 10.3, 4.4)	4.00 (dd, 10.0, 3.8)	69.6	70.0	69.5	70.4
6	2.67 (d, 17.8), 2.36 (dd, 17.8, 10.7)	1.86, 2.81 (dd, 14.8, 1.7)	2.66 (dd, 18.0, 10.4) 2.75 (dd, 18.0, 1.5)	2.16, 2.86	42.4	42.2	43.0	43.2
7					214.1	213.4	213.0	212.4
8	3.27 (ddd, 10.1, 8.2, 5.0)	3.43	3.24 (ddd, 10.3, 8.4, 5.0)	3.45	52.6	52.4	52.8	52.2
9	5.53 (br d, 10.2)	4.87 (br d, 9.8	5.88 (br d, 10.2)	5.00 (br d, 9.8)	122.7	120.2	124.0	121.9
10					138.5	138.9	138.3	138.5
11	1.57 (dd, 13.9, 11.1), 2.10	1.83, 2.13	2.27 (br d, 13.7), 1.55	1.66, 1.93	47.3	46.5	47.8	46.7
12	1.38	n.a.	1.46	n.a.	30.3	30.3	31.3	31.3
13	1.43, 1.51	n.a.	1.54, 1.81 (tm, 12.1)	n.a.	36.8	36.8	37.6	37.6
14	3.62 (ddd, 7.6, 5.0, 2.5)	3.59	3.84 (ddd, 13.6, 5.1, 2.6)	3.74 (dd, 10.6, 2.9)	75.7	75.5	75.9	75.9
15	3.88 (dd, 9.6, 2.5)	n.a.	4.20 (dd, 9.6, 2.5)	n.a.	71.0	71.0	71.7	71.7
16	3.40	n.a.	3.59 (ddd, 11.2, 9.5, 4.6)	n.a.	73.6	74.0	73.7	73.6
17	1.45, 2.11	n.a.	1.67 (m), 1.93	n.a.	32.8	32.0	33.1	32.4
18	2.05	n.a.	2.19 (m)	n.a.	33.2	33.2	33.8	33.8
19					98.5	97.8	98.9	98.5
20					189.5	196.8	190.6	197.6
21					166.9	165.2	167.7	165.8
23	eq: 3.89 ax: 3.51 (dt, 13.5, 3.6)	eq: 4.39 (br d, 13.8) ax: 3.07 (dt, 13.8, 3.0)	eq: 3.79 (dt, 13.6, 3.3) ax: 3.51 (ddd, 13.6, 10.6, 5.9)	3.24, 4.56 (m)	43.7	39.1	44.0	39.5
24	1.49, 1.74	n.a.	n.a.	n.a.	24.5	24.2	24.4	24.6
25	1.30, 1.77	n.a.	0.88, 1.49	n.a.	20.0	21.2	20.2	20.2
26	1.85, 2.33	n.a.	1.60, 2.12	n.a.	26.3	27.1	26.4	27.6
26a	4.96	4.29 (m)	5.15 (dd, 6.6, 2.1)	4.64 (m)	52.6	56.8	52.9	57.1
1′	-	-	-	-	132.5	132.4	132.9	132.9
2′	4.93	4.97	4.94 (dm, 9.0)	5.05	128.1	128.6	128.3	129.1
3′	2.31	n.a.	2.11	n.a.	34.9	34.6	35.2	35.2
4′	0.94, 2.03	n.a.	0.90, 1.92	n.a.	35.0	34.5	35.4	35.5
5′	3.01 (ddd, 11.2, 8.8, 4.4)	n.a.	2.85 (m)	n.a.	84.2	84.2	84.6	84.6
6′	3.44	3.59	3.44 (ddd, 11.4, 8.6, 4.9)	n.a.	73.5	73.3	73.9	74.2
7′	1.35, 1.99	n.a.	ax: 1.38 (qm, 12.2), eq: 2.01	n.a.	31.2	31.2	31.8	31.8
8′	1.06, 1.64	n.a.	ax: 0.91; eq: 1.45 (dm, 12.2)	n.a.	30.8	30.7	31.2	31.1
α	2.25 (dt, 14.6, 6.7), 2.50 (dt, 14.6, 6.1)	n.a.	2.58 (dt, 14.2, 6.6), 2.41 (dt, 14.2, 7.8)	2.38, 2.61	35.4	34.5	36.1	35.0
β	5.67 (ddt, 17.0, 10.1, 7.1)	5.70	5.75 (ddt, 17.0, 10.1, 7.1)	5.79	135.5	135.6	135.9	136.1
γ	4.98, 5.01 (dm, 17.0)	5.07 (dm, 17.0), n.a.	5.02 (dm, 10.3), 5.06 (dq, 17.0, 2.0)	5.13 (dq, 17.0, 1.9), 5.03	116.8	116.7	117.0	116.8
CH_3-4	0.84 (d, 6.8)	n.a.	0.77 (d, 7.4)	0.83 (d, 7.4)	9.7	10.0	9.9	10.3
CH_3-10	1.74 (br s)	1.70 (br s)	1.60 (d, 1.3)	n.a.	20.3	20.0	20.2	20.7
CH_3-12	0.84 (d, 6.8)	n.a.	0.87 (d, 6.4)	n.a.	17.8	17.8	17.8	17.8
CH_3-18	1.06 (d, 6.3)	0.96 (d, 6.4)	1.26 (d, 6.6)	1.12 (d, 6.6)	16.2	15.9	16.6	16.4
CH_3-1′	1.66 (br s)	n.a.	1.50 (d, 1.1)	n.a.	14.7	14.7	14.6	14.6
CH_3O-14	3.37 (s)	3.31 (s)	3.32 (s)	3.22 (s)	57.7	57.1	57.6	57.1
CH_3O-16	3.37 (s)	3.39 (s)	3.14 (s)	3.07 (s)	56.2	56.2	55.7	55.6
CH_3O-5′	3.41 (s)	3.41 (s)	3.11 (s)	3.12 (s)	56.6	56.6	56.3	56.3
OH-19	6.16	4.20	6.34	4.80				

[a] Values for which no multiplicities are given were obtained from 2D correlations; n.a. = not assigned.

1170, 1091, 1050 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HREIMS *m/z* 803.4802 [M$^{+\cdot}$], $C_{44}H_{69}NO_{12}$ requires 803.4814 (ΔM 1.5 ppm).

X-ray Analysis. Single crystals suitable for X-ray diffraction studies were grown from a solution in CH_3CN-H_2O (70:30); $C_{44}H_{69}NO_{12}$, M_r 804.0, orthorhombic, space group $P2_12_12_1$ (No. 19), $a = 9.5560(6)$ Å, $b = 18.1254(16)$ Å, $c = 26.136(5)$ Å, $V = 4526.9(10)$ Å^3, $Z = 4$, $D_c = 1.180$ Mg/m^3, $F(000) = 1744$, μ(Mo Kα) = 0.085 mm^{-1}, crystal size 0.66 × 0.54 × 0.31 mm.

Data Collection and Reduction. A single crystal was mounted and immersed in a stream of nitrogen gas [$T = 122(1)$ K]. Data were collected using graphite-monochromated Mo Kα radiation ($\lambda = 0.71073$ Å) on a KappaCCD diffractometer. Data collection and cell refinement were performed using COLLECT[16] and DIRAX.[17] Data reduction was performed using EvalCCD.[18] Correction for absorption was performed using Gaussian integration[19] as included in maXus.[20]

Structure Solution and Refinement. Positions of all non-hydrogen atoms were found by direct methods.[21,22] Full-matrix least-squares refinements[23] were performed on F^2, minimizing $\sum w(F_o^2 - kF_c^2)^2$, with anisotropic displacement parameters of the non-hydrogen atoms. The positions of hydrogen atoms were located in subsequent difference electron density maps. The hydrogen atoms bonded to the chiral centers were included in observed position and refined with fixed isotropic displacement parameters ($U_{iso} = 1.2U_{eq}$ for CH). The rest of the hydrogen atoms were included in calculated positions with fixed isotropic displacement parameters ($U_{iso} = 1.2U_{eq}$ for CH and CH_2, $U_{iso} = 1.5U_{eq}$ for OH and CH_3). Refinement (564 parameters, 10 262 unique reflections) converged at $R_F = 0.033$, $wR_F^2 = 0.073$ [9381 reflections with $F_o > 4\sigma(F_o)$; $w^{-1} = [\sigma^2(F_o^2) + (0.0282P)^2 + 1.5197P]$, where $P = (F_o^2 + 2F_c^2)/3$; $S = 1.085$]. The residual electron density varied between −0.17 and 0.27 e Å^{-3} (non-centrosymmetric space group), but the absolute configuration cannot be determined reliably [Flack = 0.3(5)].[24] Complex scattering factors for neutral atoms were taken from International Tables for Crystallography as incorporated in SHELXL97.[23,25]

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Supporting Information Available: 600 MHz ^{1}H and 150 MHz ^{13}C NMR spectra for compound **2** in chloroform-*d* and benzene-d_6. This information is available free of charge via the Internet at http://pubs.acs.org.

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