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Some transformations of tacrolimus, an immunosuppressive drug

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ABSTRACT

Transformations of the macrocyclic lactone tacrolimus (**1**), an important immunosuppressive drug produced by *Streptomyces* species, are described. These transformation products are primarily of interest as reference substances for drug impurity analyses. Upon action of acid (*p*-toluenesulfonic acid in toluene), tacrolimus is dehydrated by loss of water from the β-hydroxyketone moiety with partial inversion of configuration at C-8, resulting in formation of 5-deoxy-$\Delta^{5,6}$-tacrolimus and 5-deoxy-$\Delta^{5,6}$-8-epitacrolimus. The structure of the latter was determined by single-crystal X-ray crystallography. The same products are formed upon action of free radicals (iodine in boiling toluene), along with formation of 8-epitacrolimus. The latter is converted by *p*-toluenesulfonic acid to 5-deoxy-$\Delta^{5,6}$-8-epitacrolimus. Treatment of tacrolimus with weak base (1,5-diazabicyclo[4.3.0]nonene) gives, in addition to 8-epitacrolimus, the open-chain acid corresponding to 5-deoxy-$\Delta^{5,6}$-tacrolimus, a rare non-cyclic derivative of tacrolimus. Strong base (*t*-butoxide) causes pronounced degradation of the molecule. Thermolysis of tacrolimus leads to ring expansion by an apparent [3,3]-sigmatropic rearrangement of the allylic ester moiety with subsequent loss of water from the β-hydroxyketone moiety. ^{1}H and ^{13}C NMR spectra of the obtained compounds, complicated by the presence of amide bond rotamers and ketal moiety tautomers, were assigned by extensive use of 2D NMR techniques.

1. Introduction

Tacrolimus (**1**), a 23-membered macrolide lactone, is a natural product originally isolated from the bacterium *Streptomyces tsukubaensis* (Tanaka et al., 1987). It is used as an immunosuppressant to prevent graft rejection after organ transplantations (Bowman and Brennan, 2008; Busuttil and Lake, 2004; Fortune and Couriel, 2009; Penninga et al., 2010; Scott et al., 2003; Vicari-Christensen et al., 2009) and also in the treatment of autoimmune diseases (Ruemmele et al., 2008; Yeoman et al., 2010) and atopic dermatitis (Kalavala and Dohil, 2011). Furthermore, it is being investigated as treatment of rheumatoid arthritis (Lee et al., 2010). Tacrolimus is a highly functionalized molecule with L-pipecolic acid moiety adjacent to a masked tricarbonyl functionality, 14 stereocenters, three double bonds, and a number of free hydroxy groups and other functionalities. Restricted rotation of the amide bond in the pipecolic acid moiety results in a solvent-dependent equilibrium between *cis* and *trans* rotamers in solution, which can be detected by NMR spectroscopy (Karuso et al., 1990; Mierke et al., 1991; Petros et al., 1993; Tanaka et al., 1987). Furthermore, especially in polar solvents, a different kind of equilibrium exists between tacrolimus tautomers with respect to the cyclic ketal moiety (Akashi et al., 1996; Baumann et al., 1995; Gailliot et al., 1994; Namiki et al., 1995; Namiki et al., 1993; Schueler et al., 1993).

Tacrolimus acts by binding to the cytosolic immunophilin protein FKBP12. The bimolecular complex inhibits calcineurin and thereby hinders the transcription of T-cell activation genes such as interleukin-2 (Griffith et al., 1995; Kissinger et al., 1995; Moore et al., 1991; Sewell et al., 1994; Wilson et al., 1995). Tacrolimus is structurally related to a number of other L-pipecolic acid derived macrolides such as ascomycin, pimecrolimus, and rapamycin (sirolimus) (Bulusu et al., 2011; Graziani, 2009; Nghiem et al., 2002). These compounds all show immunosuppressive activity by targeting immunophilins. Although the structures of tacrolimus, ascomycin and pimecrolimus are closely related, their pharmacological profiles are different. This may be related to the fact, that even small changes such as inversion of a single stereocenter engenders considerable changes in the conformation of the molecule (Skytte et al., 2010; Wagner et al., 1996). In this article we describe a number of derivatives of tacrolimus formed by simple reactions. These compounds are intended to mimic probable tacrolimus degradation products formed during manufacturing or storage. The semi-synthetic compounds can be produced in much larger amounts compared to degradation products isolated in trace

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amounts from stability studies facilitating the identification and structure elucidation. Subsequently it will be possible to identify degradation products observed in stability studies by comparison (HPLC, NMR) with the synthesized model compounds. The synthesized products were purified by HPLC and identified primarily by NMR spectroscopy and LC-MS analysis, supported, where necessary, by single crystal X-ray diffraction.

2. Experimental

2.1. Materials and methods

Optical rotations were measured with a Jasco DIP-370 polarimeter. UV spectra were recorded with a Thermo Scientific NanoDrop 2000 spectrophotometer, and IR spectra with a Perkin–Elmer Spectrum One FTIR spectrophotometer. NMR spectra were recorded at 25 °C on a Bruker Avance 400 or 600 MHz spectrometer using benzene-d_6 as solvent and TMS as internal reference. Reactions were monitored by analytical HPLC on a Shimadzu system consisting of a LC-10AT liquid chromatography, a SPD-M10A photo-diode array detector, a FCV-10AL pump, and a CTO-10AC column oven, using a Phenomenex Luna C18 column (3 μm, 150 × 4.6 mm i.d.) operated at 50 °C, detection wavelength 210 nm. Mobile phases A and B consisted of 5% of MeCN and 0.1% of formic acid in water, and 5% of water and 0.1% of formic acid in MeCN, respectively. Products were purified by preparative HPLC on a Waters system consisting of a Model 590 pump and a Lambda-Max Model 481 detector, using a Supelco Discovery C18 column (5 μm, 250 × 21.2 mm i.d.) and mixtures of MeCN and water as mobile phases. LC-MS was performed on an Agilent 1100 system equipped with a photo-diode array detector and an Agilent MSD ion-trap mass spectrometer, using a Phenomenex Luna C18 column (3 μm, 100 × 2.0 mm i.d.). HRESIMS spectra were acquired with a Bruker micrOTOF-Q II mass spectrometer equipped with an electro-spray ionization source. Tacrolimus (**1**) was available from Veloxis Pharmaceuticals.

2.2. Reaction of tacrolimus with p-toluenesulphonic acid (Tos)

Tacrolimus (**1**, 500 mg, 0.61 mmol) and Tos (monohydrate, 11.6 mg, 0.061 mmol) were dissolved in toluene (30 ml) and heated under reflux for 1 h. The solution was washed with water (30 ml) and then with brine (30 ml), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The product was purified by preparative reversed-phase HPLC (isocratic elution, MeCN–H_2O 65:35) to give **2** (63 mg, 13%) as colorless crystals and **3** (225 mg, 47%) as white amorphous solid.

2.3. Reaction of 8-epitacrolimus with Tos

8-Epitacrolimus (**4**, 44 mg, 0.05 mmol) and Tos (monohydrate, 1.0 mg, 0.005 mmol) was dissolved in toluene (10 ml) and heated under reflux for 2 h. The solution was diluted with toluene (5 ml), washed with water (15 ml) and then with brine (15 ml), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The product was purified by preparative reversed-phase HPLC (isocratic elution, MeCN–H_2O 65:35) and recrystallized from MeCN–H_2O 7:3 to give **2** (21 mg, 49%) as colorless crystals.

2.4. Reaction of tacrolimus with 1,5-diazabicyclo[4.3.0]nonene (DBN)

Tacrolimus (**1**, 1.0 g, 1.2 mmol) was dissolved in CH_2Cl_2 (100 ml). The solution was cooled to 0 °C on an ice bath. A solution of DBN (302 mg, 2.4 mmol) in CH_2Cl_2 (40 ml) was added and the resulting solution was stirred overnight. The reaction mixture was poured into water (120 ml) containing AcOH (2.4 mmol). The layers were separated and the organic layer was 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 (isocratic elution, MeCN–H_2O 60:40) to give **4** as colorless crystals (147 mg, 15%) and **5** as colorless oil (28.5 mg, 3%).

2.5. Reaction of tacrolimus with sodium t-butoxide

Tacrolimus (**1**, 250 mg, 0.30 mmol) was added to sodium *t*-butoxide (61 mg, 0.64 mmol) in *t*-butanol (25 ml), and the solution was stirred at 65 °C for 1.5 h. The reaction mixture was quenched with water (40 ml) and extracted with EtOAc (3 × 25 ml). The combined organic layer was washed with brine (15 ml), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The product was recrystallized from heptane to give **8** (36 mg, 60%) as colorless crystals.

2.6. Thermal rearrangement of tacrolimus

Tacrolimus (**1**, 500 mg, 0.61 mmol) was dissolved in *o*-xylene (25 ml). The solution was refluxed for 24 h, cooled to room temperature, and concentrated *in vacuo*. Preparative reversed-phase HPLC (isocratic elution, MeCN–H_2O 65:35) yielded **6** as white amorphous solid (95 mg, 19%) and **7** as colorless oil (25 mg, 5%).

2.7. Iodine-catalyzed transformations of tacrolimus

Tacrolimus (**1**, 500 mg, 0.61 mmol) was dissolved in distilled toluene (15 ml). Iodine (5 mg, 0.02 mmol) was added, and the reaction mixture was refluxed for 6.5 h in daylight. After cooling and diluting with toluene (15 ml), the solution was washed with saturated sodium thiosulphate (30 ml), water (30 ml), and brine (30 ml), dried over Na_2SO_4, and concentrated *in vacuo*. Reversed-phase HPLC (isocratic elution, MeCN–H_2O 65:35) gave three compounds: **2** (colorless crystals, 33 mg, 7%), **3** (white amorphous solid, 50 mg, 10%), and **4** (colorless crystals, 41 mg, 8%).

2.8. Compound characteristics

2.8.1. 5-Deoxy-$\Delta^{5,6}$-8-epitacrolimus (3S,4R,8S,9E,12S,14S,15R,16S,18R,19R,26aS)-14,16-Dimethoxy-15,19-epoxy-19-hydroxy-3-[(1E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethenyl]-8-(2-propen-1-yl)-8,11,12,13,14,15,16,17,18,19,24,25,26,26a-tetradecahydro-4,10,12,18-tetramethyl-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone (2):

Colorless prisms; mp 186–187 °C (from MeCN–H_2O 7:3); $[\alpha]_D^{25}$ + 50.9° (*c* 0.32, $CHCl_3$); UV (MeCN) λ_{max} (ε) 201 nm (26,300); IR (film) ν_{max} 3432, 2934, 1749, 1650, 1626, 1450, 1193, 1173, 1089, 986, 752 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 786.4790 ($[M + H]^+$), $C_{44}H_{68}NO_{11}^+$ requires 786.4787 (ΔM 0.4 ppm).

2.8.2. 5-Deoxy-$\Delta^{5,6}$-tacrolimus (3S,4R,8R,9E,12S,14S,15R,16S,18R,19R,26aS)-14,16-Dimethoxy-15,19-epoxy-19-hydroxy-3-[(1E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethenyl]-8-(2-propen-1-yl)-8,11,12,13,14,15,16,17,18,19,24,25,26,26a-tetradecahydro-4,10,12,18-tetramethyl-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone (3)

White amorphous solid; $[\alpha]_D^{25}$ −91.2° (*c* 0.40, $CHCl_3$); UV (MeCN) λ_{max} (ε) 197 nm (47300; IR (film) ν_{max} 3446, 2934, 1741, 1689, 1648, 1450, 1195, 1173, 1091, 988, 758 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 786.4780 ($[M + H]^+$), $C_{44}H_{68}NO_{11}^+$ requires 786.4787 (ΔM 0.8 ppm).

Table 1
^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data[a] for enones **2** and **3** in benzene-d_6.

	5-Deoxy-$\Delta^{5,6}$-8-epitacrolimus (**2**)				5-Deoxy-$\Delta^{5,6}$-tacrolimus (**3**)			
No.	^{1}H NMR data		^{13}C NMR data		^{1}H NMR data		^{13}C NMR data	
	Major rotamer (*cis*)	Minor rotamer (*trans*)	Major rotamer (*cis*)	Minor rotamer (*trans*)	Major rotamer (*cis*)	Minor rotamer (*trans*)	Major rotamer (*cis*)	Minor rotamer (*trans*)
1			169.8	169.3			169.7	169.3
3	5.26 (d, 4.2)	5.26 (d, 4.2)	80.1	82.0	5.24 (d, 3.0)	5.32 (d, 3.6)	80.6	81.2
4	2.54–2.57 (m)	2.71–2.75 (m)	38.7	39.7	2.41–2.43 (m)	2.45–2.49 (m)	39.6	40.6
5	6.84 (dd, 15.6, 6.0)	6.99 (dd, 15.6, 9.0)	148.6	146.7	6.91 (dd, 15.6, 6.6)	6.94 (dd, 15.6, 8.4)	148.1	147.0
6	6.26 (dd, 15.6, 1.2)	6.69 (dd, 15.6, 0.6)	130.6	128.4	6.25 (dd, 15.6, 1.2)	6.43 (dd, 15.6, 1.2)	128.6	129.7
7			200.7	197.7			199.5	196.8
8	3.67 (dt, 9.6, 7.2)	3.56–3.59 (m)	50.1	53.7	3.64 (m)	3.37–3.39 (m)	52.3	52.2
9	5.02 (dd, 9.6, 1.2)	5.94 (br d, 9.6)	122.9	124.2	5.20 (d, 9.6)	5.11 (d, 10.2)	124.5	124.0
10			137.0	139.4			138.9	139.5
11	1.99–2.02 (m), 1.61–1.63 (m)	2.23–2.26 (m), 1.47–1.49 (m)	46.5	48.4	2.04 (dd, 12.6, 3.6), 1.72–1.74 (m)	2.14 (dd, 13.8, 6.0), 1.91–1.94 (m)	49.5	50.0
12	1.70 (m)	1.90–1.91 (m)	28.7	32.3	1.56–1.58 (m)	1.83–1.85 (m)	27.9	26.3
13	1.92–1.95 (m), 0.93–0.97 (m)	1.77–1.80 (m), 1.51 (m)	35.0	37.9	1.70 (m), 0.99 (ddd, 13.8, 11.4, 2.4)	1.72 (m), 1.35–1.37 (m)	34.0	36.4
14	3.71 (br d, 9.6)	3.84 (ddd, 10.8, 5.4, 2.4)	76.8	76.3	3.66 (dd, 9.6, 3.6)	3.79 (ddd, 11.4, 4.2, 2.4)	76.7	76.7
15	3.89 (d, 9.0)	4.23 (dd, 9.6, 2.4)	74.2	72.3	3.81 (d, 9.0)	4.20 (dd, 9.6, 2.4)	74.1	72.9
16	3.41–3.44 (m)	3.60 (ddd, 11.4, 9.6, 4.8)	74.8	74.1	3.35–3.37 (m)	3.59(ddd, 11.4, 9.6, 4.8)	74.7	74.5
17	1.85 (dt, 12.0, 4.2), 1.68 (m)	1.92–1.95 (m), 1.70 (m)	35.1	33.5	1.90 (m), 1.59 (m)	1.91–1.94 (m), 1.72 (m)	33.2	33.4
18	2.71–2.75 (m)	2.22–2.23 (m)	35.6	34.1	2.68 (ddq, 10.8, 6.6, 6.6)	2.47 (m)	35.4	34.9
19			99.5	99.2			99.2	99.7
20			197.9	191.3			198.1	193.4
21			166.8	168.5			165.6	166.9
23	4.71 (br d, 14.4), 3.23 (m)	3.76–3.79 (m), 3.49–3.52 (m)	39.6	44.5	4.65 (br d, 13.2), 3.09 (m)	3.72 (br d, 15.0), 3.37–3.39 (m)	40.0	44.7
24	1.24–1.27 (m), 1.24–1.27 (m)	1.05–1.08 (m), 0.93–0.97 (m)	24.9	24.9	1.24–1.26 (m), 1.17–1.18 (m)	1.24–1.26 (m), 1.12 (m)	25.0	25.1
25	1.37–1.40 (m), 1.16–1.18 (m)	n.a.	22.0	20.6	1.37–1.39 (m), 1.29–1.31 (m)	1.37–1.39 (m), 1.17–1.18 (m)	21.7	20.9
26	2.19–2.22 (m), 1.95–1.97 (m)	2.06–2.12 (m), 1.61–1.63 (m)	27.5	26.9	2.07–2.08 (m), 2.02–2.03 (m)	2.09–2.10 (m), 1.47 (m)	27.4	27.1
26a	4.48 (br d, 6.6)	5.28 (dd, 6.6, 2.4)	57.0	52.9	4.74 (br d, 4.8)	5.24 (dd, 6.6, 2.4)	57.1	53.4
1′			130.5	129.7			130.6	129.9
2′	4.97 (br d, 9.0)	4.92 (br d, 8.4)	132.4	134.8	5.04 (d, 10.2)	5.06 (d, 7.8)	132.7	134.4
3′	2.06–2.12 (m)	1.97–1.99 (m)	35.5	35.5	2.07–2.08 (m)	2.02–2.03 (m)	35.7	35.7
4′	1.88–1 90 (m), 0.88–0.90 (m)	n.a.	32.2	32.4	1.91 (m), 0.90–0.94 (m)	1.83–1.85 (m), 0.85 (m)	35.5	35.1
5′	2.85 (ddd, 11.4, 8.4, 4.2)	2.76 (ddd, 11.4, 9.0, 4.2)	84.9	84.8	2.84 (ddd, 11.4, 9.0, 4.2)	2.80 (m)	84.9	84.9
6′	3.40–3.42 (m)	3.38–3.40 (m)	74.2	74.0	3.40–3.43 (m)	3.40–3.43 (m)	74.2	74.1
7′	n.a.	n.a.	32.2	32.1	1.95–2.00 (m), 1.35–1.37 (m)	1.95–2.00 (m), 1.35–1.37 (m)	32.2	32.2
8′	1.37–1.40 (m), 0.86–0.87 (m)	1.37–1.39 (m), 0.79–0.83 (m)	31.3	31.0	1.41 (m), 0.86 (m)	1.41 (m), 0.81 (m)	31.2	31.0
α	2.71–2.75 (m), 2.38–2.43 (m)	2.54–2.57 (m), 2.43–2.47 (m)	36.0	36.9	2.61 (ddd, 13.8, 7.2, 7.2), 2.32 (ddd, 13.8, 7.2, 7.2)	2.77–2.80 (m), 2.39 (ddd, 13.8, 7.2, 7.2)	37.1	35.9
β	5.80 (ddt, 17.4, 10.2, 7.2)	5.76 (ddt, 16.8, 10.2, 7.2)	136.9	136.1	5.80 (ddt, 17.4, 10.2, 7.2)	5.87 (ddt, 16.8, 10.2, 6.6)	136.5	137.1
γ	5.06 (ddt, 17.4, 1.8, 1.8), 4.98 (ddt, 10.2, 1.2, 1.2)	5.10 (ddt, 16.8, 1.8, 1.8), 5.00 (ddt, 10.2, 0.6, 0.6)	116.7	117.1	5.08 (dd, 17.4, 1.8), 5.00 (ddt, 10.2, 1.2, 1.2)	5.08 (dd, 17.4, 1.8), 5.00 (ddt, 10.2, 1.2, 1.2)	116.9	116.6
CH_3-4	0.74 (d, 7.2)	0.76 (d, 7.8)	13.2	15.2	0.83 (d, 7.2)	0.86 (d, 7.2)	13.8	15.0
CH_3-10	1.62 (d, 0.6)	1.65 (d, 1.2)	20.7	20.8	1.65 (s)	1.70 (s)	16.0	16.3
CH_3-12	0.91 (d, 6.6)	0.86 (d, 6.6)	21.5	18.2	0.89 (d, 6.6)	0.82 (d, 6.6)	21.4	19.5
CH_3-18	1.21 (d, 6.6)	1.31 (d, 6.6)	16.8	17.0	1.16 (d, 6.6)	1.20 (d, 6.6)	16.8	16.8
CH_3-1′	1.41 (d, 1.2)	1.42 (d, 1.2)	14.5	14.6	1.43 (d, 1.2)	1.47 (d, 1.2)	14.8	14.7
CH_3O-14	3.19 (s)	3.31 (s)	57.3	57.9	3.19 (s)	3.26 (s)	57.3	57.9
CH_3O-16	3.07 (s)	3.14 (s)	55.9	56.0	3.09 (s)	3.15 (s)	56.0	56.0
CH_3O-5′	3.09 (s)	3.04 (s)	56.7	56.5	3.08 (s)	3.07 (s)	56.6	56.5
OH-19	5.58 (d, 1.2)	6.49 (d, 1.8)						

[a] Chemical shifts (δ values) with proton multiplicities and coupling constants (Hz) in parentheses, n.a. = not assigned.

2.8.3. 2,3-Seco-$\Delta^{5,6}$-tacrolimus (S)-1-[2-[(2R,3R,5S,6R)-6-[(1S,3S,5E, 7R,9E,11R,12S,13E)-7-(2-propen-1-yl)-12-hydroxy-14-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methoxy-3,5,11,13-tetramethyl-8-oxotetradeca-5,9,13-trien-1-yl]-2-hydroxy-5-methoxy-3-methyltetra hydro-2H-pyran-2-yl]-2-oxoacetyl]piperidine-2-carboxylic acid (5)

Colorless oil; $[\alpha]_D^{25}$ −55.7° (*c* 0.29, $CHCl_3$); UV (MeCN) λ_{max} (ε) 230 nm (11100); IR (film) ν_{max} 3384, 2933, 1729, 1641, 1451, 1216, 1088, 1049, 1027, 753 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRESIMS *m/z* 804.4887 ($[M+H]^+$), $C_{44}H_{70}NO_{12}^+$ requires 804.4893 (ΔM 0.7 ppm).

2.8.4. Tacrolimus rearrangement product (3S,4E,6S,7S,10R,11E,14R,16S,17R,18S,20R,21R,28aS)-7,21-Dihy droxy-16,18-dimethoxy-17,21-epoxy-7,8,10,13,14,15,16,17,18,19, 20,21,26,27,28,28a-hexadecahydro-3-[(1R,3R,4S)-4-hydroxy-3-methoxycyclohexyl]-4,6,12,14,20-pentamethyl-10-(2-propen-1-yl)-3H-pyrido[2,1-c][1,4]oxaazacyclopentacosine-1,9,22,23(6H,25H)-tetrone (6)

White amorphous solid; $[\alpha]_D^{25}$ −75.6° (*c* 0.35, $CHCl_3$); UV (MeCN) λ_{max} (ε) 230 nm (94200); IR (film) ν_{max} 3473, 2932, 1717, 1648, 1452, 1380, 1098, 1038, 754 cm^{-1}; ^1H and ^{13}C NMR, see Table 3; HRESIMS *m/z* 804.4887 ($[M+H]^+$), $C_{44}H_{70}NO_{12}^+$ requires 804.4893 (ΔM 0.7 ppm).

2.8.5. Tacrolimus rearrangement product, dehydrated (3S,4E,6S,7S,10R,11E,14R,16S,17R,18S,20R,21R,28aS)-21-Hydroxy-16,18-dimethoxy-17,21-epoxy-10,13,14,15,16,17,18,19,20,21,26,27, 28,28a-tetradecahydro-3-[(1R,3R,4S)-4-hydroxy-3-methoxycyclo hexyl]-4,6,12,14,20-pentamethyl-10-(2-propen-1-yl)-3H-pyrido [2,1-c][1,4]oxaazacyclopentacosine-1,9,22,23(6H,25H)-tetrone (7)

Colorless oil; $[\alpha]_D^{25}$ −4.2° (*c* 0.32, $CHCl_3$); UV (MeCN) λ_{max} (ε) 206 nm (56400); IR (film) ν_{max} 3460, 2936, 1735, 1648, 1453, 1381, 1195, 1096, 1035, 754 cm^{-1}; ^1H and ^{13}C NMR, see Table 3; HRESIMS *m/z* 786.4783 ($[M+H]^+$), $C_{44}H_{68}NO_{11}^+$ requires 786.4787 (ΔM 0.5 ppm).

2.8.6. (E)-3-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-2-methyl-2-propenal (8)

Colorless solid; $[\alpha]_D^{25}$ −46.5° (*c* 0.31, $CHCl_3$); UV (MeCN) λ_{max} (ε) 230 nm (31200); IR (film) ν_{max} 3396, 2933, 1686, 1451, 1246, 1088, 1053, 1034, 913, 772 cm^{-1}; ^1H and ^{13}C NMR, see Table S1; HRESIMS *m/z* 199.1329 ($[M+H]^+$), $C_{11}H_{19}O_3^+$ requires 199.1329 (ΔM 0.0 ppm).

2.9. X-ray crystallographic analysis of 5-deoxy-$\Delta^{5,6}$-8-epitacrolimus (2)

Single crystals suitable for X-ray diffraction studies were grown from a solution in MeCN–H_2O 7:3, $C_{44}H_{67}NO_{11}$, Mr. 786.0, orthorhombic, space group $P2_12_12_1$ (No. 19), a = 9.6590(13) Å, b = 18.766(3) Å, c = 24.5685(14) Å, V = 4453.3(9) Å3, Z = 4, D_c = 1.172 Mg/m^3, $F(000)$ = 1704, μ(Mo Kα) = 0.083 mm^{-1}, crystal size 0.38 × 0.15 × 0.15 mm.

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 (λ = 0.71073 Å) on a KappaCCD diffractometer. Data collection and cell refinement were performed using COLLECT (COLLECT, 1999) and DIRAX (Duisenberg, 1992). Data reduction was performed using EvalCCD (Duisenberg, 1998). Correction for absorption was performed using Gaussian integration (Coppens, 1970) as included in maXus (Mackay et al., 1999).

Positions of all non-hydrogen atoms were found by direct methods (SHELXS) (Sheldrick, 2008). Full-matrix least-squares refinements (SHELXL) (Sheldrick, 2008) were performed on F^2, minimizing $\Sigma w(F_o^2 - kF_c^2)^2$, with anisotropic displacement parameters of the non-hydrogen atoms. The position of hydrogen atoms were located in subsequent difference electron density maps and included in calculated positions, except for the hydrogen atoms bonded to methine carbon atoms and to the oxygen atoms. These hydrogen atoms were refined 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 (570 parameters, 10253 unique reflections) converged at R_F = 0.039, wR_F^2 = 0.081 [9093 reflections with $F_o > 4\sigma(F_o)$; $w^{-1} = [\sigma^2(F_o^2) + (0.0328P)^2 + 1.3370P]$, where $P = (F_o^2 + 2F_c^2)/3$; $S = 1.099$]. The residual electron density varied between −0.19 and 0.23 e $Å^{-3}$. The molecule belongs to a non-centrosymmetric space group but the absolute configuration could not be determined [Flack = 0.3(6)] (Flack, 1983). Complex scattering factors for neutral atoms were taken from International Tables for Crystallography as incorporated in SHELXL97 (Sheldrick, 2008; Wilson, 1995). Fractional atomic coordinates, a list of anisotropic displacement parameters, and a complete list of geometrical data have been deposited in the Cambridge Crystallographic Data Centre (CCDC: 844390). Copies of the data can be obtained, free of charge, on application to the director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

3. Results and discussion

Reactions described in this work were performed in order to mimic formation of likely transformation products formed in trace amounts during manufacturing of the drug substance tacrolimus (**1**), preparation of pharmaceutical formulations and solid dosage forms of this drug, and long-time storage of the dosage forms. The availability of reference samples of tacrolimus transformation products facilitates impurity identification and quantification in pharmaceutical products and allows for precautionary steps towards minimizing formation of such impurities. Thus, tacrolimus was subjected to acid- and base-catalyzed reactions, heat, oxidative conditions and free radicals. These reaction conditions were expected to lead to different kinds of dehydration reactions, epimerizations, rearrangements and isomerization of double bonds. Of the seven transformation products isolated and identified in this work, three were characterized for the first time (**2**, **3** and **5**). The degradation pathways are shown in Fig. 1.

Treatment of tacrolimus (**1**) with *p*-toluenesulfonic acid (Tos) in toluene under reflux for 1 h yielded a mixture of two compounds less polar than tacrolimus in a ratio of 1:2 (reversed phase HPLC, relative retention times of 1.57 and 1.53, respectively), resolved and purified by preparative HPLC. Both products had identical masses (*m/z* 808, $[M+Na]^+$, or 784, $[M-H]^-$) and similar ^1H and ^{13}C NMR spectra. The MS data indicated a dehydration of the tacrolimus molecule in both cases. This was confirmed by ^1H NMR data (Table 1). Thus, appearance of two new olefinic signals at low field proved formation of a new double bond next to the carbonyl group at C-7 in both products. In addition, treatment with acid was expected to cause epimerization at the α-position to the carbonyl functionality via reversible acid-catalyzed enolization. The close similarity of the NMR spectra of the two isomers strongly suggested that they differed in the configuration of C-8 but the relative configurations could not be determined from the NMR spectra alone. However, it was possible to crystallize the minor isomer, which had the longest HPLC retention time, and from single-crystal X-ray diffractometry the structure was established to be the novel α,β-unsaturated ketone **2** with inverted configuration at C-8 (Fig. 2). Hence, the major product was the novel α,β-unsaturated ketone **3**. The enone **2** was likewise formed as the only product in the reaction of 8-epitacrolimus (**4**) with Tos. The epimer **4** is available by treatment of **1** with 1,5-diazabicyclo[4.3.0]nonene (DBN) (Skytte et al., 2010).

Table 2
^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data[a,b] for carboxylic acid **5** in benzene-d_6.

No.	^{1}H NMR data			^{13}C NMR data		
	Rotamer/tautomer 1	Rotamer/tautomer 2	Rotamer/tautomer 3	Rotamer/ tautomer 1	Rotamer/ tautomer 2	Rotamer/ tautomer 3
1				160.6 or 164.6 or 165.2 or 165.4 or 166.6 or 167.0 or 168.0 or 168.4 (all isomers)		
3	3.69–3.72 (m)	3.69–3.72 (m)	3.65 (d, 7.2)	81.1	81.1	81.0
4	2.40–2.46 (m) (all isomers)			41.4	41.7	41.0
5	6.89 (dd, 8.4, 15.6)	6.92 (dd, 7.2, 15.6)	6.93 (dd, 6.6, 15.6)	149.6	149.7	149.2
6	6.15 (dd, 15.6, 1.8)	6.08 (br d, 15.6)	6.39 (br d, 15.6)	128.4 or 128.5	128.4 or 128.5	130.3
7				195.1 or 197.8 or 198.5 or 199.2 or 199.4 or 199.5 or 201.3 or 201.6 or 205.7 (all isomers)		
8	3.45–3.57 (m) (all isomers)			51.1 or 51.4	51.1 or 51.4	51.0
9	5.17 (d, 9.6) or 5.15 (d, 9.6)	5.17 (d, 9.6) or 5.15 (d, 9.6)	5.01–5.02 (m)	125.3 or 125.4	125.3 or 125.4	124.9
10				138.2	138.0	138.1
11	2.20–2.22 (m) or 1.98–2.02 (m) or 1.91–1.93 (m) (all isomers)			49.4 or 50.0 (all isomers)		
12	1.75–1.78 (m)	1.71–1.74 (m)	1.68–1.71 (m)	28.2	28.2	28.2
13	1.93–1.97 (m) or 1.64–1.74 (m) or 1.52–1.55 (m) (all isomers)			37.7	37.6	38.0
14	3.80–3.84 (m) (all isomers)			77.2	77.0	77.5
15	4.03 (dd, 1.8, 9.6)	3.97 (d, 9.6)	3.92 (dd, 3.0, 10.2)	74.1 or 74.2 or 74.3 or 74.5 or 74.8 (all isomers)		
16	3.45–3.57 (m)	3.45–3.57 (m)	3.40–3.43 (m)	74.1 or 74.2 or 74.3 or 74.5 or 74.8 (all isomers)		
17	1.91–1.93 (m) or 1.62–1.65 (m) (all isomers)			33.3	32.9	33.7 or 34.5
18	2.34–2.36 (m)	2.54–2.58 (m)	2.54–2.58 (m) or 2.24–2.26 (m)	34.7	35.5	32.6 or 35.4
19				98.9 or 99.3 or 99.6 or 100.8 (all isomers)		
20				195.1 or 197.8 or 198.5 or 199.2 or 199.4 or 199.5 or 201.3 or 201.6 or 205.7 (all isomers)		
21				160.6 or 164.6 or 165.2 or 165.4 or 166.6 or 167.0 or 168.0 or 168.4 (all isomers)		
23	4.63 (br d, 13.2), 2.37–2.39 (m)	4.49 (br d, 11.4), 2.37–2.39 (m)	4.69 (br d, 14.4), 2.37–2.39 (m)	39.8	43.1	39.9
24	n.a.	n.a.	n.a.	21.5 or 21.7	24.2	n.a.
25	n.a.	n.a.	n.a.	21.5 or 21.7	21.5 or 21.7	21.0
26	2.20–2.22 (m), 2.06–2.08 (m)	2.18–2.20 (m), 1.56–1.58 (m)	2.36–2.38 (m), 1.84–1.86 (m)	28.2	27.1	28.7
26a	4.87 (m)	5.49 (br d, 6.0)	5.21 (br d, 4.8)	57.0	52.2	n.a.
1′				135.8	135.8	135.8
2′	5.09–5.12 (m) or 5.04–5.06 (m) (all isomers)			132.3 or 132.1	132.3 or 132.1	132.2
3′	2.14–2.20 (m) (all isomers)			35.6	35.6	35.6
4′	1.93–1.97 (m) (all isomers), 0.95–1.03 (m) (all isomers)			35.6	35.6	35.6
5′	2.93–3.02 (m) (all isomers)			84.9	84.6	85.0
6′	3.45–3.57 (m) (all isomers)			74.1 or 74.2 or 74.3 or 74.5 or 74.8 (all isomers)		
7′	1.61–1.65 (m) (all isomers), 1.40–1.43 (m) (all isomers)			n.a.	n.a.	n.a.
8′	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
α	2.61–2.67 (m) (all isomers), 2.32–2.42 (m) (all isomers)			36.8	36.8	37.0
β	5.78–5.85 (m) (all isomers)			136.8	136.8	137.2
γ	5.09–5.12 (m) or 5.02–5.04 (m) (all isomers)			116.9	116.9	116.9
CH_3-4	1.12 (d, 6.6)	1.11 (d, 6.6)	1.06 (d, 7.2)	16.1 or 16.2	16.1 or 16.2	16.3
CH_3-10	1.67 (s)	1.60 (s)	1.71 (s)	16.9 or 17.0 or 17.1 (all isomers)		
CH_3-12	0.89 (d, 6.0)	0.92 (d, 5.4)	0.84 (d, 6.0)	19.6	19.9	18.9
CH_3-18	1.24 (d, 6.6)	1.20 (d, 6.0)	1.35 (d, 7.2) or 1.33 (d, 7.2)	16.8	16.7	17.3
CH_3-1′	1.52 (s)	1.55 (s)	1.50 (s)	12.3	12.1	12.5
CH_3O-14	3.35 (s) or 3.33 (s) or 3.22 (s) (all isomers)			58.1	58.1	57.6
CH_3O-16	3.17 (s) or 3.13 (s) or 3.12 (s) (all isomers)			56.0 or 56.1 (all isomers)		
CH_3O-5′	3.25 (s) or 3.21 (s) or 3.10 (s) (all isomers)			55.9 or 56.6 or 57.6 (all isomers)		

[a] Chemical shifts (δ values) with proton multiplicities and coupling constants (Hz) in parentheses, n.a. = not assigned.
[b] To facilitate comparison between spectra, the atom numbering system of tacrolimus (**1**) was preserved in this compound.

Amide bond rotamers of tacrolimus and analogs can be distinguished by means of differences in ^{1}H NMR and ^{13}C NMR chemical shifts, primarily in the positions adjacent to the nitrogen atom in the pipecolic acid moiety (Askin et al., 1990; Karuso et al., 1990; Schueler et al., 1993). Differences in shielding (Askin et al., 1990) and torsion angle (Karuso et al., 1990; Schueler et al., 1993) cause higher chemical shift values of C-26a in the *cis* rotamers (amide carbonyl oxygen atom syn to C-1) relative to the *trans* rotamers (amide carbonyl oxygen atom anti to C-1), the opposite being true for the chemical shifts of C-23. For the enone **2** the ratio between *cis* and *trans* rotamers was about 2:1 in benzene-d_6, while for the enone **3** in the same solvent the ratio 6:5 was observed.

We found that treatment of **1** with DBN in dichloromethane at ambient temperature overnight gave not only the previously reported equilibrium mixture of **1** and **4** (Skytte et al., 2010), but also another product with a considerable shorter retention time on reversed-phase HPLC (relative retention time 0.41). Isolation of this product by repeated preparative HPLC yielded a colorless compound with *m/z* 826 ($[M + Na]^+$, or 802, $[M - H]^-$) and ^{1}H and ^{13}C NMR data resembling those of the enone **3**. From 1D and 2D NMR data (^{1}H, ^{13}C, DEPT135, COSY, HMBC, HSQC) the structure was determined as the open-chain form of **3**, i.e., the novel carboxylic acid **5**. This is based, among others, on the absence of HMBC correlations between C-1 and H-3, observed for tacrolimus itself

Table 3
^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data[a,b] for rearrangement products **6** and **7** in benzene-d_6.

Rearrangement product **6**					Rearrangement product **7**	
No.	^{1}H NMR data		^{13}C NMR data		^{1}H NMR data	^{13}C NMR data
	Major rotamer (*trans*)	Minor rotamer (*cis*)	Major rotamer (*trans*)	Minor rotamer (*cis*)	*trans*-Rotamer	*trans*-Rotamer
1			169.5	171.4		169.8
3	5.38 (dd, 10.2, 1.2)	4.98–5.01 (m)	127.9	129.5	5.38 (dd, 10.2, 1.8)	129.4
4	2.65–2.68 (m)	2.55–2.58 (m)	38.5	38.7	3.00–3.03 (m)	35.0
5	4.15 (dt, 6.6, 2.4)	3.73–3.76 (m)	71.6	72.4	6.94 (dd, 15.6, 4.8)	150.1
6	2.59–2.60 (m), 2.46–2.51 (m)	3.01–3.04 (m), 2.21–2.23 (m)	44.9	47.9	6.16 (dd, 15.6, 1.8)	124.5
7			211.0	212.6		198.6
8	3.15–3.17 (m)	3.56 (ddd, 9.6, 7.8, 6.0)	53.4	51.9	3.32 (dt, 7.8, 7.8)	52.1
9	4.83 (br d, 10.2)	5.33 (d, 9.6)	124.3	124.6	5.04 (d, 9.0)	125.1
10			139.5	137.5 or 136.6		139.3
11	1.94–1.96 (m), 1.76–1.77 (m)	2.01–2.05 (m), 1.81–1.86 (m)	50.1	50.6	2.00–2.06 (m), 1.66–1.68 (m)	50.4
12	1.25–1.27 (m)	1.41–1.45 (m)	26.5	26.4	1.72–1.76 (m)	26.8
13	1.57–1.59 (m), 1.09–1.12 (m)	1.60–1.62 (m), 1.20–1.21 (m)	35.5	34.5	1.36–1.39 (m), 1.01 (dt, 12.6, 3.6)	34.4
14	3.80 (ddd, 12.0, 5.4, 2.4)	3.73–3.76 (m)	76.3	76.3	3.79 (ddd, 12.0, 5.4, 1.8)	76.7
15	4.13 (dd, 9.6, 2.4)	3.94 (dd, 9.6, 1.8)	73.0	73.3	4.11 (dd, 9.0, 1.8)	73.2
16	3.66–3.70 (m)	3.41–3.46 (m)	74.1	74.1	3.66 (ddd, 10.8, 9.6, 4.2)	74.3
17	2.09 (ddd, 12.6, 4.2, 4.2), 1.71–1.75 (m)	1.81–1.86 (m), 1.57–1.59 (m)	33.3	32.7	2.13 (dt, 12.6, 4.2), 1.72–1.76 (m)	33.1
18	2.55–2.58 (m)	2.55–2.58 (m)	35.3	35.3	2.67 (ddt, 4.2, 6.6, 6.6)	35.7
19			99.1	99.0		99.1
20			198.4	195.2		196.3
21			166.3	165.8		165.8
23	3.66–3.70 (m), 3.34–3.36 (m)	4.65 (dd, 13.8, 3.0), 3.29–3.30 (m)	44.8	39.5	3.48 (br d, 13.8), 2.98 (dt, 13.8, 2.4)	45.7
24	1.26–1.29 (m), 1.26–1.29 (m)	1.26–1.29 (m), 1.24–1.25 (m)	25.4	24.6	1.72–1.76 (m), 1.33 (dddd, 16.2, 12.6, 12.6, 3.6)	25.4
25	1.41–1.45 (m), 1.26–1.29 (m)	1.41–1.45 (m), 1.26–1.29 (m)	21.3	21.2	1.46–1.48 (m), 1.41–1.43 (m)	22.2
26	2.18–2.20 (m), 1.26–1.29 (m)	2.18–2.20 (m), 1.68–1.71 (m)	26.6	26.9	2.24 (br d, 13.8), 1.16–1.18 (m)	26.3
26a	5.23 (br d, 3.6)	4.62 (br d, 6.6)	52.8	57.2	5.23 (d, 6.0)	52.6
1′			137.5 or 136.6	134.5		137.2
2′	4.72 (d, 8.4)	4.77 (d, 4.8)	82.2 or 82.0	82.2 or 82.0	4.48 (d, 9.6)	82.6
3′	1.87–1.90 (m)	1.46–1.48 (m)	40.0	38.9	1.97 (ddd, 9.0, 3.0, 3.0)	40.1
4′	2.44 (br d, 11.4), 0.79–0.83 (m)	2.12–2.15 (m), 0.91–0.97 (m)	31.8	29.8	2.59 (br d, 12.6), 0.74 (q, 12.0)	32.4
5′	3.01–3.04 (m)	2.75 (ddd, 11.4, 9.0, 4.2)	85.0	85.1	3.07–3.11 (m)	85.2
6′	3.41–3.46 (m)	3.39 (ddd, 11.4, 8.4, 4.8)	74.4	74.5	3.46 (ddd, 11.4, 8.4, 4.2)	74.3
7′	2.01–2.05 (m), 1.37–1.39 (m)	1.94–1.96 (m), 1.25–1.27 (m)	31.8	32.0	2.00–2.06 (m), 1.36–1.39 (m)	31.8
8′	1.41–1.45 (m), 0.79–0.83 (m)	1.32–1.35 (m), 1.03–1.05 (m)	27.8	27.6	1.36–1.39 (m), 0.69 (ddt, 13.2, 13.2, 3.6)	26.5
α	2.62–2.64 (m), 2.25–2.28 (m)	2.46–2.51 (m), 2.21–2.23 (m)	35.5	37.5	2.50 (ddd, 14.4, 7.2, 7.2), 2.28 (ddd, 14.4, 7.2, 7.2)	37.5
β	5.74 (ddt, 16.8, 9.6, 7.2)	5.76 (ddt, 16.2, 9.6, 7.2)	136.6	136.2	5.76 (ddt, 16.8, 10.2, 7.2)	136.3
γ	5.03 (dq, 16.8, 1.8), 4.98–5.01 (m)	5.03 (dq, 16.8, 1.8), 4.98–5.01 (m)	116.9	117.2	5.05 (dd, 16.8, 1.8), 5.00 (ddd, 10.2, 1.2, 1.2)	117.0
CH_3-4	0.88 (d, 7.2)	1.26 (s)	17.9	17.8	0.92 (d, 6.6)	18.1
CH_3-10	1.53 (d, 0.6)	1.63 (d, 0.6)	15.6	15.8	1.41 (s)	15.3
CH_3-12	0.78 (d, 6.6)	0.76 (d, 6.6)	19.6	19.8	0.81 (d, 6.0)	20.1
CH_3-18	1.19 (d, 6.6)	1.05 (d, 6.6)	16.9	16.5	1.16 (d, 6.6)	17.0
CH_3-1′	1.64 (d, 1.2)	1.57 (d, 1.2)	18.7	16.8	1.67 (d, 1.8)	18.6
CH_3O-14	3.19 (s)	3.20 (s)	58.4	57.8	3.17 (s)	58.9
CH_3O-16	3.31 (s)	3.07 (s)	56.4 or 56.1	56.1	3.34 (s)	55.8
CH_3O-5′	3.32 (s)	3.18 (s)	56.4 or 56.1	56.7	3.38 (s)	56.4

[a] Chemical shifts (δ values) with proton multiplicities and coupling constants (Hz) in parentheses, n.a. = not assigned.
[b] To facilitate comparison between spectra, the atom numbering system of tacrolimus (**1**) was preserved in these compounds.

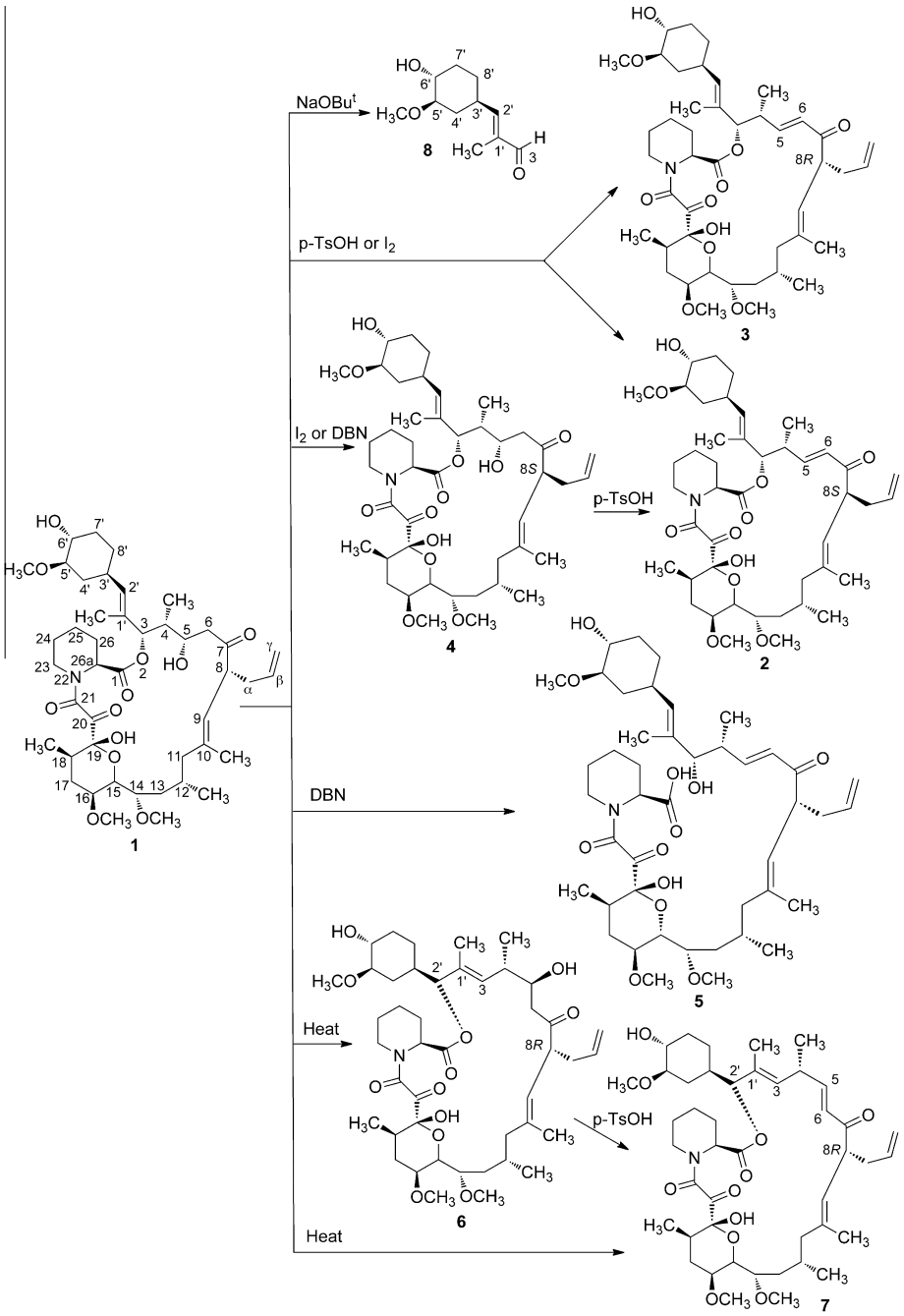

Fig. 1. Overview of the degradation pathways of tacrolimus.

and the macrocyclic compounds mentioned above. However, the stereochemistry of **5** could not be confirmed from NMR data, but we propose the structure **5** as the most plausible as NMR data resembled those of compound **3** more than those of compound **2**. The isolated compound was a mixture of at least four species, presumably amide bond rotamers as well as tautomers not separable by HPLC, in a ratio of 1:10:20:24, which complicated interpretation of the NMR spectra. Nevertheless, extensive use of 2D NMR correlations (COSY, NOESY, HSQC, HMBC) enabled assignment of most of the signals of three species present in the solution of **5** in benzene-d_6 (Table 2).

A thermal rearrangement of the allylic ester part of tacrolimus to give **6** was previously reported to take place upon reflux in *o*-xylene (b.p. 144 °C) for 25 h. Uncatalyzed thermal rearrangements of allylic esters usually requires very high temperatures, but for tacrolimus the assumed [3,3]-sigmatropic shift was believed to occur under the relatively mild conditions due to favorable conformational preorientation of the molecule for this reaction (Ok et al., 1990). Reflux in toluene (b.p. 111 °C) was not sufficient to induce the rearrangement reaction. Heating of **1** in refluxing xylene resulted in fact in two main products (Ok et al., 1990), one slightly more polar and one less polar than tacrolimus (reversed-phase HPLC, relative retention times 0.83 and 1.55, respectively), along with a number of minor impurities that were not further analyzed. The compound with the shortest retention time was identified as the previously reported rearrangement product **6** based on MS (pseudomolecular ion isobaric with that of **1**, *m/z* 826, $[M + Na]^+$, or 802, $[M - H]^-$) and NMR data (Table 3). The ratio between *cis* and *trans* amide bond rotamers of **6** was 3:5. In the reaction product with the relative retention time of 1.55, water had been lost (*m/z*

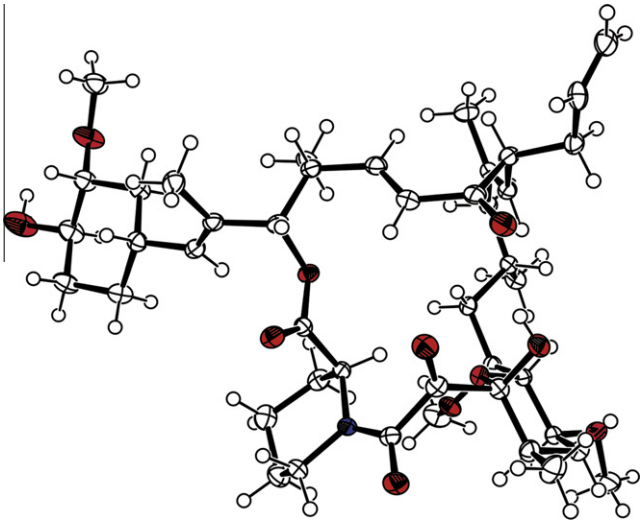

Fig. 2. ORTEP drawing of the crystal structure of compound **2**. Displacement ellipsoids are drawn at 50% probability level. Hydrogen atoms are drawn as spheres of arbitrary size. Oxygen atoms are colored red and nitrogen atoms are colored blue (atoms will appear gray in print). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

808, $[M + Na]^+$, or 784, $[M - H]^-$). NMR data (Table 3) established this compound to be **7**, a dehydration product of **6**. A large excess of the *trans* amide bond rotamer was observed in the ^1H and ^{13}C NMR spectra of **7**. A reaction time of 25 h resulted in about 50% conversion of tacrolimus to the regioisomer **6** and only a small amount of the enone **7** (reversed-phase HPLC). Extended reaction time in *o*-xylene (42 h) resulted in an increased formation of the dehydrated product **7**, which is in compliance with results obtained by Ok et al.(1990) Reaction of **6** with Tos in toluene under reflux yielded **7** as the only product.

Treatment of tacrolimus with the strong base, sodium *t*-butoxide in *t*-butanol at 65 °C for 3 h resulted in degradation of the molecule with formation of the aldehyde **8** as the only isolable product. The structure was confirmed by NMR data (see Appendix A, Supplementary data, Table S1). Formation of this compound has already been reported from hydrolysis of tacrolimus with 1 M NaOH (Tanaka et al., 1987).

Tacrolimus contains two trisubstituted double bonds, which are potentially isomerizable through exposure to light. To mimic this putative reaction under accelerated conditions, iodine was used as a reagent (Sonnet, 1980). Refluxing of **1** in toluene for 6.5 h with a catalytic amount of iodine yielded three products less polar than tacrolimus (reversed-phase HPLC). None of the isolated products had isomerized double bond. The product with the shortest retention time displayed *m/z* 826 ($[M + Na]^+$) and was identical with 8-epitacrolimus (**4**) as shown by ^1H and ^{13}C NMR spectra. The two products with the longest relative retention times (*m/z* 808, $[M + Na]^+$, or 784, $[M - H]^-$) were identical with **2** and **3**, formed in the reaction of **1** with Tos.

The susceptibility of tacrolimus towards oxidation was investigated through treatment with H_2O_2. Tacrolimus turned out to be very stable towards oxidation. Even prolonged treatment with a large excess of H_2O_2 (30% solution, several days) did not produce any oxidation products.

In conclusion, transformations of tacrolimus can be summarized as follows. Acids and free radicals cause dehydration of the β-hydroxyketone moiety with formation of α,β-unsaturated ketones as well as epimerization at C-8, i.e., next to the enolizable carbonyl function. The latter transformation is also observed upon action of a weak base (DBN) along with dehydration at C-5 and lactone group hydrolysis to give the free carboxylic acid **5**, whereas strong bases cause extensive degradation of the molecule. All products were characterized by ^1H and ^{13}C NMR spectroscopy, including assignment of rotamers of the amide group observed in solution. Also, ^1H and ^{13}C NMR spectra of the previously reported ring expansion products **6** and **7** were assigned by extensive use of 2D NMR techniques. These reactions and products are of interest in connection with production and handling of pharmaceuticals containing tacrolimus.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2012.12.001.

References

Akashi, T., Nefuji, T., Yoshida, M., Hosoda, J., 1996. Quantitative determination of tautomeric FK-506 by reversed-phase liquid chromatography. J. Pharm. Biomed. Anal. 14, 339–346.
Askin, D., Reamer, R.A., Joe, D., Volante, R.P., Shinkai, I., 1990. Synthesis of the novel sarcosine and proline (FK-525) analogs of FK-506: rearrangement of the allylic ester system. J. Org. Chem. 55, 5448–5450.
Baumann, K., Oberhauser, B., Grassberger, M.A., Haidl, G., Schulz, G., 1995. Synthesis and oxidative cleavage of the major equilibrium products of ascomycin and FK 506. Tetrahedron Lett. 36, 2231–2234.
Bowman, L.J., Brennan, D.C., 2008. The role of tacrolimus in renal transplantation. Expert Opin. Pharmacother. 9, 635–643.
Bulusu, M.A., Baumann, K., Stuetz, A., 2011. Chemistry of the immunomodulatory macrolide ascomycin and related analogues. Fortschr. Chem. Org. Naturst. 94, 59–126.
Busuttil, R.W., Lake, J.R., 2004. Role of tacrolimus in the evolution of liver transplantation. Transplantation 77, S44–51.
COLLECT; Nonius BV: Delft, The Netherlands, 1999.
Coppens, P., 1970. In: Ahmed, F.R., Hall, S.R., Huber, C.P. (Eds.), Crystallographic Computing. Munksgaard, Copenhagen, pp. 255–270.
Duisenberg, A.J.M., 1992. Indexing in single-crystal diffractometry with an obstinate list of reflections. J. Appl. Crystallogr. 25, 92–96.
Duisenberg, A.J.M., 1998. Reflections on Area Detectors: ab initio Calculation of Single-Crystal X-Ray Reflection Contours. Ph.D. Thesis, University of Utrecht, Utrecht, The Netherlands.
Flack, H.D., 1983. On enantiomorph-polarity estimation. Acta Crystallogr. Sect. A 39, 876–881.
Fortune, K., Couriel, D., 2009. Tacrolimus in hematopoietic stem cell transplantation. Expert Opin. Drug Metab. Toxicol. 5, 835–841.
Gailliot, F.P., Natishan, T.K., Ballard, J.M., Reamer, R.A., Kuczynski, D., McManemin, G.J., Egan, R.S., Buckland, B.C., 1994. Isolation and characterization of the major equilibrium product of FK-520. J. Antibiot. 47, 806–811.
Graziani, E.I., 2009. Recent advances in the chemistry, biosynthesis and pharmacology of rapamycin analogs. Nat. Prod. Rep. 26, 602–609.
Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K., Navia, M.A., 1995. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. Cell 82, 507–522.
Kalavala, M., Dohil, M.A., 2011. Calcineurin inhibitors in pediatric atopic dermatitis: a review of current evidence. Am. J. Clin. Dermatol. 12, 15–24.
Karuso, P., Kessler, H., Mierke, D.F., 1990. Solution structure of FK506 from nuclear magnetic resonance and molecular dynamics. J. Am. Chem. Soc. 112, 9434–9436.
Kissinger, C.R., Parge, H.E., Knighton, D.R., Lewis, C.T., Pelletier, L.A., Tempczyk, A.E., Bacquet, R., Villafranca, J.E., 1995. Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. Nature 378, 641–644.
Lee, Y.H., Woo, J.H., Choi, S.J., Ji, J.D., Bae, S.C., Song, G.G., 2010. Tacrolimus for the treatment of active rheumatoid arthritis: a systematic review and meta-analysis of randomized controlled trials. Scand. J. Rheumatol. 39, 271–278.
Mackay, S., Gilmore, C.J., Edwards, C., Stewart, N., Shankland, K., 1999. MaXus Computer Program for the Solution and Refinement of Crystal Structures. Bruker Nonius, Mac-Science, The University of Glasgow, The Netherlands, Japan, UK.

Mierke, D.F., Schmieder, P., Karuso, P., Kessler, H., 1991. Conformational analysis of the cis- and trans-isomers of FK506 by NMR and molecular dynamics. Helv. Chim. Acta 74, 1027–1047.

Moore, J.M., Peattie, D.A., Fitzgibbon, M.J., Thomson, J.A., 1991. Solution structure of the major binding protein for the immunosuppressant FK506. Nature 351, 248–250.

Namiki, Y., Kihara, N., Koda, S., Hane, K., Yasuda, T., 1993. Tautomeric phenomenon of a novel potent immunosuppressant (FK 506) in solution. I. Isolation and structure determination of tautomeric compounds. J. Antibiot. 46, 1149–1155.

Namiki, Y., Fujiwara, A., Kihara, N., Koda, S., Hane, K., Yasuda, T., 1995. Factors affecting tautomeric phenomenon of a novel potent immunosuppressant (FK506) on the design for injectable formulation. Drug Dev. Ind. Pharm. 21, 809–822.

Nghiem, P., Pearson, G., Langley, R.G., 2002. Tacrolimus and pimecrolimus: from clever prokaryotes to inhibiting calcineurin and treating atopic dermatitis. J. Am. Acad. Dermatol. 46, 228–241.

Ok, H., Arison, B.H., Ball, R.G., Beattie, T.R., Fisher, M.H., Wyvratt, M.J., 1990. Thermal rearrangement of the immunosuppressant FK-506. Tetrahedron Lett. 31, 6477–6480.

Penninga, L., Møller, C.H., Gustafsson, F., Steinbrüchel, D.A., Gluud, C., 2010. Tacrolimus versus cyclosporine as primary immunosuppression after heart transplantation: systematic review with meta-analyses and trial sequential analyses of randomised trials. Eur. J. Clin. Pharmacol. 66, 1177–1187.

Petros, A.M., Luly, J.R., Liang, H., Fesik, S.W., 1993. Conformation of an FK506 analog in aqueous solution is similar to the FKBP-bound conformation of FK506. J. Am. Chem. Soc. 115, 9920–9924.

Ruemmele, F.M., Moes, N., de Serre, N.P., Rieux-Laucat, F., Goulet, O., 2008. Clinical and molecular aspects of autoimmune enteropathy and immune dysregulation, polyendocrinopathy autoimmune enteropathy X-linked syndrome. Curr. Opin. Gastroenterol. 24, 742–748.

Schueler, W., Christians, U., Schmieder, P., Schiebel, H.M., Holze, I., Sewing, K.F., Kessler, H., 1993. Structural investigations of 13-O-demethyl-FK506 and its isomers generated by in vitro metabolism of FK506 using human-liver microsomes. Helv. Chim. Acta. 76, 2288–2302.

Scott, L.J., McKeage, K., Keam, S.J., Plosker, G.L., 2003. Tacrolimus: a further update of its use in the management of organ transplantation. Drugs 63, 1247–1297.

Sewell, T.J., Lam, E., Martin, M.M., Leszyk, J., Weidner, J., Calaycay, J., Griffin, P., Williams, H., Hung, S., Cryan, J., Sigal, N.H., Wiederrecht, G.J., 1994. Inhibition of calcineurin by a novel FK-506-binding protein. J. Biol. Chem. 269, 21094–21102.

Sheldrick, G.M., 2008. A short history of SHELX. Acta Crystallogr. Sect. A 64, 112–122.

Skytte, D.M., Frydenvang, K., Hansen, L., Nielsen, P.G., Jaroszewski, J.W., 2010. Synthesis and characterization of an epimer of tacrolimus, an immunosuppressive drug. J. Nat. Prod. 73, 776–779.

Sonnet, P.E., 1980. Olefin inversion. Tetrahedron 34, 557–604.

Tanaka, H., Kuroda, A., Marusawa, H., Hatanaka, H., Kino, T., Goto, T., Hashimoto, M., Taga, T., 1987. Structure of FK506, a novel immunosuppressant isolated from Streptomyces. J. Am. Chem. Soc. 109, 5031–5033.

Vicari-Christensen, M., Repper, S., Basile, S., Young, D., 2009. Tacrolimus: review of pharmacokinetics, pharmacodynamics, and pharmacogenetics to facilitate practitioners' understanding and offer strategies for educating patients and promoting adherence. Prog. Transplant. 19, 277–284.

Wagner, R., Rhoades, T.A., Or, Y.S., Lane, B.C., Luly, J.R., 1996. Selective epimerization and skeletal resection in the ascomycin framework: a study of the biological consequences of lactam rotamer selection. Tetrahedron 52, 9643–9654.

Wilson, A.J.C. (Ed.), 1995. International Tables for Crystallography. Kluwer Academic Publishers, Dordrecht, The Netherlands, vol. C, Tables 4.2.6.8 and 6.1.1.4.

Wilson, K.P., Yamashita, M.M., Sintchak, M.D., Rotstein, S.H., Murcko, M.A., Boger, J., Thomson, J.A., Fitzgibbon, M.J., Black, J.R., Navia, M.A., 1995. Comparative x-ray structures of the major binding protein for the immunosuppressant FK506 (Tacrolimus) in unliganded from and in complex with FK506 and rapamycin. Acta Crystallogr. D51, 511–521.

Yeoman, A.D., Longhi, M.S., Heneghan, M.A., 2010. Review article: the modern management of autoimmune hepatitis. Aliment Pharmacol. Ther. 31, 771–787.