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COMMUNICATION

Degradation Products of Mycophenolate Mofetil in Aqueous Solution

Elvira Hooijmaaijer, Michael Brandl,* Janis Nelson, and David Lustig

Roche Bioscience, 3401 Hillview Avenue, Palo Alto, California 94304

ABSTRACT

*The thermal and peroxide-catalyzed degradation products of mycophenolate mofetil (**1**) were studied in aqueous solution at pH 2.0, 3.5, 6.0, and 8.2. The major thermal degradation product observed was mycophenolic acid (**2**). At pH 6.0 and 8.2, **2** was the only product observed in the absence of peroxide, while at pH 2.0 and 3.5, the lactone analogue of mycophenolic acid (**5**), a hydroxylactone due to oxygenation of the double bond (**6**), and an unidentified product were formed. Compound **6** degraded to 4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydro-isobenzofuran-5-carbaldehyde (**9**) on prolonged storage and was present in the sample stressed at pH 2. Mycophenolic acid (**2**), the* N*-oxide of mycophenolate mofetil (**3**), the hydroxylactone of mycophenolic acid (**6**), and the erythro form of 4-methoxy-5-methyl-2-(2-methyl-5-oxo-tetrahydro-furan-2-yl)-3,6-dihydro-2H-1,7-dioxa-as-indacen-8-one (**8**) were observed in the presence of hydrogen peroxide at pH 3.5, 6.0, and 8.2. In addition, at pH 8.2, 4-hydroxy-4-(4-methoxy-5-methyl-8-oxo-2,3,6,8-tetrahydro-1,7-dioxa-as-indacen-2-yl)-pentanoic acid (**7**) was seen. Peroxide-stressed samples at pH 2.0 gave no major degradation peaks, but a small amount of the hydroxylactone of mycophenolic acid (**6**) was formed.*

INTRODUCTION

Mycophenolate mofetil, **1**, is the morpholinoethyl ester (1) of mycophenolic acid, **2**, which has been shown to have a broad spectrum of activities (2–7). Oral capsule, oral suspension, and injectable dosage forms have been approved. Its chemical name is 2-(4-morpholino)ethyl(E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzo-furanyl)-4-methyl-4-hexenoate.

A limited chemical reactivity study of **1** in aqueous solution was reported when prodrug selection was com-

* To whom correspondence should be addressed. Telephone: (650) 354-7406. Fax: (650) 855-6428.

pleted (1). To validate the specificity of analytical methods for the registration of the various formulations of **1**, the degradation products formed by thermal and peroxide stressing of solutions of **1** were studied. This report details the results of these studies.

MATERIALS AND METHODS

Materials

Mycophenolate mofetil (**1**), the *N*-oxide of mycophenolate mofetil (**3**), and the lactone of mycophenolic acid **(8)** were from the Institute of Organic Chemistry, Syntex Research. The mycophenolic acid (**2**) was obtained from Calbiochem. Citric acid, potassium acetate, potassium chloride, potassium carbonate, and potassium dihydrogen phosphate were obtained from Mallinckrodt (analytical grade) and were used without further purification. Water was purified using a Barnstead Nanopure system. Hydrogen peroxide (30%), trifluoroacetic acid, acetonitrile, and phosphoric acid were from Aldrich, Pierce, Burdick and Jackson, and Mallinckrodt, respectively.

The degradation products of **1** (see Fig. 1) were prepared by stressing **1** with heat or hydrogen peroxide in aqueous solutions. The specific details are described below.

Preparation of Compound 7

The erythro form of the 4-hydroxy-4-(4-methoxy-5-methyl-8-oxo-2,3,6,8-tetrahydro-1,7-dioxa-as-indacen-2-yl)-pentanoic acid (**7**) was prepared by reacting **1** thermally and in the presence of hydrogen peroxide (2,8–10). A sample of 0.3 mg/ml of **1** in pH 3.5 buffer was stressed at 60°C for 7 days, and the compound was collected by high-performance liquid chromatography (HPLC). Alternatively, 10 ml of the 0.3 mg/ml solution of **1** in pH 3.5 buffer was mixed with 1 ml 30% hydrogen peroxide solution. The compound was collected by HPLC after reacting for 8 days. The ^{1}H nuclear magnetic resonance (NMR) and mass spectra agree with those previously reported (9,10).

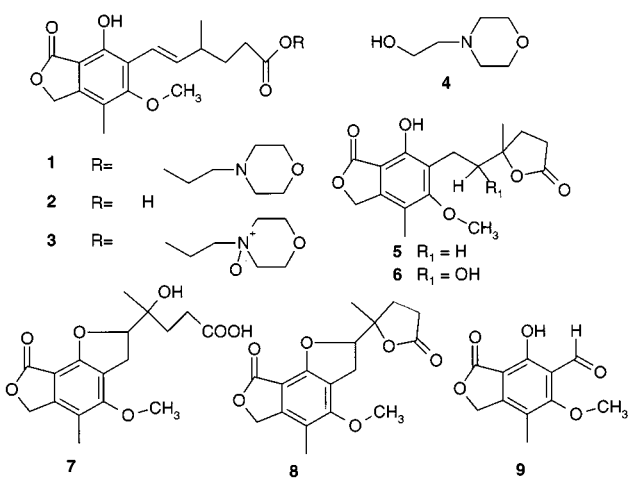

Figure 1. Structures of mycophenolate mofetil and its degradation products.

Preparation of Compound 8

The erythro form of 4-methoxy-5-methyl-2-(2-methyl-5-oxo-tetrahydro-furan-2-yl)-3,6-dihydro-2H-1,7-dioxa-as-indacen-8-one (**8**) was prepared by a reaction of **1** with peroxide at pH 8.2 (2,9). A 3 mg/ml solution of **1** in acetonitrile was diluted 100-fold with pH 8.2 buffer. Then, 10 ml of this solution was mixed with 250 µl 30% peroxide solution. The peak was collected by HPLC after 8 days. Compound **8** was also obtained when 100 ml of a 1 mg/ml solution of **1** in pH 8 buffer solution was diluted with an equivalent volume of acetonitrile and 5 ml of 30% hydrogen peroxide solution was added. The compound was collected by HPLC after 6 days. The ^{1}H NMR and mass spectra were in agreement with that previously reported (9). ^{13}C NMR chemical shifts were δ 11.02, δ 20.64, δ 28.45, δ 29.60, δ 30.87, δ 59.20, δ 59.20, δ 69.07, δ 85.70, δ 87.44, δ 115.43, δ 115.61, δ 147.37, δ 157.14, δ 159.78, δ 168.58, δ 175.95, and δ 197.50.

Preparation of Compound 9

The 4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydro-isobenzofuran-5-carbaldehyde (**9**) was obtained from **6** on prolonged storage. ^{1}H NMR chemical shifts were δ 1.56, δ 2.17, δ 3.98, and δ 5.20.

Instrumentation

The pH measurements were made with a Radiometer model PHM 84 or PHM 64 Research pH meter equipped with a Sensorex model SG900C or Radiometer model 2410C combination electrode. The HPLC system consisted of an HP1050 HPLC and an Applied Biosystems model 757 ultraviolet (UV) absorbance detector. The detector was interfaced with a Macintosh SE30 computer, and the data analyzed with Dynamax software from Rainin. A Gilson fraction collector, model 201, was used.

The ^{1}H and ^{13}C NMR experiments were performed on a Bruker AMX500 NMR spectrophotometer, typically in deuterated chloroform solution referenced to chloroform at δ 7.26 and δ 77.0 for ^{1}H and ^{13}C spectra, respectively.

Mass spectrometry (MS) was performed on a Finnigan TSQ 700 triple-stage quadrupole mass spectrometer equipped with a Finnigan Electrospray Ionization Source and a Finnigan Atmospheric Pressure Chemical Ionization Source.

High-Performance Liquid Chromatography Method

Two methods were developed for HPLC. Method 1 was used to determine the degradation profiles and the isolation of most of the degradation products. Method 1 used a Zorbax Rx C8, 250 × 9.4 mm column with an isocratic mobile phase consisting of 35/65 acetonitrile/0.1% trifluoroacetic acid. A flow rate of 2.5 ml/min with a column temperature of 45°C and detection at 250 nm were used. Method 2 was used to isolate **8.** It differed from method 1 in that the mobile phase consisted of 32/68 acetonitrile/25 mM ammonium acetate pH 5.4, and the flow rate was 4.7 ml/min.

Degradation Profiles

Buffers were prepared from hydrochloric acid (pH 2.0), citric acid (pH 3.5), sodium dihydrogen phosphate (pH 6.0), and sodium hydrogen phosphate (pH 8.2) and adjusted with sodium hydroxide or hydrochloric acid. Generally, reactions that were completed in less than 1 day were injected directly on the HPLC using a thermostated autosampler. For slower reactions, the solutions were flame sealed in 1- or 2-ml clear glass ampoules and placed in ovens. Samples were removed at predetermined time intervals and refrigerated until assayed. Drug concentrations were approximately 0.02 mg/ml for pH 6.0 and pH 8.2 and 0.1 mg/ml for pH 2.0 and pH 3.5. The peroxide concentration for pH 6.0 and 8.2 studies was 0.75% (v/v) of the final solution and 6% (v/v) for the pH 2.0 and 3.5 studies.

Identification of Degradation Products

The degradation products were collected using HPLC; solvents were subsequently removed by rotary evaporation and lyophilization. The compounds were analyzed by ^{1}H NMR and MS. Main beam MS, in which the mass analyzer was scanned from 100 to 1000 amu, was used to survey all ions formed. Once molecular weights were determined, tandem MS-MS was performed to obtain structural information.

To determine the structure of 4-hydroxy-4-(4-methoxy-5-methyl-8-oxo-2,3,6,8-tetrahydro-1,7-dioxa-as-indacen-2-yl)-pentanoic acid (**7**), a ^{1}H NMR experiment was performed with a solution of **2** in pH 8.2 buffer with peroxide. An initial sample was taken to be able to subtract the signals of the other components in the sample, and several time points were taken to measure the growing degradation product that eluted at 8.7 minutes with HPLC method 2.

The identity of the lactone analogue of mycophenolic acid (**5**) and the *N*-oxide of mycophenolate mofetil (**3**) were verified by injection of an authentic sample.

RESULTS AND DISCUSSION

Thermal Degradation of Compound 1 at pH 2.0, 3.5, 6.0, and 8.2

At pH 6.0 and 8.2, solutions of **1** hydrolyze to mycophenolic acid (**2**) and 2-morpholino-ethanol (**4**), which is not detected by the HPLC method (see Fig. 2) (1). At the more-acidic pH values, **2** was still the major degradation product; however, since ester hydrolysis is slower in these conditions, additional peaks were observed. At pH 2.0, these were **5**, **6**, and **9**, and at pH 3.5, **6** and **8** were seen. An additional product eluting just prior to **1** was

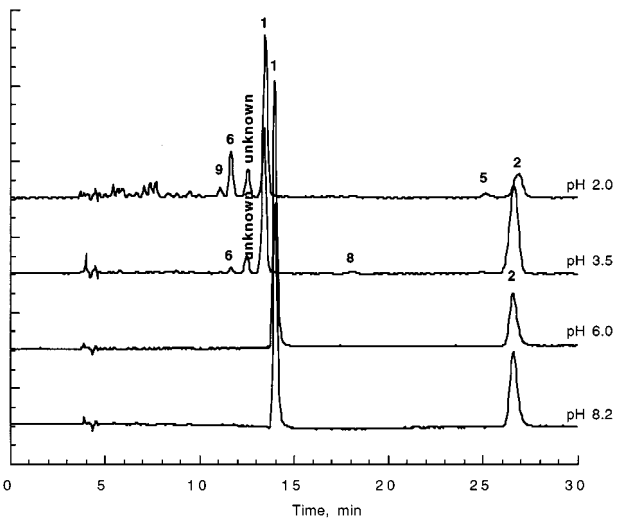

Figure 2. HPLC chromatograms of samples of RS-61443-000 stored at (a) pH 8.2, 40°C (65% remaining); (b) pH 6.0, 40°C (73% remaining); (c) pH 3.5, 60°C (38% remaining); and (d) pH 2.0, 60°C (44% remaining).

Table 1

*Percentages of Products Observed by HPLC from the Thermal Degradation of **1** in Solution*

			% Observed[a]						
			Compound:	**1**	**2**	**5**	**6**	**8**	**9**	**Unknown**
pH	Reaction Time (hr)	T (°C)	Retention Time (min):	13.8	26.6	25.1	11.7	18.1	11.1	12.5
2.0	48	60		44	14	2	10		1	7
3.5	120	60		39	49		1	2		5
6.0	8.0	40		54	50					
8.2	8.5	40		54	44					

[a] Percentage of the initial area of **1** observed by HP:C with detection at 250 nm.

not identified. Table 1 gives values for the percentage of products formed in thermally stressed samples of **1**.

Except for **5**, which forms from the intramolecular addition of the carboxylic group to the olefin, the additional products are a result of the oxidation of **1**. These products have been found in microbial modifications of **2** (9,10) and arise from the oxidation of the olefinic group. The initial oxidation product cyclizes once or twice to form **6** or **8**, respectively. Since only the erythro compound was formed, the initial oxidation most probably proceeds through an epoxide intermediate (9). Formation of the aldehyde (**9**) must involve oxidation alpha to the aromatic ring with subsequent cleavage of the carbon-carbon bond. A possible mechanism is shown in Fig. 3.

Degradation of Compound 1 at pH 2.0, 3.5, 6.0, and 8.2 in the Presence of Hydrogen Peroxide

Figure 4 shows chromatograms of **1** degraded in the presence of hydrogen peroxide. Samples in pH 8.2 and 6.0 buffer were reactive in the presence of hydrogen peroxide. Five degradation products were observed at 25°C by HPLC. At pH 8.2, these compounds were identified as **2**, **3**, **6**, **7**, and **8**. At pH 6.0, **2**, **3**, **6,** and **8** were observed. Compound **7** converts to **8** on isolation, which can be reasonably explained by cyclization to form a lactone in acidic solution. Solutions containing peroxide at pH 2.0 and pH 3.5 were less reactive; therefore, these samples were stressed at 40°C. Samples at pH 3.5 showed the same products that were observed at pH 6.0, but the mass balance was lower. Samples stressed at pH 2.0 showed a few minor products, two of which were identified as **6** and **7**. An unidentified compound eluting at approximately 8 min was detected at pH 2.0, 3.5, and 6.0. At pH 2.0, six other minor compounds were detected as well. Table 2 gives values for the percentage of degrada-

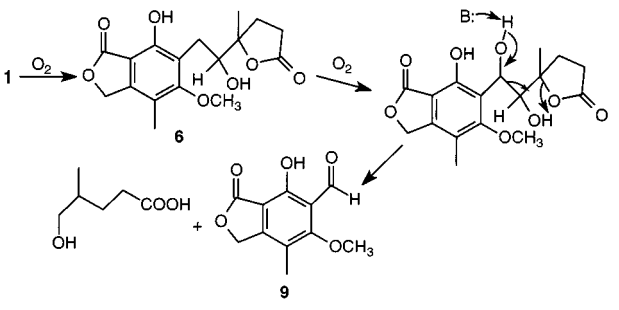

Figure 3. Possible mechanism for the formation of **9**.

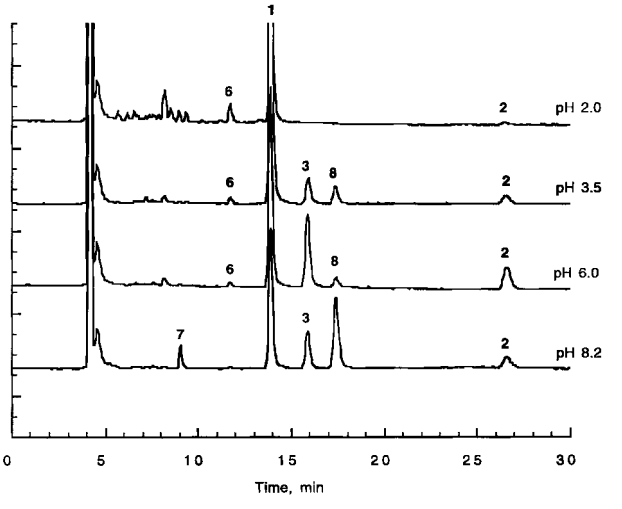

Figure 4. HPLC chromatograms of RS-61443-000 degraded in the presence of peroxide at (a) pH 8.2, 25°C (41% remaining); (b) pH 6.0, 25°C (56% remaining); (c) pH 3.5, 39°C (74% remaining); and (d) pH 2.0, 39°C (50% remaining).

Table 2

*Percentages of Products Observed by HPLC form the Degradation of **1** in Solution in the Presence of Hydrogen Peroxide*

			% Observed[a]					
			Compound:	**1**	**2**	**3**	**6**	**7**	**8**
pH	Reaction Time (hr)	T (°C)	Retention Time (min):	13.8	26.6	15.9	11.7	9.0	17.4
2.0	16	40		50	1		4	1	
3.5	20	40		72	4	7	1		5
6.0	8.0	25		54	11	23	1	1	3
8.2	4.5	25		42	5	12	1	5	26

[a] Percentage of the initial area of **1** observed by HPLC with detection at 250 nm.

tion products of **1** formed in the presence of hydrogen peroxide and their retention times.

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